Cancer Research

Epigenetically Deregulated microRNA-375 Is Involved in a Positive Feedback Loop with Estrogen Receptor α in Breast Cancer Cells

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Abstract

Estrogen receptor α (ERα) upregulation causes abnormal cell proliferation in about two thirds of breast cancers, yet understanding of the underlying mechanisms remains incomplete. Here, we show that high expression of the microRNA miR-375 in ERα-positive breast cell lines is a key driver of their proliferation. miR-375 overexpression was caused by loss of epigenetic marks including H3K9me2 and local DNA hypomethylation, dissociation of the transcriptional repressor CTCF from the miR-375 promoter, and interactions of ERα with regulatory regions of miR-375. Inhibiting miR-375 in ERα-positive MCF-7 cells resulted in reduced ERα activation and cell proliferation. A combination of expression profiling from tumor samples and miRNA target prediction identified RASD1 as a potential miR-375 target. Mechanistic investigations revealed that miR-375 regulates RASD1 by targeting the 3′ untranslated region in RASD1 mRNA. Additionally, we found that RASD1 negatively regulates ERα expression. Our findings define a forward feedback pathway in control of ERα expression, highlighting new strategies to treat ERα-positive invasive breast tumors. *Cancer Res; 70(22); 9175–84. ©2010 AACR.*

Introduction

Breast cancer is the leading cause of cancer death in women worldwide (1). Although it is a heterogeneous disease, two thirds of breast cancers share the common feature of being dependent on the presence and interaction of estrogen with the nuclear estrogen receptor α (ERα) protein (2, 3). Approximately 70% of invasive breast cancers express ERα in actively proliferating cells. It has become evident that ERα is upregulated in luminal mammary epithelial cells during early stages of tumorigenesis and its overexpression is an important stimulatory factor for the proliferation of mammary cells, leading to cell division and eventually to tumor development. The obvious role of ERα signaling in orchestrating the expression of genes involved in growth-related pathways has established ERα as an important therapeutic target in breast cancer treatment (4). However, our understanding of the molecular mechanisms underlying deregulation of this signaling pathway is scarce.

MicroRNAs (miRNA) are endogenous small noncoding RNAs of 20 to 23 nucleotides, which are involved in post-transcriptional control of gene expression (5). Due to their sequence complementarities to the 3′ untranslated region (UTR) of many mRNAs, miRNAs are able to recognize target transcripts and promote translation inhibition or mRNA destabilization and degradation, both resulting in reduced expression of target genes (6). miRNAs are assumed to directly control the expression of a large portion of the human genome and are thus involved in the regulation of major cellular activities, such as metabolism, differentiation, proliferation, and apoptosis (6, 7). The observations that all these processes are altered in cancer (8) and that miRNA expression is deregulated in a variety of cancer types (9) suggest that miRNA expression has a profound influence on carcinogenesis.

We hypothesized that miRNAs might play an important role in the upregulation of ERα in breast cancer. We

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identified an upregulated miRNA in ERα-positive breast cancer cells that was able to enhance ERα signaling activity through the regulation of its target, RASD1. We also show that the level of miR-375 expression in breast cell lines was dependent on epigenetic marks adjacent to its coding region. Therefore, our study brings significant insight into our knowledge of the mechanisms underlying ERα deregulation in breast cancer.

Materials and Methods

Cell culture and transfection

The BT474, ZR7530, T47D, MCF-10A, MCF-12A, and SK-BR3 cell lines were obtained from the American Type Culture Collection, where they are regularly verified by genotypic and phenotypic tests. The HEK293T, MCF-7, MDA-MB-231, MDA-MB-435, and HDQ-P1 cell lines were provided by Prof. Lichter (German Cancer Research Center, Heidelberg, Germany) and were authenticated by short tandem repeat profiling analysis. Following resuscitation and every six months, cell lines were tested for the presence of contamination using multiplex cell contamination test provided by the German Cancer Research Center (DKFZ) core facility (10). The expression status of ERα in the cell lines was confirmed by immunoblotting before they were used in the experiments. Cells were cultured under standard conditions. Before experimental use, MCF-7 cells were grown for 96 hours in phenol red-free DMEM with 4.5 g/L D-glucose (Invitrogen) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum prepared as described previously (11). Transfection with siRNAs and pre- and anti-miR was performed using siPORT NeoFX (Applied Biosystems) following the supplier's protocol. Plasmid transfection was performed with Effectene (Qiagen) as specified by the manufacturer.

Array-based miRNA profiling

RNA was extracted using the miRNeasy kit (Qiagen). miRNA profiles were generated by using the Geniom Biochip miRNA and RT Analyzer (febit). The array contained seven replicates of each human miRNA as annotated in the Sanger miRBase 11.0. Briefly, 3 μg of total RNA containing small RNAs were labeled using the FlashTag RNA kit (Genisphere). Array hybridization and washing procedures were performed in the RT Analyzer device as recommended by the supplier and signal intensities were calculated using the Geniom Wizard Software (febit). All further statistical analyses were carried out using R. Following background correction, the seven replicate intensity values of each miRNA were summarized by their median value. To normalize the data of different arrays, the variance stabilizing normalization (12) was applied by the R "vsn" package, such that the miRNA profiles were homoscedastic. This normalization transformed the background subtracted raw data, ensuring that the variance was almost constant. Differentially expressed miRNAs between cell line models were identified by using the *t* test procedure within significance analysis of microarrays (13).

Immunoblots

Primary antibodies against ERα (NCL-L-6F11, Novocastra), actin (20-33, Sigma), and horseradish peroxidase–conjugated secondary antibodies were used as previously described (14).

Cell proliferation and apoptosis assays

Cell proliferation and apoptosis were measured using Cell TiterGlo Luminescent Cell Viability and Caspase-Glo 3/7 assays (Promega), respectively, following the manufacturer's instructions. RNA transfections were carried out in a 96-well plate (6×10^3 cells/well) in a final RNA concentration of 100 nmol/L per well in five replicates. For cell counting, 72 hours posttransfection of cells in 6-well plates (3×10^5 cells/well), the cells were trypsinized and living cells were counted by a cell viability analyzer (Beckman Coulter).

Estrogen responsive element Firefly luciferase reporter gene assay

MCF-7 cells were reverse transfected in five replicates with pre-miRs and anti-miRs. After 24 hours, cells were cotransfected with Firefly and Renilla luciferase reporters. Twenty-four hours later, reporter activities were assayed with the Dual Luciferase Reporter Assay System (Promega). Firefly activity was normalized to the Renilla signals.

Bisulfite sequencing

Genomic DNA was extracted using the AllPrep DNA/RNA Kit (Qiagen). One microgram total genomic DNA was treated with sodium bisulfite using the EpiTect Kit (Qiagen). CpG islands were amplified from the bisulfite-converted DNA by PCR. Amplicons were cloned and sequenced. The quality of the bisulfite-converted sequences was analyzed with the BiQAnalyzer software (15).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (16). Antibodies specific for H3K9me2 (ab1220, Abcam) and H3K4me2 (07-030, Upstate), acetylated H3 (06-598, Upstate) and H4 (06-599, Upstate), ZEB1 (H-102, sc25388X, Santa Cruz), polymerase II (4H8, ab5408, Abcam), CTCF (ab70303, Abcam), and ERα (HC-20, sc543X, Santa Cruz) were purchased from the indicated suppliers. Immunoprecipitates were eluted into 25 μL of TE buffer [10 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA]. One microliter of the DNA was used for a 10 μL PCR reaction using the Absolute QPCR SYBR Green Mix (Thermo Scientific) and a Roche LightCycler 480. Enrichments were calculated as percentage of the input.

Patient samples

Normal breast and tumor samples were obtained with the informed consent of patients after approval of the Institutional Review Board at Tehran University of Medical Sciences, Shahid Beheshti University of Medical Sciences, and University of Welfare Sciences and Rehabilitation, Tehran, Iran. Clinical information of patients is provided in Supplementary Table S2. For simplification purposes, the

Table 1. Differentially expressed miRNAs in mammary cell lines

ERα+ compared with ERα- cancer cells		ERα+ compared with noncancer cells	
miRNA	**Fold-change***	**miRNA**	**Fold-change***
	5% most upregulated		
hsa-miR-203	7.4	hsa-miR-200a	7.7
hsa-miR-375	**5.4**	**hsa-miR-375**	**6.9**
hsa-miR-205	4.6	hsa-miR-200b	5.4
hsa-miR-148a	4.3	hsa-miR-203	4.9
hsa-miR-615-3p	4.0	hsa-miR-200b*	4.3
hsa-miR-196a	3.9	hsa-miR-196a	4.2
hsa-miR-200c	2.9	hsa-miR-615-3p	3.5
hsa-miR-421	2.8	hsa-miR-429	3.5
	5% most downregulated		
hsa-miR-146b-5p	–3.0	hsa-miR-34c-5p	–3.7
hsa-miR-29a	–3.3	hsa-miR-29a	–3.9
hsa-miR-31*	–4.0	hsa-miR-146b-5p	–4.5
hsa-miR-146a	–4.9	hsa-miR-224	–5.3
hsa-miR-155	–6.4	hsa-miR-31*	–6.5
hsa-miR-31	–6.7	hsa-miR-221	–8.6
hsa-miR-221	–8.2	hsa-miR-31	–9.3
hsa-miR-222	–9.7	hsa-miR-222	–10.3

*Fold-change is log transformed (base 2).

sample diagnosed with fibrocystic changes (tumor 7) is referred to as a tumor in the main text and figures.

Gene expression profiling

Gene expression profiling was performed using Human Sentrix-6 v2 BeadChip arrays (Illumina). Microarray hybridization, scanning, and data analysis are described in the Supplementary Data.

Luciferase reporter assay for miRNA target identification

HEK293T and MCF-7 cells were reverse transfected in five replicates with synthetic RNAs in a final concentration of 50 nmol/L. After 24 hours, cells were cotransfected with 50 ng RASD1 Firefly luciferase and 10 ng Actin-RL Renilla luciferase reporter constructs.[12] Luciferase activities were measured 24 hours later using the Dual Luciferase Reporter Assay System (Promega). Firefly activity was normalized to Renilla signal.

Quantitative reverse transcriptase-PCR

To avoid contamination by genomic DNA, 1 μg total RNA was subjected to *DNase I* digestion (1 U/μL; amplification grade DNase I, Invitrogen) for 10 minutes at 25°C, followed by heat inactivation at 75°C for 5 minutes. First-strand cDNA-synthesis and quantitative PCR were performed as previously described (17). ERα, RASD1, and GAPDH primers were provided by QuantiTect Primer Assays (Qiagen).

Quantitative reverse transcription-PCR (qRT-PCR) analysis of miRNAs was performed using TaqMan MicroRNA Reverse Transcription Kit and TaqMan gene-specific MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. All measurements were performed in triplicate. The expression of miR-375 was normalized to RNU6B and RNU66.

Statistical analyses

Unless otherwise noted, data are presented as mean ± SE from three to five independent experiments. Student's *t*-test was used for comparisons.

Results

Reciprocal regulation between miR-375 and ERα

In an initial attempt to identify miRNAs involved in the regulation of ERα pathway, we performed miRNA profiling of eight human mammary cell lines (Supplementary Table S1). We compared the miRNA expression profile of ERα-positive to ERα-negative cell lines as well as to nontumorigenic immortalized cells (Table 1). As expected, miR-221 and miR-222, both reported as negative modulators of ERα activity (18), were found among the most significantly downregulated miRNAs. As we aimed at the identification of miRNAs that positively regulate ERα activity, we looked for upregulated miRNAs in ERα-positive cell lines. Strikingly,

[12] D. Nickles and M. Boutros, unpublished.

miR-375 was identified as the second most significantly upregulated miRNA in ERα-positive cells when compared with both ERα-negative and nontumorigenic cell lines (Table 1). The specific overexpression of miR-375 in ERα-positive breast cancer cells was further validated by real-time PCR analysis, which included additional ERα-positive cell lines (Fig. 1A).

To assess a possible role of miR-375 in ERα signaling, we tested the effect of its ectopic expression in MCF-7 cells transiently transfected with an estrogen responsive element (ERE)-controlled Firefly luciferase vector. Overexpression of miR-375 resulted in a >2-fold induction of luciferase activity, whereas its inhibition resulted in decreased ERα activity (Fig. 1B). Similarly, ERα protein levels decreased after diminishing the level of endogenous miR-375 with a synthetic anti-miR (Fig. 1C).

Given the high endogenous level of miR-375 in MCF-7 cells, we sought to evaluate the potential contribution of miR-375 to the proliferation of these cells. We therefore blocked miR-375 activity with anti-miR-375 in MCF-7 breast cancer cells. Compared with the control experiments (transfection with anti-miR-control), cell proliferation decreased in miR-375–inhibited cells to almost 50%, 72 hours after transfection (Fig. 1D and E). However, inhibition of miR-375 did not result in an induction of caspase activation (Fig. 1F), suggesting that the antiproliferative effect of miR-375 inhibition is not due to the induction of apoptosis.

Interestingly, we found that miR-375 expression was also dependent on the expression of ERα, as transfection with ERα siRNA led to a 50% decrease in the expression level of miR-375 (Fig. 1G). Therefore, our data indicate a reciprocal regulatory connection between miR-375 and ERα.

Epigenetic marks determine the transcriptional state of the miR-375 locus

We next looked for the mechanisms regulating miR-375 expression. Analyzing the genomic region spanning the *miR-375* gene, we identified two large CpG-rich regions (CpG islands; Fig. 2A). The expression of genes (including miRNA genes) possessing CpG islands in the vicinity of their

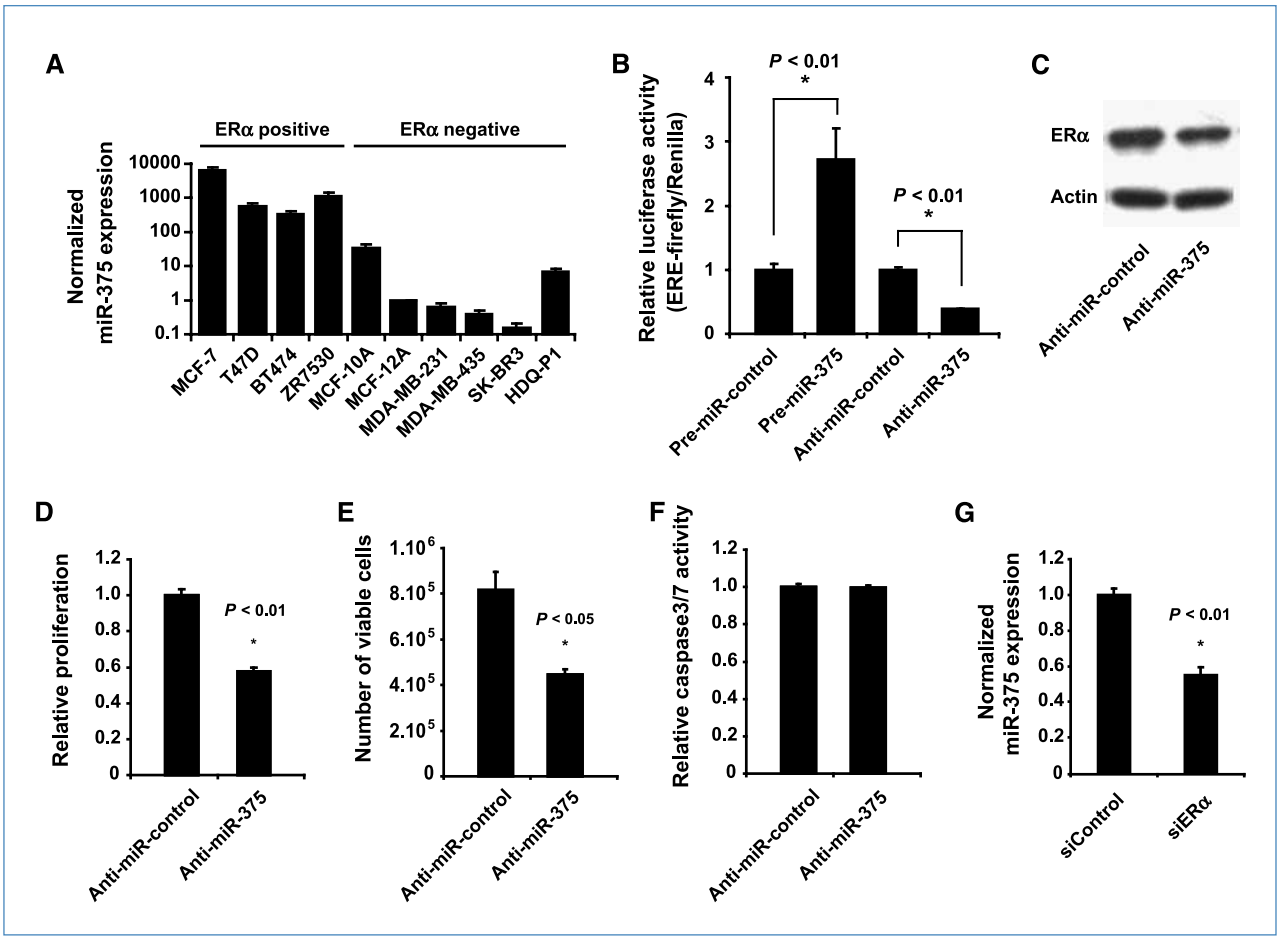

Figure 1. Reciprocal regulation between miR-375 and ERα and the effect of miR-375 on proliferation of MCF-7 cells. A, miR-375 expression in breast cell lines measured by qRT-PCR. The results are presented as mean of three measurements ± SD. B and C, effect of miR-375 modulation on ERα transcriptional activity (B) and protein expression in MCF-7 cells (C). D, proliferation of MCF-7 cells after inhibition of miR-375. Cell proliferation was measured using Cell TiterGlo Luminescent Cell Viability and using a cell viability analyzer (E). F, induction of apoptosis was assayed by Caspase-Glo 3/7 assay. Cell proliferation and apoptosis were measured in five replicates. G, effect of ERα knockdown on the expression of miR-375 in MCF-7 cells measured by qRT-PCR. Values are presented as mean of three measurements ± SD.

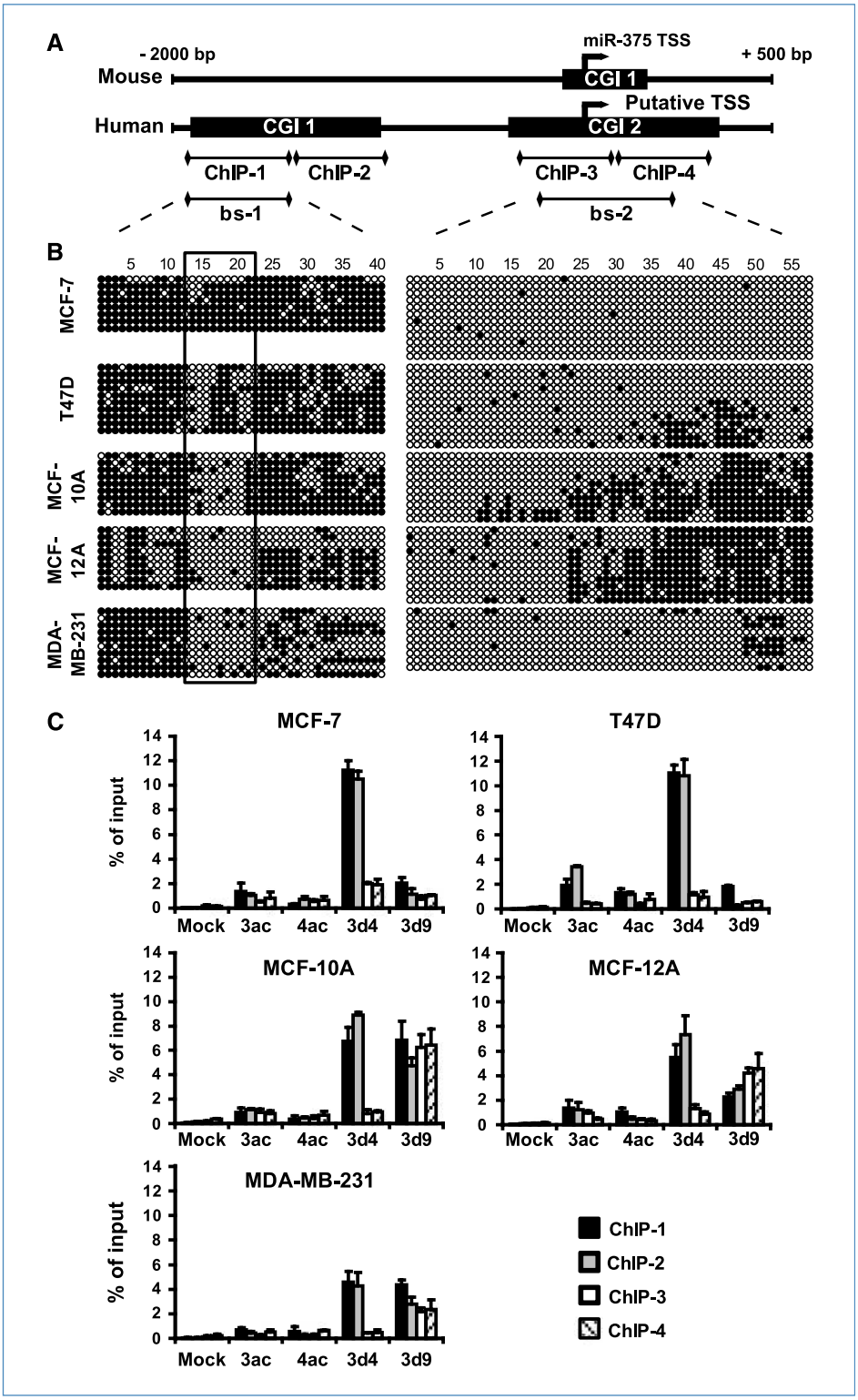

Figure 2. Epigenetic marks determine the transcriptional state of the *miR-375* locus. A, comparison of the *miR-375* locus in human and mouse. CpG-rich regions (CGI 1 and 2) are shown. Arrows, transcription start site (TSS) of miR-375. The locations of two primer pairs used for bisulfite sequencing (bs-1 and bs-2) and the four primer pairs employed for the ChIP analysis (ChIP-1, ChIP-2, ChIP-3, ChIP-4) are depicted. B, bisulfite sequencing of the CGIs in five breast cell lines. Black and open circles, methylated and unmethylated CpGs, respectively. Quadrangle, region with specific hypermethylation in MCF-7 and T47D cells. C, ChIP analysis of the *miR-375* locus in cell lines. Cross-linked chromatin of each cell line was immunoprecipitated with antibodies specific for acetylated histone H3 (3ac), acetylated histone H4 (4ac), dimethylated lysine 4 of histone H3 (3d4), and dimethylated lysine 9 of histone H3 (3d9). Purified DNA was amplified with the four ChIP primer pairs (see A). Results are shown as percentage of the input (normalized against input). Diagrams show the results of three independent experiments ± SD.

transcription start site tends to correlate with epigenetic marks (such as DNA methylation patterns) at these islands (19, 20). Therefore, we investigated the epigenetic regulation of the *miR-375* locus. The more distal CpG island (CpG island 1; CGI 1) has a size of approximately 700 bp. A second CpG island (CpG island 2; CGI 2) spans approximately 850 bp and contains at its most distal part a region homologous to the *miR-375* promoter identified in mouse (ref. 21; Fig. 2A and

Supplementary Fig. S1). We analyzed the epigenetic modification pattern of the miR-375 locus in MCF-7 and T47D cells (cells with high miR-375 expression), as well as in MCF-10A, MCF-12A, and MDA-MB-231 cells (cells with low miR-375 expression). Bisulfite sequencing results showed that CGI 1 is methylated in the cell lines showing high expression of miR-375, whereas MCF-10A, MCF-12A, and MDA-MB-231 cells showed specific hypomethylation in the distal part of this region (Fig. 2B). In contrast, CGI 2 was mostly unmethylated in MCF-7, T47D, and MDA-MB-231 cells, whereas MCF-10A and MCF-12A showed strong DNA methylation in the proximal part of the region (Fig. 2B).

To characterize the chromatin state of the *miR-375* locus, we employed four different antibodies recognizing distinct covalent histone modifications in a ChIP experiment. ChIP analysis revealed a peak of histone H3 dimethylated at lysine 4 (H3K4me2), a marker of active transcription, in the CGI 1 in all cell lines analyzed (Fig. 2C). Repressive histone H3 lysine 9 dimethylation (H3K9me2) was found throughout the locus in the three cell lines with low miR-375 expression, whereas H3K9me2 levels were found to be low in both CGIs in MCF-7 and T47D. H3 and H4 acetylation, a marker of active transcription, was generally low and was only slightly enriched in CGI 1 in T47D cells (Fig. 2C).

Together, these findings led us to conclude that an active miR-375 epiallele is characterized by a fully methylated CGI 1, an unmethylated CGI 2 spanning the gene body, H3K4me2 enrichment in the CGI 1, and low overall H3K9me2 levels. The repressed epiallele is characterized by local hypomethylation around CpG 18 of the CGI 1 (see box in Fig. 2B and Supplementary Fig. S1), a methylated gene body (with the exception of the MDA-MB-231 cell line), H3K4me2 enrichment in the CGI 1, and overall high levels of H3K9me2. These results suggest that H3K9 methylation is a major repressive mark of the *miR-375* locus.

Transcriptional repressors bind to the *miR-375* locus

The bisulfite sequencing data indicated that one feature of the repressed epiallele of miR-375 is local hypomethylation around CpG 18 of CGI 1, which in the case of MCF-12A and MDA-MB-231 cells also became detectable throughout the CGI (Fig. 2B). Analysis of the miR-375 locus with MatInspector software (22) revealed the presence of consensus binding sites for the CCCTC-binding factor (CTCF) protein in this locus and especially in the hypomethylated region (Supplementary Fig. S1). CTCF is a highly conserved multifunctional zinc finger protein involved in transcriptional repression and activation, insulation, imprinting, and X-inactivation that binds preferentially to unmethylated DNA (23, 24). CTCF is a very widely expressed factor that is abundant in many breast cancer cell lines, including MDA-MB-231 and MCF-7, but also in nontumorigenic breast cell lines like MCF-12A (25). Moreover, we identified several Z- and E-boxes that are potential binding sites for ZEB1, a transcriptional repressor that has been found to be involved in the regulation of several cancer-associated genes (refs. 26, 27; Supplementary Fig. S1). ZEB1 has been described to be expressed in MCF-10A and MDA-MB-231 cells; however, almost no expression was reported for the MCF-7 and T47D cell lines (26). We therefore performed ChIP with antibodies against CTCF and ZEB1 in MCF-7, MCF-12A, and MDA-MB-231 cells. ChIP was also performed with antibodies against ERα and the largest subunit of RNA polymerase II to obtain information about ERα-binding and active or paused transcription events. We confirmed the binding of CTCF not only to the predicted binding site around CpG 18 (ChIP-1) in MCF-12A cells but also to sites in the proximal region of CGI 1 (ChIP-2) and in CGI 2 (ChIP-3 and ChIP-4; Fig. 3). In MDA-MB-231 cells, a similar pattern was found, although peak binding was observed in all but the region amplified by the ChIP-1 primers. In contrast, MCF-7 cells showed a weak enrichment for CTCF-immunoprecipitated DNA only in the proximal region of CGI 1. These results suggest that CTCF regulates the miR-375 locus by interacting with several hypomethylated binding sites, creating a higher-order chromatin structure that prevents active transcription. RNA polymerase II (POLII) was detected in most regions with CTCF enrichment, presumably representing paused polymerase molecules interacting with CTCF (Fig. 3). Consistently, low levels of RNA POLII were detected in the miR-375 coding region (ChIP-4) in MCF-12A and MDA-MB-231 cells. ZEB1 binding was restricted to CGI 2 in the cell lines with low miR-375 levels, which correlates well with the presence of E-boxes in the ChIP-3 region (Fig. 3 and Supplementary Fig. S1). We found no ZEB1 binding in MCF-7 cells but very prominent peaks of ERα and RNA POLII in the miR-375 coding region (ChIP-4), adjacent to the putative miRNA promoter (Figs. 3 and 2A). Collectively, these findings support a role of CTCF and ZEB1 in the repression and ERα in the activation of miR-375 expression. The binding of ERα to the putative miRNA promoter further supports our preceding findings on a key role of ERα in miR-375 overexpression in MCF-7 cells and indicates the existence of a positive feedback regulation between these molecules.

RASD1 is a functional target of miR-375 and negatively regulates ERα

We expanded our functional analyses by measuring the expression of miR-375 in nine pairs of primary breast carcinomas and adjacent normal tissues from breast cancer patients using quantitative real-time PCR. Although not specific to ERα-positive tumors (Supplementary Table S2), miR-375 was upregulated (up to 150-fold) in seven of nine analyzed tumors (Fig. 4A). To identify miR-375 targets, mRNA expression profiles of tumor and normal breast tissue specimens of four patients showing differential miR-375 expression were analyzed by microarrays. We identified 125 genes commonly downregulated in tumors overexpressing miR-375 (Supplementary Table S3). In parallel, 144 potential miR-375 targets were predicted using the TargetScan algorithm (28). Combining microarray profiling and target prediction data, we identified two genes, Ras dexamethasone-induced 1 (*RASD1*) and early B-cell factor 3 (*EBF3*), as potential miR-375 targets (Fig. 4B). We cloned segments of the 3′UTRs of both genes into luciferase reporter vectors and performed luciferase assays upon overexpression and inhibition of miR-375 in HEK293T cells (that do not express endogenous miR-375)

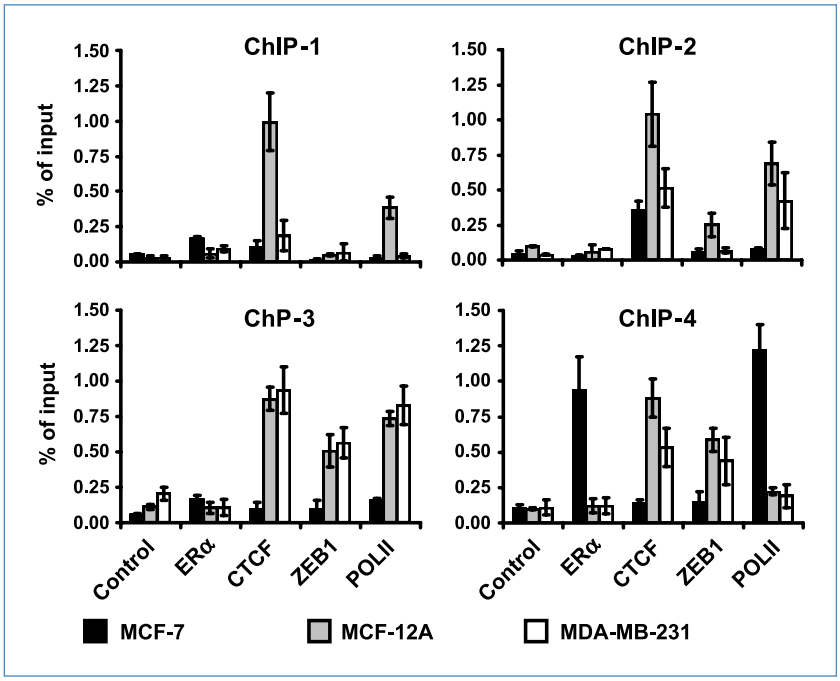

Figure 3. Transcriptional repressors bind to the *miR-375* locus. Cross-linked chromatin was immunoprecipitated with antibodies specific for ERα, CTCF, ZEB1, and RNA POLII. Purified DNA was amplified with the four ChIP primer pairs (see Fig. 2A). Results are shown as percentage of the input (normalized against input). Diagrams show the results of three independent experiments ± SD.

and in MCF-7 cells, respectively. The *EBF3* luciferase construct showed no sensitivity to miR-375 (data not shown). In contrast, reporter assays for RASD1 showed significant changes in luciferase activity (Fig. 4C). The observation that modulation of miR-375 caused consistent expression changes in the *RASD1*-luciferase construct in both cell lines strongly suggests that RASD1 is a functional target of miR-375. Because of the lack of appropriate antibodies, the effect of miR-375 on RASD1 protein levels could not be evaluated. However, inhibition of miR-375 in MCF-7 cells resulted in >2.5-fold induction in *RASD1* mRNA levels, as measured by qRT-PCR (Fig. 4D).

It has been reported that RASD1 is able to suppress the growth of breast cancer cells and to inhibit clonogenic growth of MCF-7 cells (29). This fact, combined with our preceding findings, led us to hypothesize that RASD1 could function as a negative regulator of ERα. Therefore, we performed RASD1 loss- and gain-of-function experiments in MCF-7 cells and analyzed the effect on ERα protein. Whereas silencing of RASD1 by specific siRNAs gave rise to increased ERα levels, overexpression of RASD1 had the opposite effect and led to downregulation of ERα (Fig. 4E). These effects were further confirmed by qRT-PCR (Fig. 4F), in which overexpression of RASD1 resulted in downregulation of *ERα* mRNA levels. In contrast, silencing of RASD1 resulted in increased *ERα* mRNA levels in a manner similar to that observed for protein levels. Together, these observations provided evidence for a negative regulation of ERα by the miR-375 target RASD1.

Discussion

Emerging evidence emphasizes a fundamental role for miRNAs in different steps of tumor formation and progression. In the current study, we show that miR-375 functions as an activator for ERα signaling in breast cancer cells and its inhibition gives rise to an attenuated ERα activity and eventually decreased cell proliferation.

Interestingly, miR-375 was not identified as a breast cancer–associated miRNA in previous studies analyzing miRNA expression patterns. In some studies, this was due to the fact that miR-375 was not present on the microarrays (30, 31). In other reports, the experimental settings and the parameters applied in the data analysis did not identify a differential expression of miR-375 between breast cancer cell lines (32, 33). Notably, our screening revealed that miR-375 was overexpressed specifically in ERα-positive breast cancer cells. To date, only a limited number of miRNAs with a regulative connection to the ERα pathway have been discovered (18, 34–37). All these miRNAs act as inhibitors of ERα signaling pathways. In contrast, we identified miR-375 as the first miRNA with the capacity to enhance ERα signaling in breast cells and, thus, to promote cell proliferation. In agreement with the proliferative activity of miR-375, Poy and colleagues reported on an impaired proliferation of pancreatic β-cells in miR-375 knockout mice (38). miR-375 was also identified as a potential marker for cell proliferation in a study of miRNA profiling of patients with Barrett's-associated adenocarcinoma (39). We also observed overexpression of miR-375 in ERα-negative primary tumors. These observations suggest that miR-375 might modulate cell proliferation through other mechanisms than ERα signaling, as well. Indeed, the activity of miR-375 seems strongly dependent on the cellular context, as its overexpression in gastric carcinoma cells led to a decreased number of viable cells by induction of apoptosis (40). More work is required to dissect in even more detail the molecular networks, which are influenced by miR-375.

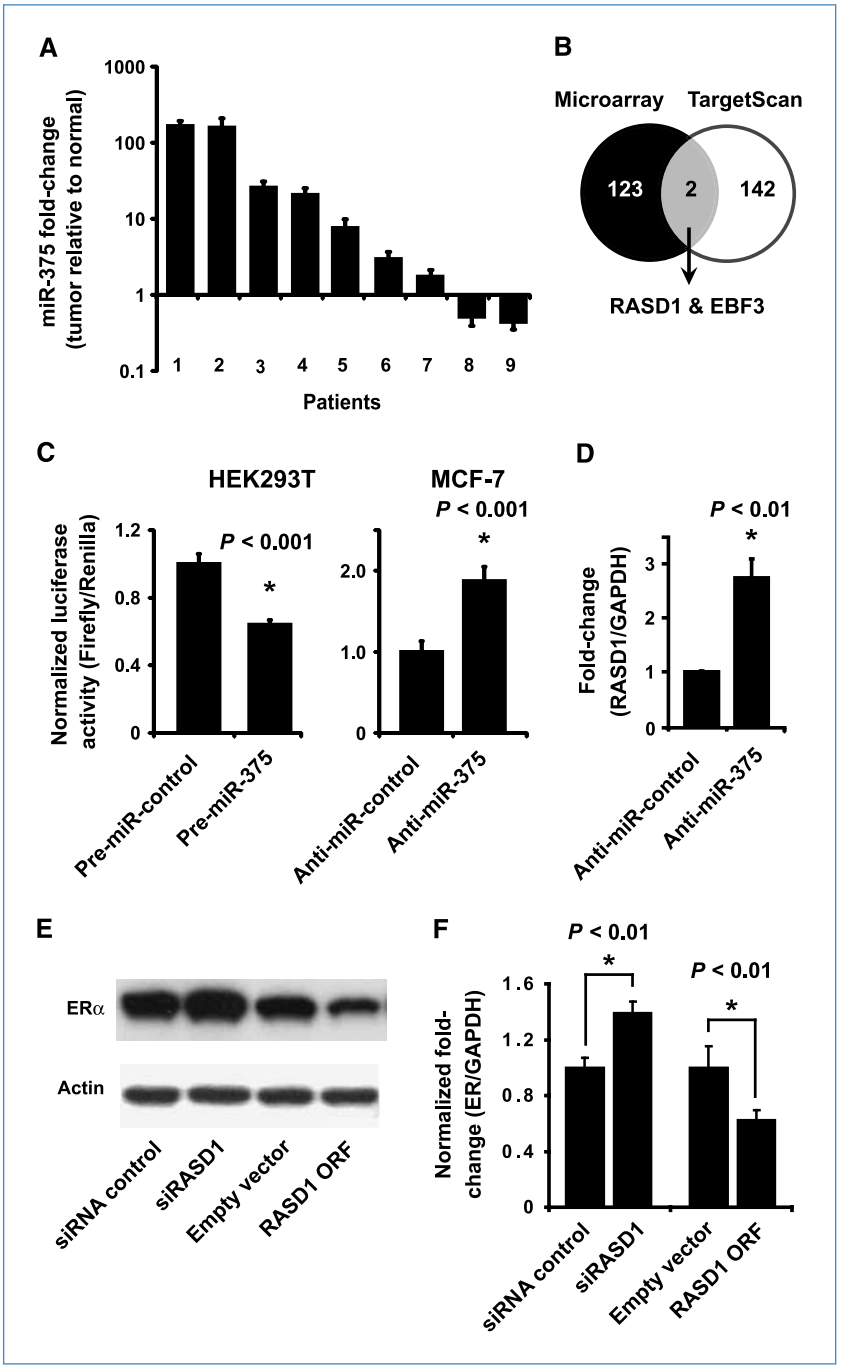

Figure 4. RASD1 is a functional target of miR-375 and negatively regulates ERα. A, miR-375 expression in breast cancer patients measured by qRT-PCR. Values are represented as the ratio of miR-375 expression in tumors versus matched normal tissues. B, comparison between 125 genes commonly downregulated in patients 1, 2, 6, 7 (who showed overexpression of miR-375), and 144 predicted targets of miR-375. C, relative luciferase activity in HEK293T and MCF-7 cells transfected with the *RASD1* 3′ UTR reporter construct. Luciferase activity was measured in five replicates in three independent experiments. D, RASD1 expression in MCF-7 cells after transfection with anti-miR-375 measured by qRT-PCR. Values are presented as mean of three measurements ± SD. E and F, effect of RASD1 silencing and overexpression on ERα protein (E) and mRNA levels (F). Values are presented as mean of three measurements ± SD.

miRNA genes have been previously described to be epigenetically regulated by DNA methylation (19). We found that DNA methylation patterns in a distal part of CGI 1, which contains binding sites for the insulator protein CTCF, are crucial for the expression of miR-375. We suggest that hypomethylation of this region is necessary for CTCF recruitment and subsequent silencing of the *miR-375* locus. CTCF binding also correlated with the repressive mark H3K9me2, which was found throughout the locus in cells with low miR-375 expression. Additionally, we detected the binding of the transcriptional repressor ZEB1, which has been reported to regulate the expression of the miR-200 family (41), at a region encompassing the putative *miR-375* promoter. Thus, our data indicate the presence of a repressive chromatin structure at the miRNA locus in cells with low miR-375 expression. Such a repressive structure would either directly prevent transcription or inhibit the interaction of the *miR-375* promoter region with activating factors and enhancer elements

(insulation). However, the *miR-375* locus maintains the potential to be reactivated, as indicated by a peak of H3K4me2 on the distal part of CGI 1 in cell lines with a silenced locus.

A role of CTCF as tumor suppressor has previously been suggested, as CTCF has been found to inhibit cell growth and induce cell cycle arrest. CTCF has a repressive role in the regulation of several prominent oncogenes and also seems to be important in preventing epigenetic silencing of growth suppressor genes (24). Our findings are in agreement with recent data showing that CTCF confines estrogen receptor action on a genome-wide scale (42). Furthermore, the CTCF antagonist BORIS, which seems to interact in a methylation-independent way with CTCF-binding sites (43), has been found to be aberrantly expressed in breast tumors, which correlated in many cases with overexpression of ERα (44).

Previous studies have described a negative feedback loop between ERα and several miRNAs that are induced upon estrogenic stimulation and that downregulate ERα (45, 46). The present work suggests the existence of a positive loop between ERα and miR-375. We showed that ERα binds near the putative promoter of miR-375 and silencing of ERα by siRNAs diminished the expression of miR-375 in MCF-7 cells. The modulation of ERα activity by miR-375 is achieved through the repression of RASD1, which has been reported as an antiproliferative factor in MCF-7 cells (29). Our data suggest that RASD1 interferes with the proliferation of MCF-7 cells through the downregulation of ERα. Inhibition of miR-375 in ERα-positive breast cancers may be a promising strategy for clinical therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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