miR-200a Regulates SIRT1 Expression and Epithelial to Mesenchymal Transition (EMT)-like Transformation in Mammary Epithelial Cells*

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Evidence supports a critical role for microRNAs (miRNAs) in regulation of tissue-specific differentiation and development. Signifying a disruption of these programs, expression profiling has revealed extensive miRNA dysregulation in tumors compared with healthy tissue. The miR-200 family has been established as a key regulator of epithelial phenotype and, as such, is deeply involved in epithelial to mesenchymal transition (EMT) processes in breast cancer. However, the effects of the miR-200 family on transformation of normal mammary epithelial cells have yet to be fully characterized. By examining a TGF-β-driven model of transformation of normal mammary epithelium, we demonstrate that the class III histone deacetylase silent information regulator 1 (SIRT1), a proposed oncoprotein in breast cancer, is overexpressed upon EMT-like transformation and that epigenetic silencing of miR-200a contributes at least in part to the overexpression of SIRT1. We have established the SIRT1 transcript as subject to regulation by miR-200a, through miR-200a targeting of SIRT1 3'-UTR. We also observed SIRT1 and miR-200a participation in a negative feedback regulatory loop. Restoration of miR-200a or the knockdown of SIRT1 prevented transformation of normal mammary epithelial cells evidenced by decreased anchorage-independent growth and decreased cell migration. Finally, we observed SIRT1 overexpression in association with decreased miR-200a in breast cancer patient samples. These observations provide further evidence for a critical tumor suppressive role of the miR-200 family in breast epithelium in addition to identifying a novel regulatory mechanism, which may contribute to SIRT1 up-regulation in breast cancer.

SIRT1 is a class III histone deacetylase and plays important roles in aging, obesity, and cancer (1, 2). Dramatic up-regulation of SIRT1 has been observed in various cancers including breast, prostate, and ovarian cancers, implicating a role for SIRT1 in tumorigenesis (3–5). SIRT1 functions by deacetylating histone (e.g. H3-Lys9 and H4-Lys16) and non-histone proteins (e.g. p300 and Ku70) in an NAD+-dependent manner, thus modifying gene expression and modulating protein activity (1, 6). Previous studies have illustrated several mechanisms of SIRT1-dependent gene silencing in addition to histone deacetylation. It was shown that at sites of DNA damage, SIRT1 recruits DNA methyltransferases (DNMTs) to promoter regions leading to hypermethylation and potential silencing of tumor suppressor genes (e.g. E-cadherin) (7). It is also known that SIRT1 facilitates transcriptional repression of tumor suppressor genes by modulating histone methyltransferase SUV39h1, the key enzyme responsible for histone H3 methyltransferase (H3-Lys9-me3) in regions of heterochromatin (8). SIRT1 induction of tumor suppressor gene silencing promotes the initiation and progression of tumors as well as drug resistance (1, 9, 10). Studies from our laboratory and others show that inhibition of SIRT1 by pharmacological inhibitors or genetic depletion reduces estrogen-dependent signaling pathways in breast cancer cells (11, 12). The inhibition of SIRT1 in breast and prostate cancer cell lines has resulted in acetylation of p53 and subsequent growth arrest and apoptosis, while not affecting viability of several non-cancer epithelial cell lines (13, 14). Although several inhibitors of sirtuins have been described (reviewed in Ref. 15), and the potential value that SIRT1 inhibition may possess for cancer therapy has been recognized, there are no ongoing clinical trials of SIRT1 inhibitors for cancer therapy because of serious concerns, e.g. stability and toxicity. These deficiencies have lead to the search for new molecules that regulate SIRT1 expression.

SIRT1 expression can be mediated at the transcriptional level and several mechanisms involved in dysregulation of SIRT1 in cancer cells have been proposed (16). Tumor suppressors p53 and HIC1 (hypermethylated in cancer 1) can bind to the SIRT1 promoter and form a complex with SIRT1, leading to inhibition of SIRT1 transcription (17, 18). In cancer cells, inactivation of these tumor suppressor genes by genetic or epigenetic mechanisms leads to up-regulation of SIRT1 transcription. However, this is not the sole mechanism for overexpression of SIRT1 in tumors. For example, the RNA binding protein HuR, a potential oncoprotein, stabilizes SIRT1 mRNA through 3'-untranslated region (3'-UTR) interactions leading to elevated SIRT1 levels (19). This suggests that the 3'-UTR of SIRT1 mRNA may also be important in governing SIRT1 expression in tumors.

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miRNAs are small non-coding RNAs (18~24 nucleotides) that elicit their regulatory effects by base pairing with specific sequences (miRNA response element) in the 3'-UTR of target mRNAs causing degradation of mRNA or inhibition of translation (20). Differential expression of miRNAs with associated oncogenic or tumor suppressive roles has been highlighted in tumorigenesis by many recent reports (21–23). These cancer-related aberrant miRNA levels may result from copy number variation (24), defects in processing (24), or epigenetic silencing (25).

Several miRNAs, including miR-34a, miR-132, and miR-199a, directly bind to the 3'-UTR of SIRT1 mRNA leading to suppression of SIRT1 expression in colon, adipocyte, and cardiac tissues, respectively (26–28). However, array-based miRNA profiling suggests that these miRNAs may not be expressed in mammary epithelial cells or differentially expressed in breast tumors (29–31), indicating that any miRNAs that regulate SIRT1 expression may do so in a tissue-dependent manner.

The miR-200 family has been highlighted for its importance in tumor progression and metastasis, specifically in the maintenance of the epithelial phenotype. The miR-200 family consists of two clusters: miR-200b, miR-200a, and miR-429 located on chromosome 1; and miR-200c and miR-141 located on chromosome 12. Both clusters are encoded as polycistronic transcripts and contain predicted CpG islands in their promoters (32). ZEB1 and SIP1, transcriptional repressors of E-cadherin and inducers of EMT (33, 34), have been thoroughly investigated as targets of miR-200 family members. As such, enforced expression of miR-200 family members prevented TGF-β induced EMT, whereas inhibition of the miR-200 family resulted in EMT (35). It is known that in several mesenchymal-phenotype breast cancer cell lines, the miR-200 family has been found down-regulated (35). In breast cancer, in addition to targeting ZEB1 and SIP1, miR-200 family has been shown to target phospholipase C-γ1 and BMI1, reducing EGF-driven motility and cancer stem cell self-renewal, respectively (36, 37).

Levels of miR-200 family members are regulated by genetic and/or epigenetic mechanisms. miR-200c is suppressed transcriptionally by ZEB1/SIP1, creating negative feedback loops that act in switch-like manners to regulate EMT/Mesenchymal to Epithelial Transition (MET)-like processes (32, 38). Also, loss or reduced expression of miR-200c in breast cancer cell lines has been attributed to down-regulation of Dicer, a key component of miRNA processing machinery (39). In addition to these genetic mechanisms, miR-200c/miR-141 silencing was shown to be associated with hypermethylation of promoter CpG islands in several aggressive, mesenchymal-phenotype breast and prostate cancer cell lines (40).

Our present work uses an in vitro model of EMT-like transformation to tackle the questions of how normal mammary epithelial cells undergo transformation and whether SIRT1 overexpression is present in such transformations. We identify that loss of miR-200a expression is associated with transformation and responsible in part for SIRT1 overexpression. This verifies another oncogenesis-related target in the miR-200 repertoire, further expanding the miR-200 tumor-suppressive role through regulation of SIRT1 in mammary epithelium.

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**EXPERIMENTAL PROCEDURES**

**Cell Culture—**MDA-MB-231 cells were maintained in DMEM with 5% FBS and 1% glutamine (Invitrogen). Cells were grown at 37 °C in an atmosphere containing 5% CO₂. Human mammary epithelial cells (HME, Lonza; Walkersville, MD) were maintained in mammary epithelial cell growth medium with bovine pituitary extract, GA-1000 (gentamicin sulfate amphoterin-cin-B), recombinant human EGF, hydrocortisone, and insulin. HME-T cells were also grown in the presence of 10% FBS. TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Drug treatments included SAHA (BioVision; Mountain View, CA) and 5-azacytidine (Sigma).

**miRNA Screening and mRNA/miRNA Expression Analysis—**Cell line total RNA was extracted with TRIzol reagent (Invitrogen). Small RNA was converted to complimentary DNA using poly-A-polymerase based First-Strand Synthesis kit (SABiosciences; Flat Lake, MD). Total RNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen). MiRNA expression was screened by qRT-PCR for an 88-miR panel, human miFinder array (SABiosciences). Subsequent miRNA analysis was performed by real-time qRT-PCR with miRNA (miR-200a, miR-141) primer assays (SABiosciences) normalizing to control U6 snRNA levels. SIRT1 and CDH1 mRNA was normalized to the housekeeping gene GAPDH (SIRT1_F, CTCCTAAAGTAAAGACCATGAC; SIRT1_R, CACAGTCTCC-AAGAAGCTCTA; CDH1_F, CAACGATGCCAATTTGGAAAACAG; CDH1_R, TCACATCCAGCAGCAGCACC; GAPDH_F, GAAGGTGAAGGTCGGAGTC; and GAPDH_R, GAAGATG- GTGATGGGATTC). Patient blood samples and fresh patient tissue samples were obtained from the Tissue Bank of the University of Maryland School of Medicine. Total RNA was isolated using a whole-blood RNA isolation kit (Zymo Research; Orange, CA), and tissue samples were extracted using an RNaseasy Lipid Tissue Kit (Qiagen; Valencia, CA). All real-time PCR was carried out with the Light Cycler 480 II (Roche Diagnostics).

**Luciferase Reporter Assays—**HEK293T cells were seeded in six-well plates (5 × 10⁵/well) and transfected with pGL3 luciferase vector containing wild-type SIRT1 3’-UTR or mutant SIRT1 3’-UTR (A mutated to G in the miR-200a response element) by Lipofectamine 2000 (Invitrogen). Cells were co-transfected with 50 nM miR-200a precursor (Ambion; Austin, TX). Luciferase activity was determined using the Dual-Luciferase assay system (Promega; Madison, WI) after 48 h of transfection. Luciferase activity was normalized to Renilla luciferase activity. Similar procedures were followed to measure promoter activity of a pGL3 (Promega) reporter construct containing miR-200a promoter (~1574 to +120) (32).

**Immunoblotting—**Whole cell lysates were prepared in cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1%SDS, 1% Nonidet P-40), and supernatants were collected by centrifugation. Equal amounts of protein were denatured in SDS sample buffer and separated on 10% polyacrylamide gels. Separated proteins were transferred to PVDF membrane and probed with antibodies (SIRT1, E-cadherin, and N-cadherin) from Santa Cruz Biotechnology (Santa Cruz, CA). Protein expression was detected by chemilumines-
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cence (ECL, Amersham Biosciences; Arlington Heights, IL). Expression of β-actin was used as a loading control. Band density analysis was performed in Adobe Photoshop CS4.

CpG Island Identification—The genomic DNA segment encompassing the miR-200a coding and promoter sequences was analyzed with the USC CpG island searcher software. The following parameters were used: minimum GC%, 55%; minimum observed CpG to expected CpG ratio, 0.65; minimum length of an island, 200 bp; and minimum gap between adjacent islands, 100 bp.

 Bisulfite Mapping and Methylation-specific PCR—Genomic DNA was prepared from cultured cells using the ZymoBead genomic DNA kit (Zymo Research; Irvine, CA). Bisulfite conversion of genomic DNA was performed using the EZ DNA methylation-direct kit (Zymo Research). Bisulfite-treated DNA was used as template for PCR with Zymo Taq Premix (Zymo Research) and sets of bisulfite sequencing primers (miR-200a promoter region, F, GTTTGGTATAG GTGGGGTTTATTG and R, CCAAACCTCAAAAA AACCAAATTA; and miR-200a coding region, F, GTTTGATTTAATATTGGTTGTA and R, AAATACCCAACCCCTTAC). Primers were designed using MethPrimer software. The following PCR conditions were used: 10 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C; and 7 min at 72 °C. PCR products were purified and cloned into pGEM-T Easy vector (Promega) and sequenced. At least five clones were sequenced. CDH1 coding regions, F, GCTAGAGGGTCACCGCGTCT; R, GTTTGATTTAATATTGGTTGTA and miR-200a are encompassing the mir-200a coding and promoter sequences.

Chromatin Immunoprecipitation—ChIP was performed as described previously (11) with anti-polycional rabbit acetyl-histone H3, anti-polycional rabbit acetyl-histone H4(Millipore; Billerica, MA), anti-polycional rabbit SIRT1(Delta Biolabs; Gilroy, CA), anti-polycional rabbit DNMT1(Santa Cruz Biotechnology), anti-polycional rabbit DNMT3A(Pierce), and anti-polycional rabbit DNMT3B (Santa Cruz Biotechnology) antibodies. Real-time PCR was performed for the proximal promoter region of the miR-200a cluster (F, CACCGCTCTCCAT-TGTC and R, CACAGGAA GTCACTTCAACC) or for CDH1 proximal promoter (F, GCTAGAGGGTCACCGCTCT; R, ACGCGTGATTTGGCTGAGGGT). Results are presented as the percent of input chromatin.

Immunohistochemistry—Formalin-fixed and paraffin-embedded human breast cancer and normal tissue samples were obtained from the Tissue Bank of the University of Maryland School of Medicine. Sections were deparaffinized and rehydrated using xylene and gradient ethanol. Antigens were retrieved by boiling in sodium citrate (10 mm, pH 6.0). Polyclonal rabbit anti-SIRT1 antibody (1:200, Delta Biolabs) was applied at 4 °C overnight followed by a biotin conjugated bovine anti-rabbit secondary antibody (1:250, Santa Cruz Biotechnology) at room temperature for 1 h. Avidin-biotin peroxidase substrate kit (Vector Laboratories; Burlingame, CA) was used to develop brown precipitate. Hematoxylin was utilized for nuclei staining. Quantitative analysis was performed on cells determined morphologically to be mammary epithelial cells by calculating the percentage of cells with positive staining from the total cells counted.

RESULTS

TGF-β-induced EMT of Normal Human Mammary Epithelial Cells Leads to SIRT1 Overexpression—We examined an in vitro model of EMT-like transformation and monitored alterations of SIRT1 expression. Normal HMEs were stably transfected with human telomerase reverse transcriptase and underwent immortalization. The immortalized cells were cultured in the presence of 10% FBS and treated with 10 ng/ml human TGF-β1 for 21 days. Treatment with TGF-β1 has been shown to induce EMT-like transformation of epithelial cells in many cell culture models (42). Fig. 1A shows that treatment with TGF-β1 leads immortalized HME cells to undergo EMT-like transformation evidenced by loss of cell-cell adhesion and alterations of morphology from a round compact shape to a spindel shape. These transformed cells were defined as HME-T. Using Western blot analysis in Fig. 1B, we found a phenotypic transition from an epithelial morphology toward mesenchymal-like properties evidenced by loss of the epithelial marker E-cadherin and overexpression of the mesenchymal marker N-cadherin in HME-T cells. Importantly, this transformation was associated with remarkable up-regulation of SIRT1, a key class III histone deacetylase that can induce epigenetic inactivation of E-cadherin in breast cancer (43).

EMT-like Transformation Leads to Loss of miR-200 Family Members—To identify dysregulated miRNAs that might play critical roles in the transformative process, we performed PCR-based miRNA array analysis and evaluated differential expression of 88 highly characterized miRNAs, many of which had been previously implicated in tumorigenesis. Analysis of miRNA arrays revealed numerous oncogenic or tumor-suppressive miRNAs (up- or down-regulated miRNAs) with differ-
ential expression in transformed cells (HME-T) as compared with parental cells (HME). Fig. 1C shows that among all miRNAs examined, miR-141 and miR-200c (members of the miR-200 family tested on this array), were the most significantly dysregulated miRNAs. miR-141 and miR-200c were down-regulated 3,100- and 1,500-fold, respectively, in transformed cells. We further confirmed the results of our array by examining miR-200 family expression by real-time qRT-PCR. The miR-200 family is encoded in two locations, on chromosome 1 (miR-200b/miR-200a/miR-429) and chromosome 12 (miR-200c/miR-141), where these individual clusters of miR-200 family members are co-transcribed. We tested expression of members from both clusters and as shown in Fig. 2A, miR-200a (chromosome 1) and miR-141 (chromosome 12) are decreased significantly upon transformation (p < 0.01, data for other members of miR-200 family not shown). These miRNAs (miR-200a and miR-141) are also found decreased in MDA-MB-231 cell line (breast cancer cell line with mesenchymal phenotype) compared with MCF-10A (a non-tumorigenic breast epithelial cell line). These results clearly demonstrate down-regulation of miR-200 family members between normal mammary epithelial cells and cells having undergone EMT-like transformation.

DNA Hypermethylation Is Associated with Loss of miR-200a in Transformed Cells—The CpG dinucleotide is clustered in 40–50% of human gene promoters (44). Methylation of CpG islands (CG-rich sequences) in these gene promoters can lead to an inactive transcriptional state and loss of gene expression (45). As miR-200a and miR-141 exist on separate chromosomes, we decided to focus our efforts on first examining dysregulation of miR-200a. We set out to determine whether DNA methylation may contribute to the dramatic down-regulation of miR-200a in our model of EMT-like transformation in HME-T cells. A 2500-bp CpG island on chromosome 1 was identified in the miR-200b/miR-200a/miR-429 cluster promoter (RefSeq accession no. AC_000133.1 strand (+), nucleotides 369401 to 371963). We performed bisulfite sequencing analysis to determine DNA methylation status of a 187-bp region within this miR-200a promoter CpG island. In Fig. 2B, the results from bisulfite sequencing revealed hypermethylation in transformed cells as well as in MDA-MB-231 cells. Promoter methylation levels were close to identical between HME-T cells (83.6%) and MDA-MB-231 cells (96.3%). These observations were further confirmed by methylation-specific PCR (data not shown). These data suggest that DNA hypermethylation of the miR-200a promoter region may contribute to miR-200a silencing upon EMT.

It was shown previously that coding region methylation may also affect gene expression (46). While examining the miR-200a promoter region CpG island, an additional 321-bp CpG island on chromosome 1 (RefSeq accession no. AC_000133.1 strand (+), nucleotides 374588–374908) was identified encompassing the coding region of miR-200a. Bisulfite sequencing of a 153-bp region within this CpG island revealed differential methylation of the miR-200a coding region between transformed cells (28.8%) and MDA-MB-231 cells (90%) (shown in Fig. 2B). These data suggest that intragenic methylation may also influence miR-200a expression in addition to promoter region methylation.

Epigenetic Therapy Can Lead to Restoration of miR-200a/miR-141 in Transformed Cells—To further address whether epigenetic mechanisms (DNA methylation and histone deacetylation) contribute to down-regulation of the miR-200 family, we treated TGF-β-transformed cells (HME-T) as well as breast cancer cells (MDA-MB-231) with epigenetic therapies...
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A, loss of miR-200a/miR-141 in transformation. Results of qRT-PCR comparing miR-200a and miR-141 between normal and transformed HME cells as well results comparing non-tumorigenic MCF-10A breast epithelial cells with breast cancer cell line MDA-MB-231 (n = 3; ± S.E.). B, methylation levels of the promoter region and coding region CpG islands of miR-200a. Empty and filled ovals represent cytosine unmethylated and cytosines methylated, respectively. The promoter region CpGs are located from nucleotides 370633 to 374945 on chromosome 1 (strand (+)), RefSeq accession no. AC_000133.1). The coding region CpGs are located from nucleotides 374693 to 374845 on chromosome 1 (strand (+)), RefSeq accession no. AC_000133.1). The filled rectangle represents the coding region of miR-200a in relation to sequencing results. Arrows represent miR-200B/A/429 relative locations on chromosome 1. The results of five clones sequenced and mean % of methylated CpGs is shown.

FIGURE 2. Epigenetic silencing of miR-200a associated with transformation. A, loss of miR-200a/miR-141 in transformation. Results of qRT-PCR comparing miR-200a and miR-141 between normal and transformed HME cells as well results comparing non-tumorigenic MCF-10A breast epithelial cells with breast cancer cell line MDA-MB-231 (n = 3; ± S.E.). B, methylation levels of the promoter region and coding region CpG islands of miR-200a. Empty and filled ovals represent cytosine unmethylated and cytosines methylated, respectively. The promoter region CpGs are located from nucleotides 370633 to 374945 on chromosome 1 (strand (+)), RefSeq accession no. AC_000133.1). The coding region CpGs are located from nucleotides 374693 to 374845 on chromosome 1 (strand (+)), RefSeq accession no. AC_000133.1). The filled rectangle represents the coding region of miR-200a in relation to sequencing results. Arrows represent miR-200B/A/429 relative locations on chromosome 1. The results of five clones sequenced and mean % of methylated CpGs is shown.

capable of reversing methylation-induced gene silencing: histone deacetylase inhibitors and DNMT inhibitors. Fig. 3A shows that treatment with SAHA (10 μM; a histone deacetylase inhibitor) or 5-azacytidine (Aza, a DNMT inhibitor; 5 μM) resulted in elevated expression of miR-200a in HME-T cells. Similarly, treatment of MDA-MB-231 cells with SAHA or Aza resulted in elevation of miR-200a levels, but to a lesser extent. Additionally, treatment of transformed cells with SAHA or Aza resulted in elevated expression of miR-200a/miR-141 in HME-T cells. Despite this treatment, miR-200a levels in HME-T cells were subsequently transfected with 50 nM miR-200a precursor for 72 h. Fig. 4B shows that miR-200a inhibits the luciferase activity of the wild-type SIRT1 3′-UTR, suggesting that miR-200a inhibits the luciferase activity of the wild-type SIRT1 3′-UTR, but mutation of the miR-200a mRNA-responsive element within the SIRT1 3′-UTR abolishes miR-200a action, suggesting that miR-200a targets one complementary sequence in the SIRT1 3′-UTR.

Because SIRT1 has the ability to induce epigenetic inactivation of tumor suppressor genes, we decided to focus on the impact of miR-200a on SIRT1 expression in EMT-like transformation. We examined the impact of miR-200a on SIRT1 mRNA expression by qRT-PCR analysis. We first observed that loss of miR-200a is associated with SIRT1 mRNA up-regulation in HME-T (transformed) cells (Fig. 3A). Similarly, treatment of MDA-MB-231 cells with either SAHA (10 μM; a histone deacetylase inhibitor) or 5-azacytidine (Aza, a DNMT inhibitor; 5 μM) resulted in elevated expression of miR-200a/miR-141 in HME-T cells (Fig. 3B). However, the full complement of miR-200a family target genes is not known. To identify new molecular targets of the miR-200 family during transformation, we explored TargetScan (release 5.1) (47) and mirBase (48) databases for SIRT1. The results of qRT-PCR confirmed that miR-200a/miR-141 is SIRT1. As miR-200a and miR-141 target identical seed sequences, we subsequently examined miR-200a and whether it can directly target SIRT1.

The ability of miR-200a to regulate the 3′-UTR of SIRT1 was evaluated via luciferase reporter assays. TargetScan (release 5.1) predicted a single miRNA-responsive element containing a conserved 7-mer exact seed match at positions 1728–1734 of SIRT1 3′-UTR as a miR-200a target (Fig. 4A). Wild-type SIRT1 3′-UTR as well as mutSIRT1 3′-UTR with mutated target sites (A to G) were cloned into a pGL3 luciferase vector. HEK293T cells, which do not express detectable levels of miR-200 family (data not shown), were co-transfected with a pGL3 luciferase vector and the pRL-TK Renilla luciferase vector. To examine the impact of miR-200a on SIRT1 3′-UTR activity, HEK293T cells were co-transfected with miR-200a precursor (Ambion) that activated miR-200a expression. Fig. 4B shows that miR-200a inhibits the luciferase activity of the wild-type SIRT1 3′-UTR, but mutation of the miR-200a mRNA-responsive element within the SIRT1 3′-UTR abolishes miR-200a action, suggesting that miR-200a targets one complementary sequence in the SIRT1 3′-UTR.

Identification of SIRT1 as New Target of miR-200a—To determine whether a possible correlation existed between SIRT1 up-regulation and miR-200a down-regulation, we probed for a mechanistic link between the observations. It is known that miR-200a targets the 3′-UTR of ZEB1 and SIP1 in breast cancer cells (35). However, the full complement of miR-200a family target genes is not known. To identify new molecular targets of the miR-200 family during transformation, we explored TargetScan (release 5.1) (47) and mirBase (48) databases to find that one of the best scoring mRNAs that can be targeted by miR-200a/miR-141 is SIRT1. As miR-200a and miR-141 target identical seed sequences, we subsequently examined miR-200a and whether it can directly target SIRT1.

Because SIRT1 has the ability to induce epigenetic inactivation of tumor suppressor genes, we decided to focus on the impact of miR-200a on SIRT1 expression in EMT-like transformation. We examined the impact of miR-200a on SIRT1 mRNA expression by qRT-PCR analysis. We first observed that loss of miR-200a is associated with SIRT1 mRNA overexpression in HME-T (transformed) cells (Fig. 3A). These data indicate that miR-200a is sufficient to inhibit SIRT1 mRNA levels. We had shown previously that treatment with epigenetic therapies resulted in an increase in miR-200a in HME-T cells (Fig. 3A). We tested whether these treatments would also result in an associated down-regulation of SIRT1 mRNA. A decrease in SIRT1 mRNA was confirmed by qRT-PCR after treatment of HME-T cells with either SAHA (10 μM for 24 h) or Aza (5 μM for 96 h) (Fig. 4C, right panel). These data indicate that miR-200a re-expression correlates with inhibition of SIRT1 expression. HEK293T cells, which show detectable levels of SIRT1 protein, were transfected with 50 nM miR-200a precursor, and Western blot analysis showed that miR-200a inhibits SIRT1 protein expression (Fig. 4D, left panel). Similar results were observed in HME-T cells (Fig. 4D, middle panel). To further
examine the impact of miR-200a on the SIRT1 3′-UTR, a SIRT1_CDS construct (without 3′-UTR, coding region only) was co-transfected with 50 nM miR-200a precursor into HEK293T cells. In Fig. 4D (right panel), Western blot analysis shows that miR-200a is unable to alter SIRT1 protein expression when cells are transfected with SIRT1 construct lacking a 3′-UTR, supporting that miR-200a reduces SIRT1 expression by targeting the SIRT1 3′-UTR.

SIRT1 Involvement in Negative Feedback Loop Regulating miR-200a Expression—Because down-regulation of miR-200a may lead to overexpression of SIRT1, we set out to determine whether the high levels of SIRT1 in HME-T cells might be involved in miR-200a epigenetic silencing. We first tested the ability of SIRT1 to regulate a miR-200a promoter luciferase reporter. Co-transfection of pGL3-miR-200a reporter and SIRT1 resulted in a significant decrease in reporter activity compared with reporter only controls (Fig. 5A). Next, we performed ChIP experiments to monitor SIRT1 recruitment to the miR-200a promoter and observed increased levels of SIRT1 occupying the miR-200a promoter in HME-T cells compared with control HME cells (Fig. 5B). As it was previously shown that SIRT1 might participate in the recruitment of DNMTs to silence tumor suppressor genes (7, 42), we also examined whether increased recruitment of SIRT1 was associated with altered recruitment of DNMTs (DNMT1, DNMT3A, or DNMT3B) to the miR-200a promoter. Similarly to SIRT1, we observed increased DNMT1, DNMT3A, and DNMT3B localized at the miR-200a pro-
moter region in HME-T cells compared with control HME cells (Fig. 5B). These data provide further support for the involvement of epigenetic mechanisms in miR-200a silencing as well as demonstrate a negative regulatory circuit wherein SIRT1 and miR-200a are involved in the negative regulation of one another in mammary epithelium.

Activation of miR-200a and Inhibition of SIRT1 Decrease Anchorage-independent Cell Growth in HME-T Cells—To demonstrate the possible function of SIRT1 and miR-200a in mammary epithelial cell transformation, HME-T cells were transfected with SIRT1 shRNA (HME-T/shSIRT1) or miR-200a precursor (HME-T/miR-200a) and examined by soft agar assay (Fig. 6A). HME-T cells showed more colony formation in a soft agar assay than control HME cells (Fig. 6B). However, HME-T/shSIRT1 and HME-T/miR-200a cells showed significantly decreased colony formation compared with HME-T cells. These results indicate that SIRT1 plays a role in HME-T anchorage-independent cell growth, therefore maintaining the transformation of these cells.

Activation of miR-200a and Inhibition of SIRT1 Decreases Cell Migration in HME-T Cells—The ability of SIRT1 and miR-200a to regulate cell migration and cell invasion was investigated by utilizing transwell migration assays. HME-T cells were much more migratory than control HME cells (Fig. 6C). HME-T cells transfected with either shRNA for SIRT1 (HME-T/shSIRT1) or miR-200a precursor (HME-T/miR-200a) showed dramatically decreased cell migration. These findings further confirmed a functional role for SIRT1 in promoting EMT-like transformation of mammary epithelial cells as well as establishing a role for miR-200a in inhibiting this process.

SIRT1 Recruitment to CDH1 Promoter Is Correlated with Epigenetic Silencing of E-cadherin Expression—SIRT1 inhibition has been shown to result in re-expression of epigenetically silenced E-cadherin in breast cancer cell lines (42). Because E-cadherin expression is required for maintenance of the epithelial phenotype in mammary epithelium, we determined whether SIRT1 has the ability to regulate E-cadherin expression in transformed HME-T cells. Fig. 7A shows that knock-
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Circulatory Levels of miR-200a and miR-141 Are Decreased in Breast Cancer Patients—miRNAs have been detected in peripheral circulation of cancer patients that distinguish tumor-bearing individuals from healthy individuals (49–51). The source for these altered circulatory miRNAs is suggested to be dysregulation of miRNAs in tumors, indicating these circulatory miRNAs may serve as surrogates for tumor miRNA levels. As a preliminary test of our in vitro findings, we examined levels of miR-200a and miR-141 in the blood of several DCIS and IDC patients. Fig. 8C shows that compared with healthy control patient blood, levels of miR-200a and miR-141 are decreased in blood from two DCIS and four IDC patients. These results suggest that a down-regulation of miR-200a and miR-141 in blood may be associated with SIRT1 overexpression in patients with breast cancer.

DISCUSSION

Previous studies have explored dysregulated miRNAs in breast cancer and have implicated tumor suppressive and oncogenic miRNAs in various stages of the disease. In our study, we examined a TGF-β-induced model of EMT-like transformation, attempting to identify differentially expressed miRNAs that were associated with the transformation of normal mammary epithelial cells. Among the miRNAs with altered expression, miR-200 family members were dramatically down-regulated upon TGF-β-induced transformation. Exploring possible mechanisms responsible for miR-200a loss, we identified epigenetic repression of miR-200a in transformed mammary epithelial cells. In addition, we examined SIRT1 levels in our transformation model, given that overexpression of SIRT1 is reported for several cancers, including breast cancer. We demonstrated transformation-associated overexpression of SIRT1 at the protein and transcript level and find that SIRT1 is a novel target for miR-200a regulation through a 3′-UTR-dependent mechanism. Indeed, re-expression of silenced miR-200a with epigenetic therapy correlated with down-regulation of SIRT1. Additionally, we showed transformation-associated altered recruitment of SIRT1, DNMT1, DNMT3A, and DNMT3B to the miR-200a promoter, further implicating epigenetic machinery in miR-200a silencing and establishing a negative feedback loop involving miR-200a and SIRT1. We also

![Figure 5. SIRT1 regulation of miR-200a expression. A, SIRT1 regulation of a miR-200a promoter luciferase reporter. Co-transfection of SIRT1 (2 μg) or control vector, with a miR-200a promoter reporter (2 μg) (-1574 to +120) and Renilla luciferase control for 48 h, after which cells were lysed, and the luciferase activity was assessed. Mean ± S.E. (n = 3). * altered SIRT1 recruitment to the miR-200a promoter. Real-time qRT-PCR results from ChIP experiments for the miR-200a promoter after IP with SIRT1, DNMT1, DNMT3A, and DNMT3B antibodies. Mean ± S.E. (n = 3). A and B, p value was determined by two-sided unpaired t test. * p < 0.05.](25999)
miR-200a Targets SIRT1 Preventing EMT in Mammary Epithelium

![Diagram](image)

**FIGURE 6.** miR-200a regulation of SIRT1 influences transformation-induced anchorage-independent growth and invasion. A, reversal of transformation-associated morphology by miR-200a addition or SIRT1 inhibition. Representative images of normal HME cells, HME-T transformed cells, HME-T transformed cells transfected with 50 nM miR-200a precursor, and HME-T transformed cells transfected with shRNA against SIRT1 for 72 h. Scale bar, 100 μm. B, colony formation of cells in soft agar assay. HME-T cells were transfected with 50 nM miR-200a precursor or SIRT1 shRNA. 10^4 cells from each cell line were seeded in each well of a six-well plate. After 3 weeks, the colonies were stained with crystal violet. Quantitative analysis was performed by averaging colony number over four random fields (10× objective), Mean ± S.E. for three independent experiments. Scale bar, 100 μm. C, miR-200a/shSIRT1 inhibit transformation-induced cell migration. Transwells with 8-μm pore size and coated with Matrigel were used. HME-T cells were transfected with 50 nM miR-200a precursor or shRNA targeting SIRT1 for 48 h. 0.5 × 10^5 cells from each cell line was used per well for overnight migration. Migrated cells were stained with crystal violet. Analysis was performed by averaging migrated cell number for four random fields (10× objective), Mean ± S.E. for two independent experiments. Scale bar, 100 μm. Asterisks indicate significance, p value determined by two-sided unpaired t test. ***, p < 0.001.

observed increased SIRT1 recruitment to the E-cadherin promoter, which correlated with methylation of the E-cadherin promoter and silencing of E-cadherin expression. Importantly, we attempted to test and establish the translation of these findings by examining SIRT1 and miR-200a expression in breast cancer patient samples. We found SIRT1 up-regulation in DCIS patient samples as well as in IDC and additionally demonstrated a decrease of miR-200a in DCIS and IDC patient samples.

Members of the miR-200 family are already known to target regulators of E-cadherin, ZEB1/SIP1, as well as regulators of stem cell self-renewal and cancer cell motility: BMI1 and phospholipase c-γ 1. We identify SIRT1 as a new target of miR-200a/miR-141, placing further emphasis on the importance of the miR-200 family as tumor-suppressive miRNA. Our observations in TGF-β transformed mammary epithelium confirm a possible role of SIRT1 in EMT-like processes in breast cancer and suggest a new mechanism of SIRT1 up-regulation in breast cancer: increased SIRT1 expression by loss of miR-200a/miR-141.

We investigated an epigenetic role in the loss of miR-200a by both monitoring DNA methylation and examining effects of epigenetic therapy on expression level and histone acetylation status. We observed hypermethylation of the miR-200a promoter region in transformed cells as well as MDA-MB-231 cells (breast cancer cells with a mesenchymal phenotype). It is well established that aberrant DNA methylation in promoter regions can silence gene expression, and it has previously been shown that treatment with TGF-β can result in the acquisition of DNA methylation and silencing of E-cadherin (52). We find that treatment with epigenetic therapies (SAHA and AzA) resulted in re-expression of miR-200a and miR-141 in transformed cells. Similarly, epigenetic therapies resulted in re-expression (although a less dramatic response) of miR-200a in MDA-MB-231 breast cancer cells. However, SAHA treatment of MDA-MB-231 cells did not result in miR-141 re-expression. MDA-MB-231 cells are highly malignant cells having undergone many genetic and epigenetic changes and would likely not respond to therapy identically to acutely transformed mammary epithelial cells. Additionally, miR-200a and miR-141 reside in clusters on separate chromosomes and, as such, may not respond identically to epigenetic therapies, as was the case with SAHA treatment of MDA-MB-231 cells.

We also discovered intragenic methylation of a CpG island overlapping the miR-200a coding region. This island showed heavy methylation in MDA-MB-231 cells and may negatively regulate miR-200a expression in breast cancer. Studies indicate that intragenic methylation may inhibit gene expression through decreasing transcriptional elongation efficiency (45). The more extensive methylation of miR-200a in MDA-MB-231 cells may also contribute to the lessened response to epigenetic therapies compared with treatment of transformed cells. To our knowledge, this might be the first report of intragenic methylation within an miRNA-encoding region. Whether a reversal of methylation in the promoter region or coding region of miR-200a is of unique relevance for response to epigenetic therapy is unknown and will be investigated further.

We also find that overexpression of SIRT1 upon EMT-like transformation results in increased recruitment to the miR-200a promoter region along with other epigenetic machinery (DNMT1/3A/3B). This suggests a negative feedback loop exists between miR-200a and SIRT1 and also provides further evidence for the involvement of epigenetic mechanisms in the silencing of miR-200a. Additionally, we find evidence that SIRT1 is involved in the epigenetic silencing of E-cadherin, a critical step in the EMT-like transformation of mammary epithelium.

We sought to explore the translational impact of our findings by examining patient tumor samples for SIRT1 expression. We were able to detect elevated levels of SIRT1 in samples of DCIS and IDC, supporting a role for SIRT1 in early and later stages of cancer progression. As a preliminary stage of testing our *in vitro* findings concerning miR-200a, we examined patient tissue samples and patient blood samples of DCIS and IDC.
We were able to demonstrate a decrease in miR-200a levels in DCIS and IDC patient tissue samples. Additionally, we observed decreased circulatory miR-200a/miR-141 levels in breast cancer patients. Serum and whole blood levels of circulating miRNAs are currently being explored in cancer research for their potential prognostic value as surrogates of tumor cell miRNA levels (49–51). Additional work needs to be done to test our in vitro findings by examining levels of miR-200 family in additional patient samples of in situ and invasive breast carcinomas.

FIGURE 7. A, SIRT1 knockdown can restore E-cadherin expression. Western blot of HME-T cells transfected with scrambled control or shRNA for SIRT1 (n = 2). B, qRT-PCR results for E-cadherin mRNA normalized to GAPDH. E-cadherin expression in HME-T (transformed) or HME (control) cells (n = 2). C, altered SIRT1 recruitment to the E-cadherin (CDH1) promoter. Real-time qRT-PCR results from ChIP experiments for the CDH1 promoter after immunoprecipitation with SIRT1 and DNMT3B antibodies. Mean ± S.E. (n = 2). D, methylation status of the CDH1 promoter. Results from methylation-specific PCR for the CDH1 promoter in HME-T (transformed) and HME-C (control) cells. Product sizes were as follows: unmethylated (U), 97 bp; methylated (M), 116 bp (n = 2). B and C, p value determined by two-sided unpaired t test. *, p < 0.05.

FIGURE 8. SIRT1 elevation is associated with miR-200a down-regulation in breast cancer patient samples. A, overexpression of SIRT1 in breast cancer patient samples. Immunohistochemistry staining of SIRT1 in DCIS and IDC patient samples or normal breast tissue (n = 5). Hematoxylin was used to visualize nuclei. 3,3-Diaminobenzidine was used to demonstrate SIRT1 staining. Quantification is performed for percentage of mammary epithelial cells with positive staining nuclei (mean ± S.D.). Scale bar, 60 µm. B, miR-200a levels are decreased in breast cancer patient samples. Results of qRT-PCR measuring miR-200a in five DCIS and five IDC patient samples relative to healthy mammary epithelial tissue. Results normalized to snRNA U6 levels. C, MiR-200a/miR-141 levels are decreased in breast cancer patient blood. Results of qRT-PCR measuring miR-200a and miR-141 levels in DCIS (n = 2) and IDC (n = 4) patient sample whole blood relative to control patient blood. Results were normalized to snRNA U6 levels. A and B, p value determined by two-sided unpaired t test. *, p < 0.05.
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SIRT1 overexpression has been demonstrated to occur through various mechanisms in cancer, including loss of negative regulation by p53 or HIC1 tumor suppressors. However, no study has demonstrated a mechanism of SIRT1 up-regulation in EMT-like processes in breast cancer. The finding of novel miR-200a/141-related dysregulation of SIRT1, in a model of transformed mammary epithelial cells, may be therapeutically relevant and reveal new ways to clinically manipulate SIRT1 expression.

REFERENCES