

A Double-Negative Feedback Loop between ZEB1-SIP1 and the microRNA-200 Family Regulates Epithelial-Mesenchymal Transition

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Abstract

Epithelial to mesenchymal transition occurs during embryologic development to allow tissue remodeling and is proposed to be a key step in the metastasis of epithelial-derived tumors. The miR-200 family of microRNAs plays a major role in specifying the epithelial phenotype by preventing expression of the transcription repressors, ZEB1/δEF1 and SIP1/ZEB2. We show here that miR-200a, miR-200b, and the related miR-429 are all encoded on a 7.5-kb polycistronic primary miRNA (pri-miR) transcript. We show that the promoter for the pri-miR is located within a 300-bp segment located 4 kb upstream of miR-200b. This promoter region is sufficient to confer expression in epithelial cells and is repressed in mesenchymal cells by ZEB1 and SIP1 through their binding to a conserved pair of ZEB-type E-box elements located proximal to the transcription start site. These findings establish a double-negative feedback loop controlling ZEB1-SIP1 and miR-200 family expression that regulates cellular phenotype and has direct relevance to the role of these factors in tumor progression. [Cancer Res 2008;68(19):7846–54]

Introduction

MicroRNAs are small noncoding RNAs that mediate posttranscriptional repression through sequence-specific binding to their mRNA targets resulting in translational inhibition and, in some cases, destruction of the target mRNA (1). It is now evident that microRNAs are important regulators of cellular differentiation and developmental pathways, but relatively little is known of the mechanisms that control expression of the microRNAs themselves. Primary miRNA transcripts (pri-miR) are transcribed by RNA Polymerase II (2) and may be derived either from their own dedicated transcripts, or from within the transcripts of known protein-coding genes. Computational modeling has predicted the promoters of these miRNA-encoding transcripts are of a similar type to protein-coding genes and typically located within the first 500 nucleotides upstream of the transcription start site (TSS; refs. 3, 4). Despite these predictions, the direct transcriptional regulation of only a few pri-miRs have been reported, including the activation of the paralogous miR-17~92 and miR-106b~25 clusters by E2F (5, 6) and regulation of the miR-223 promoter by NFIA and CAAT/enhancer binding protein α (7).

Note: C.P. Bracken, P.A. Gregory, and N. Kolesnikoff contributed equally to this study.

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The miR-200 family inhibits expression of the related transcriptional repressors ZEB1/δEF1 and SIP1/ZEB2 in epithelial cells and play a major role in preventing these factors from triggering epithelial to mesenchymal transition (EMT; refs. 8, 9). ZEB1 and SIP1 bind to paired ZEB-type E-boxes (CACCTG) within target gene promoters, such as the key epithelial gene E-cadherin, and act to repress their transcription (10–17). ZEB1 and SIP1 are directly implicated in the control of EMT (11, 12), a process underlain by characteristic changes in gene expression in which epithelial cells acquire mesenchymal characteristics. EMT has considerable relevance to tumor progression, whereby an EMT-like process is postulated to confer invasive properties to tumors and correlates with poor patient prognosis (18, 19).

Following on from the finding that the miR-200 family regulate the level of ZEB1 and SIP1, we undertook an investigation of the control of expression of the miR-200 family. We report here that the miR-200b, miR-200a, and miR-429 miRNAs are encoded by a single polycistronic transcript, and we identify a minimal promoter element that controls its expression. Using both ectopic expression and the knockdown of endogenous protein, we show that ZEB1 and SIP1 negatively regulate miR-200b ~ 200a ~ 429 transcription in Madin-Darby canine kidney (MDCK) cells and human breast cancer cells by binding paired E-box sites in close proximity to the miR-200b ~ 200a ~ 429 TSS. This interaction establishes a double negative feedback regulatory loop between ZEB1-SIP1 and the miR-200 family that has important implications for EMT and tumorigenesis.

Materials and Methods

Cell culture and generation of stable cell lines. All cell lines were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) with the exception of MDA-MB-435 and BT-549. MDA-MB-435 were maintained in α Modified Eagles Medium (Invitrogen) supplemented with 5% FBS. BT-549 were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS. The MDCK-Pez and MDCK-vector stable cell lines were generated as previously described (20). MDCK-ZEB1 and MDCK-SIP1 stable cell lines were made by stable transfection of MDCK with pcDNA3.1-HisC-ZEB1 and pcDNA3.1-HisC-SIP1 vectors. These vectors express mouse ZEB1 and SIP1 lacking their natural 3' untranslated regions (UTR) and are therefore not targeted by the miR-200 family. Neomycin-resistant cells were selected using 500 μ g/mL G418 with representative colonies that had undergone EMT isolated and expanded.

Extraction of RNA and genomic DNA and PCR analysis. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Total cell genomic DNA was extracted using the DNeasy Blood & Tissue Protocol for Purification of Total DNA (Qiagen). mRNA and miRNA analysis was carried out as previously described with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPD) and U6 levels, respectively (8). All primer pairs have been previously described (8) with the exception of the following. To amplify canine and human hypoxanthine phosphoribosyltransferase (HPRT), the primer pairs were

5'-GACCAGTCAACAGGGGACAT-3' and 5'-AACACTTCGAGGGGTCCTT-3', and 5'-GGCAGTATAATCCAAAGATGGTCA-3' and 5'-TCCTTTTCA-CCAGCAAGCTTG-3', respectively. To define the boundaries of the miR-200b~200a~429 primary transcript, the following primer sets were used (with product sizes indicated): 5'-CAGGCTGTGACCTCAGTGG-3' and 5'-ACAAAAACCAGGGAGGAAGG-3' for set A (119 bp); 5'-CCACAGCCTC-CATTGTTC-3' and 5'-AGCTCAGGCTCCTCACAAAG-3' for set B (111 bp); 5'-GGCCAAAATAAGTCAAAGC-3' and 5'-CTCGCCAGACGGTCTTAC-3' for set C (146 bp); 5'-GGACAAAGCCAGAGAACTCG-3' and 5'-AAGTTTTC-CAGCACCTTCCA-3' for set D (140 bp); 5'-CCTGGATTAGGATGCTCAGG-3'

and 5'-CCTCCAGACGCTTTGTGTGC-3' for set E (100 bp); 5'-CTGCAGGG-CACAGGTCAG-3' and 5'-GCTATGACAGAAAGGCCAAAG-3' for set F (101 bp); 5'-GGCTGGCTTCTCTCGTCGC-3' and 5'-AGTACGGTGTCTGCCT-CACC-3' for set G (100 bp); 5'-GGCCTGCTCACCCTCACT-3' and 5'-CTGCCTTACAAGGAGCAGT-3' for set H (142 bp); 5'-TCGAAACTCTC-CAGAGACG-3' and 5'-AGACTGCAAGGGTGTGAGCTT-3' for set I (109 bp); 5'-AGTGGGGCTCACTCTCCAC-3' and 5'-AGGAGGAGGAGGAGAGAAA-3' for set J (121 bp); and 5'-TGTGGTCAGCTCAGGTCAAG-3' and 5'-CCATA-CGCTTTGGTCACT-3' (140 bp) for set K. Reverse transcription-PCR (RT-PCR) products were analyzed on 2% agarose gels and visualized using ethidium bromide.

Generation of miR-200b~200a~429 promoter constructs. A -1574/+120 genomic region (relative to the putative TSS as indicated by the EST DA589106) containing the miR-200b~200a~429 promoter was amplified by PCR using *Pfu* DNA polymerase (Promega) and directionally cloned into the pGL3-basic (Promega) reporter using *Nhe I* and *Bgl II* sites generating the -1574/+120 construct. The -1574/+120 reporter was made using forward primer 5'-ACCTGCTAGCCATTCCCGACAGTCACAG-3' and reverse primer 5'-CAGGAGATCTCTGGCACAGGAAGTCAGTT-3'. Subsequent promoter truncations were amplified from this template and cloned in a similar manner using the following forward and reverse primer combinations. The -321/+120 used the same reverse primer but with forward primer 5'-GTGGGCTAGCAGAGGTGGAGAGGCGAGAGT-3'. Following 3' end truncations to +19 from the TSS used reverse primer 5'-TGGAA-GATCTGGGGCCTCGGAGGGAAGAG-3', with forward primers ATCGGCTAGCCTCGCTCACCCTCACT for -158/+19, 5'-GTGAGCTAGCC-AGGGGACACACCTGTGCG-3' for -110/+19, and 5'-GTCGGCTAGC-GAGCTCGCACCTGTGTGG-3' for -64/+19. Mutations in the ZEB-type E-box binding sites in the were made in the -321/+120 construct using the Quikchange site-directed mutagenesis kit using the primer pairs 5'-CCAGGGGACACACCGGTCGGGGGCCAGAG-3' and 5'-CTCCT-GGCCCCGACCGGTGTGTCCCTGGG-3' (E-box mut1) and 5'-GCGG-GAGCTCGCACCGGTGTGGGGCGGGGAG-3' and 5'-CTCCCCGCCCA-CACCGGTGCGAGCTCCCGC-3' (E-box mut 2).

Transfection and reporter assays. For reporter assays, cells were plated in 24-well plates and cotransfected with 5 ng pRL-TK Renilla plasmid (Promega) and 200 ng miR-200b~200a~429 promoter firefly luciferase reporter plasmids using Lipofectamine 2000 (Invitrogen). In Fig. 2C and D, 100 ng ZEB1 or SIP1 expression plasmids (pcDNA3.1-HisC-ZEB1 and pcDNA3.1-HisC-SIP1, or 50 ng of both) were also cotransfected. After 48 h of incubation, cells were either lysed in trizol and RNA extracted or lysed in passive lysis buffer (Promega) and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) using the TD-20/20 Luminometer (Turner Designs). For siRNA reporter experiments, siRNA mixtures of two canine ZEB1 (20 nmol/L) and SIP1 (20 nmol/L) siRNAs (as described in ref. 8) or mixtures of 3 human ZEB1 (60 nmol/L) and SIP1 (20 nmol/L) siRNAs (Stealth siRNAs; Invitrogen) were transfected, then retransfected 48 h later with additional plasmids as above into MDCK-Pez or MDA-MB-231 cells, respectively, using HiPerFect (Qiagen). To examine the effect of ZEB1 and SIP1 knockdown on endogenous gene expression (Fig. 3B), siRNAs were transfected as above, retransfected after 48 h, and RNA harvested 48 h later followed by real-time PCR analysis. All reporter assays are shown as relative luciferase activities (averaged ratios of Firefly luciferase: Renilla \pm SE) and are representative of multiple experiments.

Western blotting. Whole cell extracts were prepared from transfected cells by Triton X-100 lysis [50 mmol/L Hepes (pH 7.5), 150 mmol/L sodium chloride, 10 mmol/L sodium PPI, 5 mmol/L EDTA, 50 mmol/L sodium fluoride, and 1% Triton X-100 with protease inhibitor cocktail] and 50 μ g fractionated on 7.5% SDS polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes and probed with ZEB1-specific (ZEB E-20; Santa Cruz Biotechnology), SIP1-specific (21), or tubulin-specific (Abcam) antibodies. Membranes were exposed using ECL (GE Healthcare) and imaged using the LAS4000 Luminescent Image Analyzer (Fujifilm).

Chromatin immunoprecipitation. Cells were grown to 70% confluency and cross-linked with 1% formaldehyde at room temperature for 10 min. Glycine was added to a final concentration of 0.125 mol/L for 5 min at room temperature. Cells were then washed with 10 mL of PBS, scraped into 2 mL

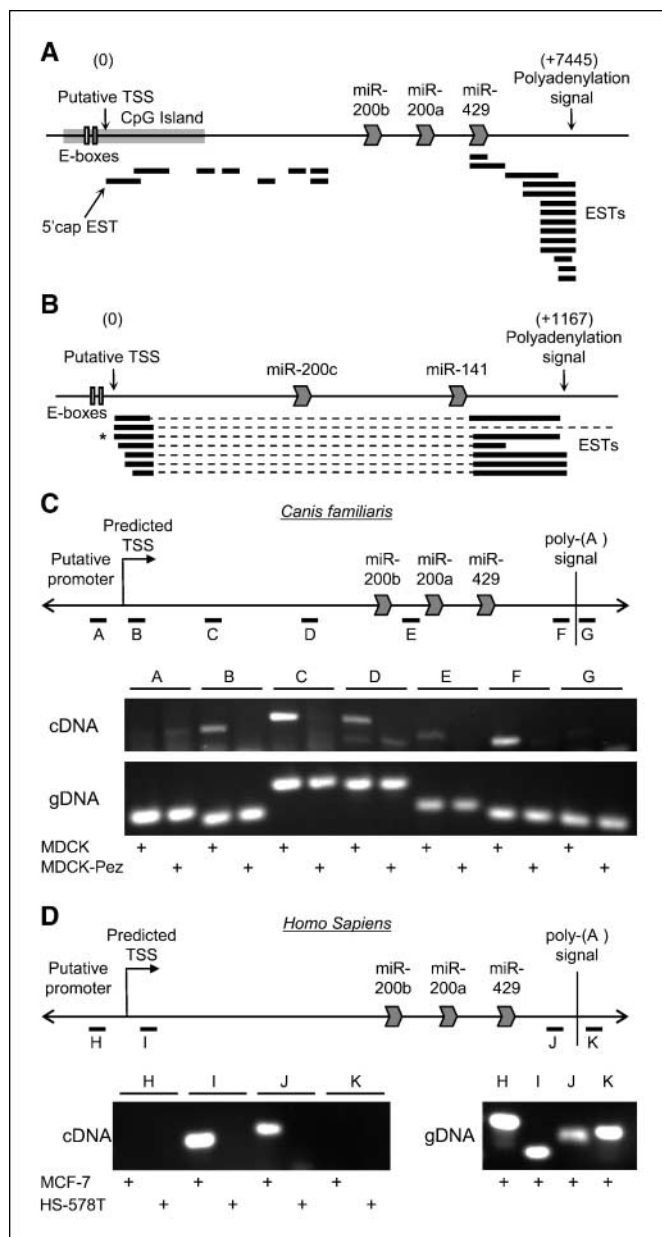


Figure 1. miR-200b, miR-200a, and miR-429 are encoded by a single polycistronic transcript. A and B, schematic representations of the miR-200b~200a~429 (A) and miR-200c~141 (B) genes indicating putative transcription start and polyadenylation signals. Black bars, reported ESTs. *, EST BX094730. C and D, transcription along the length of the putative miR-200b~200a~429 cluster was confirmed by RT-PCR, using primers targeted against regions both within and outside the dog (C) or human (D) predicted transcript. PCR was performed on genomic DNA (gDNA) as a positive control. The miR-200b~200a~429 transcript was detected in epithelial (MDCK and MCF-7) but not mesenchymal (MDCK-Pez and HS-578T) cells.

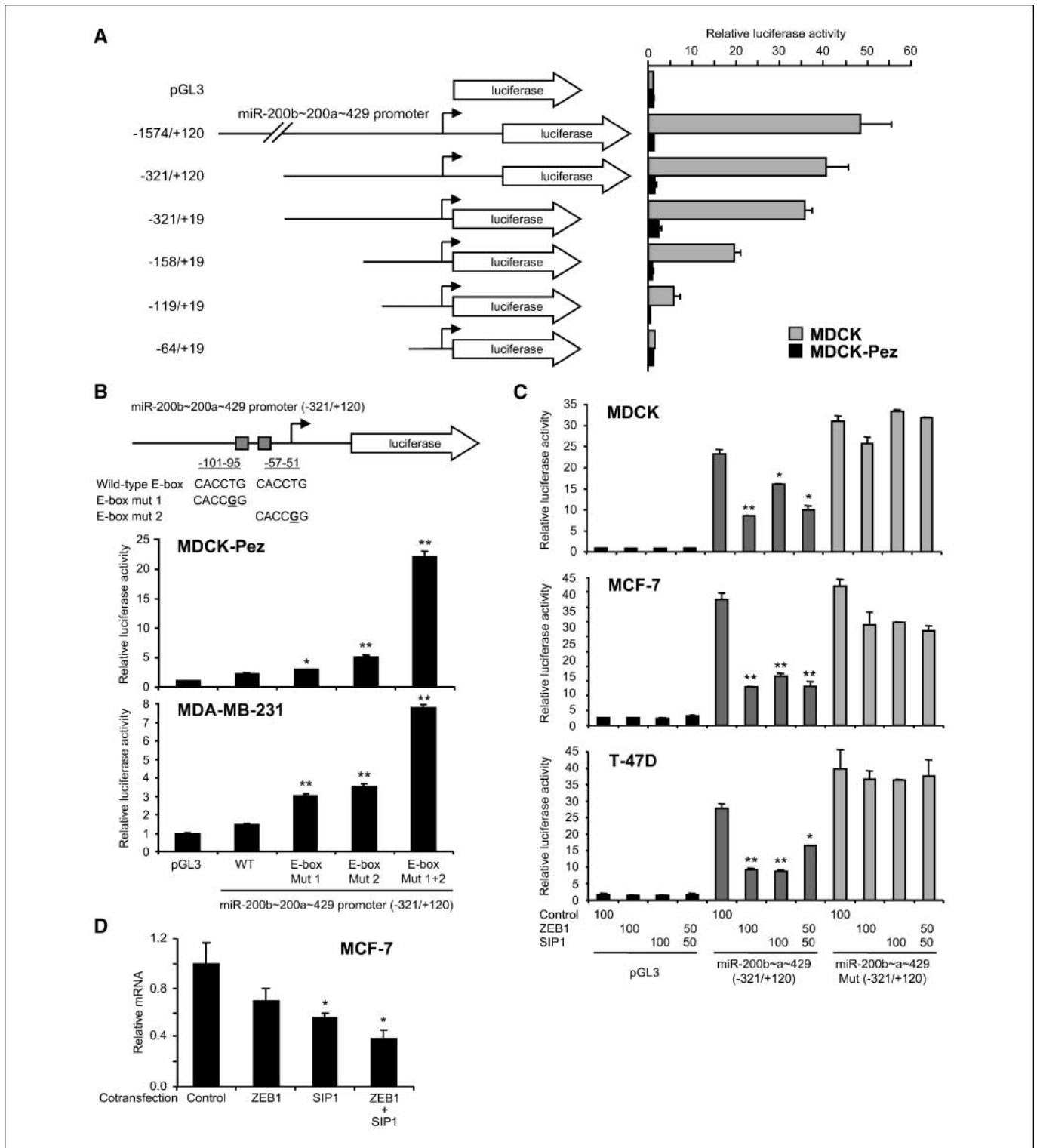


Figure 2. E-box elements and ZEB1/SIP1 expression inhibit miR-200b~200a~429 promoter activity. **A**, 5' and 3'-deletions of the human miR-200b~200a~429 promoter were cloned into a firefly luciferase reporter plasmid and transiently transfected into MDCK and MDCK-Pez cells along with a Renilla vector for normalization. Luciferase activity was measured 48 h posttransfection. **B**, a schematic diagram of the -321/+120 human miR-200b~200a~429-luciferase construct is shown with the location of the ZEB-type E-boxes, and sequence of the point mutations, also indicated. MDCK-Pez and MDA-MB-231 cells were cotransfected with Renilla and either empty vector, wild-type, or E-box mutant promoter-reporter constructs and luciferase activity determined 48 h posttransfection. Statistical significance was determined relative to wild-type promoter activity. **C**, MDCK, MCF-7, and T47-D cells were cotransfected with promoter-reporter constructs (empty vector, wild-type, or double E-box mutant) and ZEB1 and/or SIP1-expressing plasmids as indicated. Relative luciferase activity was determined 60 h posttransfection. Statistical significance was determined relative to control vector transfection. **D**, MCF-7 cells were transfected with ZEB1, SIP1, or empty pcDNA3 vector as indicated and left for 60 h before RNA extraction and qRT-PCR analysis. The pri-miR-200b~200a~429 transcript was detected using primer pair I from Fig. 1C and graphed relative to GAPDH \pm SE. Statistical significance (*, $P < 0.05$; **, $P < 0.01$) was determined relative to control vector transfection using unpaired two-tailed Student's *t* tests.

PBS, and centrifuged (1,000 rpm, 4°C for 5 min). The supernatant was removed and the pellet resuspended in 0.5 mL of lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1), protease inhibitor cocktail (SIGMA)] left on ice for 10 min, and snap frozen in liquid nitrogen. Samples were then sonicated at 4°C (6 × 20 s; Misonix Sonicator XL2007). Supernatants were recovered by centrifugation at 12,000 rpm and diluted

10-fold in dilution buffer [1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.1)]. Samples were then precleared for 2 h at 4°C with 2 µg of sheared salmon sperm DNA and 20 µL of protein A-Sepharose (GE Healthcare). Immunoprecipitations were performed overnight with ZEB1 (ZEB E-20; Santa Cruz) or IgG (Silenus) antibodies (3 µg), with the addition of BRIJ-35 detergent to a final concentration of 0.1%. The

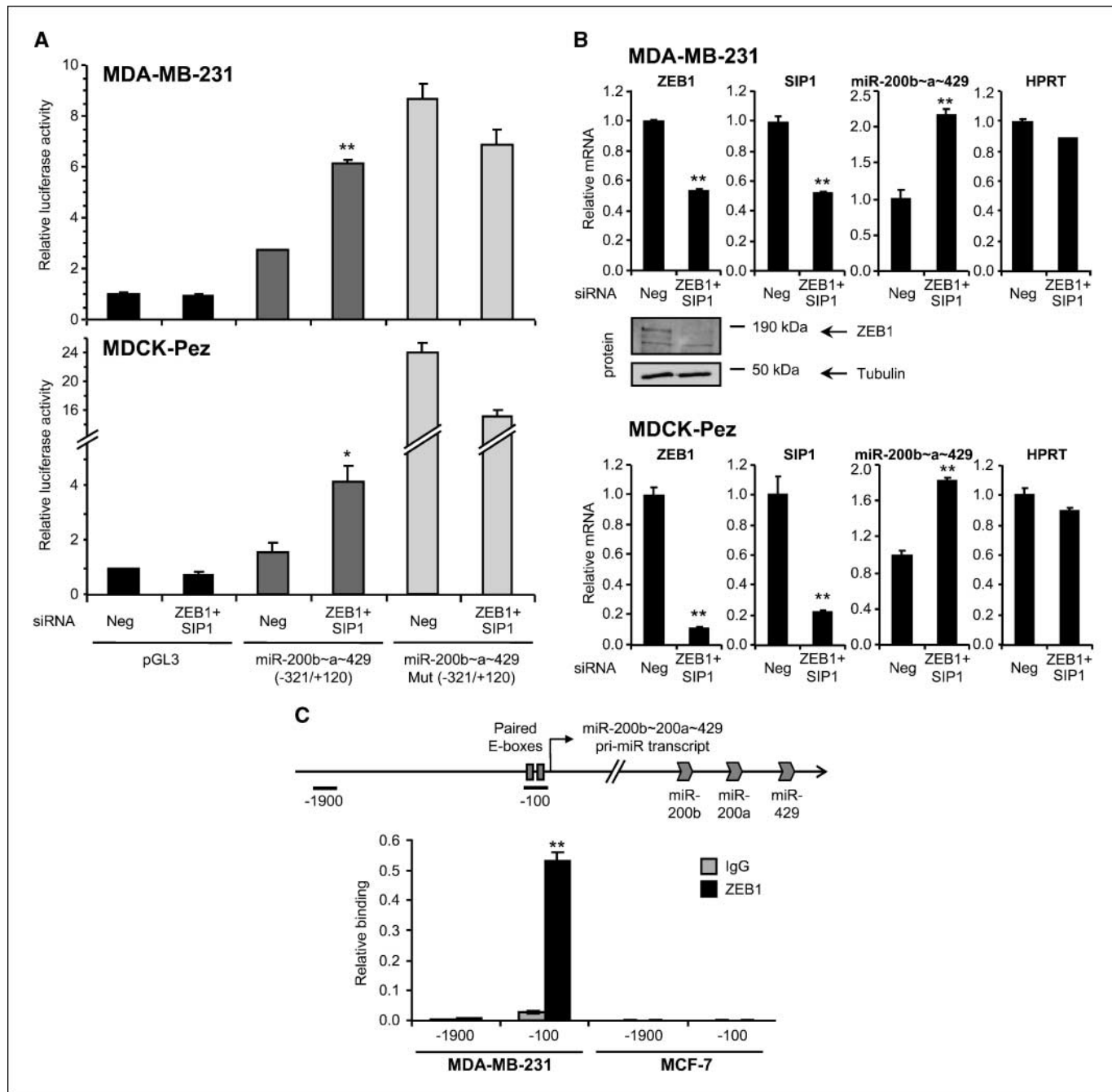


Figure 3. Endogenous ZEB1 and SIP1 directly bind and repress miR-200b~200a~429 promoter activity in mesenchymal cells. **A**, the mesenchymal cell lines MDA-MB-231 and MDCK-Pez were cotransfected with a pool of siRNAs targeting ZEB1 and SIP1, along with Renilla and either empty vector, wild-type, or E-box mutant promoter-reporter constructs as indicated. Forty-eight hours later, luciferase activity was determined. **B**, MDA-MB-231 and MDCK-Pez cells were transfected with a pool of siRNAs targeting ZEB1 and SIP1 and, 48 h later, retransfected with the same siRNAs. RNA was extracted 48 h later. The relative levels of ZEB1, SIP1, HPRT, and the miR-200b~200a~429 pri-miR (using primer pair I from Fig. 1D or primer pair F from Fig. 1C) was measured by qRT-PCR. Data are presented relative to GAPDH ± SE. Knockdown of ZEB1 protein in MDA-MB-231 cells was measured by Western Blot with tubulin as a loading control. **C**, ChIP assays were performed in MDA-MB-231 and MCF-7 cells using antibodies directed against ZEB1 or an IgG control. After immunoprecipitation, DNA was eluted and amplified by qPCR using primers designed to amplify the core promoter region flanking the E-box pair (-100) or an upstream site (-1900). Data are presented relative to PCRs using input DNA before immunoprecipitation. Statistical significance relative to controls (*, $P \leq 0.05$; **, $P \leq 0.01$) was determined using an unpaired two-tailed Student's *t* test. Neg, negative.

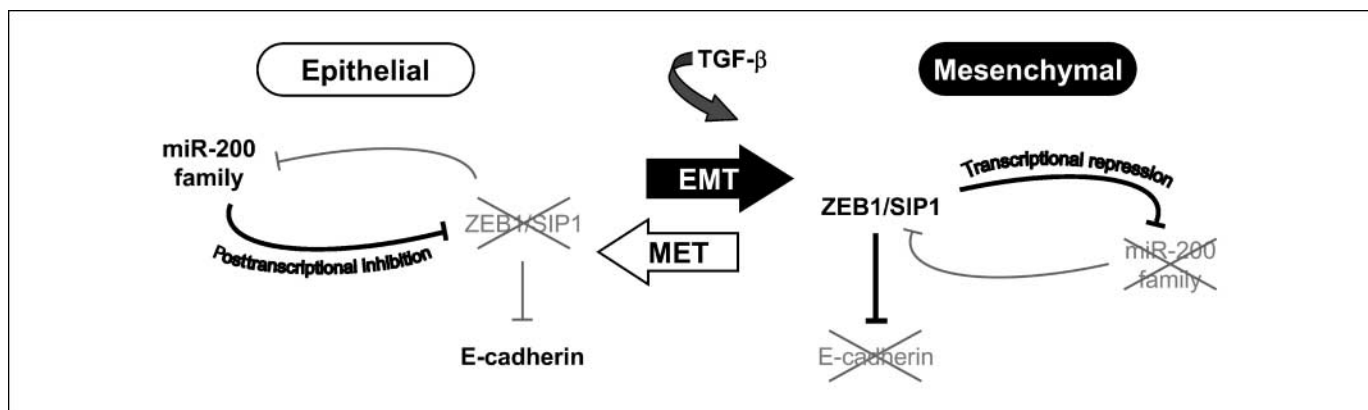


Figure 4. Model for a double negative feedback loop between ZEB1-SIP1 and the miR-200b ~ 200a ~ 429 cluster. In epithelial cells, a stable state is maintained by high levels of miR-200, which inhibits ZEB1/SIP1 and, hence, increases the expression of ZEB-repressed epithelial genes such as E-cadherin. The switch to a mesenchymal state (*EMT*) can be induced by TGF- β , which increases ZEB1-SIP1 levels. This in turn instigates and maintains a mesenchymal state through ZEB1-SIP1 binding to ZEB-type E-boxes within the miR-200b ~ 200a ~ 429 promoter and repressing miR-200 transcription. Together, this system forms a double-negative feedback loop, maintaining stable cellular states depending upon the relative levels of ZEB1-SIP1 and miR-200, yet retaining the ability to switch between these states, as exemplified by TGF- β induced EMT.

immune complexes were captured by incubation with 30 μ L of protein A-Sepharose and 2 μ g salmon sperm DNA for 1 h at 4°C. The immunoprecipitates were washed sequentially for 5 min each at 4°C in Wash Buffer 1 [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 150 mmol/L NaCl], Wash Buffer 2 [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 500 mmol/L NaCl], and Wash Buffer 3 [0.25 mol/L LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8.1)]. Beads were washed with Tris-EDTA buffer and eluted with 100 μ L of Elution Buffer (1% SDS, 0.1 mol/L NaHCO₃). Cross-links were reversed by the addition of 200 mmol/L NaCl and overnight incubation at 65°C, before DNA purification (PCR cleanup kit; QIAGEN) and qRT-PCR. The primers pairs used for PCR analysis are as follows: (–1900) 5' CAGCAGTTTTCCACCACAG 3' and 5' GAAGCTGCTCTTCTCCAAGG 3' and (–100) 5' CTGCGTCACCGTCACTGG 3' and 5' ACAACTCGCCCGTCTCTG 3'.

Fluorescent staining for E-cadherin, ZO-1, and filamentous actin. MDCK-vector, MDCK-ZEB1, and MDCK-SIP1 cells were plated onto fibronectin-coated chamber slides (BD Biosciences), and stained for E-cadherin ZO-1, and filamentous actin as previously described (8). Nuclei were visualized by costaining with 4',6-diamidino-2-phenylindole. Cells were visualized on an Olympus IX81 microscope, and pictures were taken using a Hamamatsu Orca camera. Images were analyzed with Olympus Cell^R software.

Results

Characterization of the miR-200 primary transcripts. To determine the location of the promoters that drive expression of the five members of the miR-200 family, we first sought to characterize their primary precursor transcripts. Three members of the miR-200 family (miR-200b, miR-200a, and miR-429) are clustered within a 2-kb region on chromosome 1 that is surrounded by numerous ESTs as annotated on the University of California, Santa Cruz (UCSC) genome browser (Fig. 1A; ref. 22). Ten overlapping ESTs, half of which were cloned using oligo(dT) priming, terminate just downstream of a canonical polyadenylation signal after the miR-429 hairpin. Because no other polyadenylation signal resides in this interval, we conclude the primary transcript encoding miR-429 terminates at the site 1262 bp downstream of miR-429. Upstream of miR-200b, there are eight scattered ESTs, including one (DA589106) that was cloned by a method that selects for priming from the 5' cap, indicating a potential TSS (23). This is

further supported by the presence of an overlapping conserved CpG island, a feature commonly associated with gene promoters. Taken together, these data led us to hypothesize that miR-200b, miR-200a, and miR-429 are encoded within a single independent noncoding polycistronic transcript ~7.5 kb in length, with the proximal promoter lying within a CpG island.

The miR-200c and miR-141 pre-miR hairpins are located ~400 bp apart on human chromosome 12, suggesting they are also likely to be produced also from a single primary transcript (4). Examination of the genomic location of known transcripts and ESTs in the vicinity of the microRNAs using the UCSC genome browser indicates that both microRNAs reside in the single intron of a noncoding RNA, exemplified by Genbank entry BX094730 (Fig. 1B). The close correspondence in start sites for these ESTs suggests the TSS for this RNA is ~500 bp upstream of miR-200c.

Of special interest, we noted that a conserved pair of potential binding sites for ZEB1 and SIP1 reside just upstream of the predicted TSS of both the miR-200b ~ 200a ~ 429 and miR-200c ~ 141 transcripts, raising the possibility that ZEB1 and SIP1 might regulate both genes. To test our prediction of the TSS and 3' end of the miR-200b ~ 200a ~ 429 primary transcript, we performed RT-PCR on RNA from epithelial or mesenchymal cells using primers designed to amplify segments along the length of the predicted dog and human transcripts (Fig. 1C and D). Consistent with the prediction, RT-PCR products were obtained with primers located within the predicted transcript using RNA from the canine and human epithelial cell lines, MDCK and MCF-7, but no products were obtained with primers located upstream of the predicted TSS or downstream of the putative 3' end (Fig. 1C and D). No RT-PCR products were obtained from the mesenchymal cell lines MDCK-Pez or HS-578T (Fig. 1C and D), in accordance with their lack of miR-200 family expression (8). As a control, genomic DNA was successfully amplified by all primer sets in each of the cell lines (Fig. 1C and D). To further confirm that miR-200b, miR-200a, and miR-429 are encoded on a single polycistronic transcript, we were able to amplify and sequence cDNAs containing adjacent miRNAs from MCF-7 cells (data not shown).

Characterization of the miR-200b ~ 200a ~ 429 promoter. To identify the miR-200b ~ 200a ~ 429 promoter and investigate the mechanism responsible for epithelial-specific expression of the

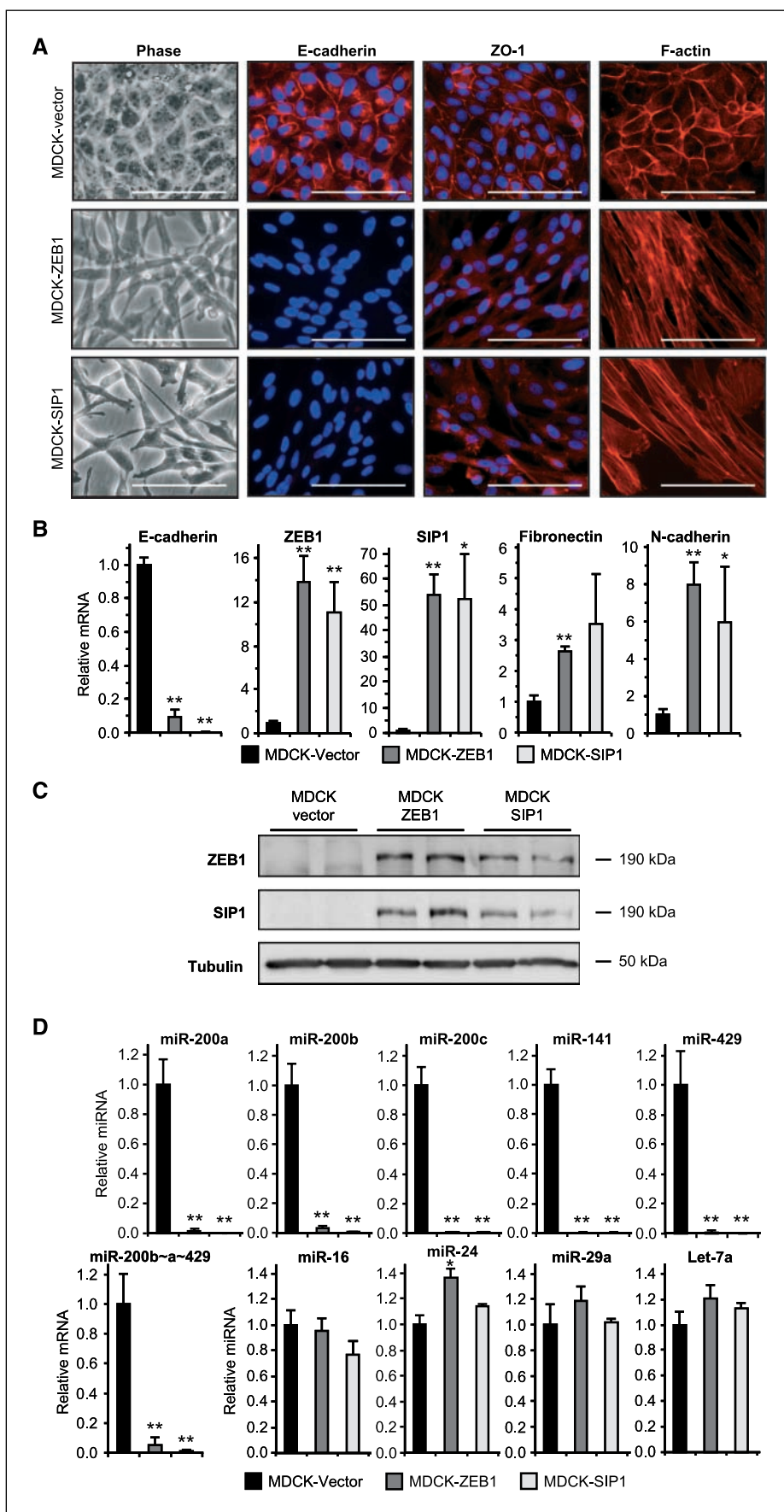


Figure 5. ZEB1 and SIP1 stable cell lines undergo EMT and repress miR-200 expression. **A**, phase contrast and immunofluorescence staining of MDCK cells stably transfected with empty vector, ZEB1, or SIP1 expression vectors. Scale bar, 100 μ m. **B**, qRT-PCR analysis of the expression of ZEB1, SIP1, and epithelial-specific (E-cadherin) or mesenchymal-specific (fibronectin, N-cadherin) genes across the stable MDCK cell lines graphed relative to GAPDH. **C**, ZEB1 and SIP1 (and tubulin control) protein levels in the MDCK stable cell lines as assessed by Western blot. **D**, expression levels of the miR-200 family (and unrelated miRNAs) as assessed by Taqman qRT-PCR in ZEB1 and SIP1 stable MDCK cells. The relative level of the miR-200b~200a~429 pri-miR was also assessed using primer pair F and shown relative to GAPDH \pm SE. Data are averages of three independent MDCK-vector and MDCK-ZEB1 clones and two independent MDCK-SIP1 clones. Statistical significance relative to controls (*, $P = <0.05$; **, $P = <0.01$) was determined using an unpaired two-tailed Student's t test.

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microRNAs, we inserted a segment of the human gene, encompassing -1574 to $+120$ relative to the putative TSS, into the pGL3-basic firefly luciferase reporter and examined its ability to drive firefly expression in MDCK and MDCK-Pez cells. This promoter region produced high luciferase activity in MDCK cells but had little activity in MDCK-Pez cells (Fig. 2A), correlating with the expression of these miRNAs in MDCK but not MDCK-Pez cells, and indicating this region of the promoter contains the regulatory elements responsible for epithelial-specific expression. To further locate the elements involved, we made a series of truncations of the promoter. Truncation of the downstream region to within 19 bp of the TSS had only a modest effect on promoter activity (Fig. 2A). Truncation of the upstream region to within 321 bp of the TSS also had little effect on activity, but further successive truncations resulted in progressive loss of activity (Fig. 2A), indicating that multiple elements between -321 and -64 contribute to the activity of the promoter. Within this region, there are multiple putative Sp1 and AP-2 α binding sites, which may contribute positively to promoter activity as well as paired E-box sites that can bind transcriptional repressors (see Fig. 2B).

E-box elements inhibit miR-200b ~ 200a ~ 429 promoter activity in mesenchymal cells. The presence of paired CACCTG (E-box) sites that are conserved across the human, mouse, rat, and canine genomes in the proximal miR-200b ~ 200a ~ 429 promoter region resembles the epithelial-specific genes *E-cadherin*, *Crumbs-3*, and *Lgl-2*, which have all been shown to be repressed by the binding of ZEB1 and SIP1 to their paired E-box elements (10, 15, 16). To determine whether the E-boxes in the miR-200b ~ 200a ~ 429 promoter confer repression of promoter activity in mesenchymal cells, single nucleotide mutations were made in one or both of these motifs in the context of the $-321/+120$ promoter (Fig. 2B), and the promoter activity was ascertained in MDCK-Pez and MDA-MB-231 cells. Mutation of either site alone had a mild activating effect (~ 2 -fold); however, the promoter became highly activated when both E-boxes were mutated, demonstrating an involvement of the paired sites in the repression of miR-200b ~ 200a ~ 429 promoter activity (Fig. 2B).

ZEB1 and SIP1 repress the miR-200b ~ 200a ~ 429 promoter in mesenchymal cells. To verify ZEB1 and SIP1 repress the miR-200b ~ 200a ~ 429 promoter, the $-321/+120$ luciferase reporter was cotransfected with ZEB1 and SIP1 into epithelial MDCK, MCF-7, and T-47D cells that lack endogenous ZEB1 and SIP1 expression. ZEB1 and SIP1 inhibited the activity of the wild-type promoter in all the cell lines tested, but had little to no effect on the $-321/+120$ mutant reporter, in which both E-boxes were mutated (Fig. 2C). Furthermore, ectopic expression of ZEB1 or SIP1 in MCF-7 cells reduced expression of the endogenous miR-200b ~ 200a ~ 429 primary transcript (Fig. 2D).

To test whether the endogenous levels of ZEB1 and SIP1 in mesenchymal cells can repress the miR-200b ~ 200a ~ 429 promoter, we cotransfected MDCK-Pez cells or MDA-MB-231 cells with siRNAs to ZEB1 and SIP1 along with the wild-type or mutant $-321/+120$ luciferase reporters. We performed simultaneous knockdown of ZEB1 and SIP1 because in our previous study, we found that knockdown of both ZEB1 and SIP1 is required for relief of E-cadherin repression, indicating a redundancy in their function (8). Knockdown of ZEB1 and SIP1 caused a 2-fold increase in activity from the wild-type promoter, but had little effect on the activity from the mutant promoter (Fig. 3A), confirming that the endogenous ZEB1 and/or SIP1 can repress the promoter in an E-box-dependent manner. The knockdown of ZEB1 and SIP1, as measured

by protein and/or mRNA levels, also caused an increase in the expression of endogenous miR-200b ~ 200a ~ 429 transcript, without affecting the levels of the unrelated HPRT transcript (Fig. 3B).

To establish that the repressive effect of ZEB1 on miR-200b ~ 200a ~ 429 transcription is a direct effect, endogenous chromatin immunoprecipitation (ChIP) assays were performed in MDA-MB-231 cells. This showed the strong and specific recruitment of endogenous ZEB1 to the paired E-box region near the TSS but not to an upstream site lacking E-boxes (Fig. 3C). As expected, no such enrichment was observed in MCF-7 cells, which are epithelial and lack ZEB1.

Epithelial versus mesenchymal phenotype is specified by a double-negative feedback loop. It was previously shown that the miR-200 family regulate EMT by targeting ZEB1 and SIP1 (8), whereas we show here that ZEB1 and SIP1 can regulate the activity of the miR-200b ~ 200a ~ 429 promoter. Furthermore, the miR-200c ~ 141 promoter has a conserved pair of E-boxes near the TSS and is similarly subject to repression by ZEB1 (24). Together, these observations indicate that ZEB1 and/or SIP1 and the miR-200 family can reciprocally regulate each other in a double-negative feedback loop (Fig. 4). Consistent with this, we previously showed that ectopic expression of miR-200a and miR-200b represses ZEB1 and SIP1 and switches mesenchymal MDCK-Pez cells to an epithelial-like phenotype (8). To test whether the reciprocal event predicted by the double-negative feedback loop model occurs, we stably transfected MDCK cells with expression vectors encoding the ZEB1 or SIP1 proteins but devoid of their natural 3'UTRs to avoid inhibition of expression by the miR-200 family. A number of the resulting clones from both the ZEB1 and SIP1 transfections displayed the hallmarks of EMT, including a change in morphology from round compact shape to spindle shaped. This was accompanied by the loss of E-cadherin and the tight junction protein ZO-1 from the plasma membrane, and a rearrangement of actin filaments from a cortical to a stress-fiber pattern (Fig. 5A). Real-time PCR confirmed that E-cadherin mRNA was strongly down-regulated and that the mesenchymal markers fibronectin and N-cadherin were induced (Fig. 5B). Interestingly, overexpression of ZEB1 led to the induction of SIP1, whereas overexpression of SIP1 induced expression of ZEB1, at both the mRNA and protein levels (Fig. 5B and C), suggesting both factors are able to elicit a full EMT reprogramming of the cell. Consistent with the model, the primary miR-200b ~ 200a ~ 429 transcript and the mature miR-200 family microRNAs were strongly down-regulated in the MDCK-ZEB1 and MDCK-SIP1 cells, whereas other unrelated microRNAs were unchanged in level (Fig. 5D).

The E-boxes in the miR-200b ~ 200a ~ 429 promoter confer repression in human breast cancer cells. We have previously shown that epithelial human breast cancer cells such as MCF-7, MDA-MB-361, and T-47D express the miR-200 family but do not express ZEB1 or SIP1, whereas mesenchymal breast cancer lines such as HS-578T, MDA-MB-231, MDA-MB-435, and BT-549 express ZEB1 and/or SIP1 but do not express the miR-200 family (8). To assess whether the feedback regulation of ZEB1 and SIP1 on the miR-200b ~ 200a ~ 429 promoter occurs in human breast cancer cells, we measured the activity of the wild-type $-321/+120$ promoter and the double E-box mutant promoter in several breast cancer cell lines. The promoter was active in the epithelial cell lines and was unaffected by mutation of the E-boxes (Fig. 6), as expected in cells that do not express ZEB1 or SIP1. In contrast, the wild-type promoter had little activity in the mesenchymal cell lines but was activated by two- to sixfold in response to mutation of the

E-boxes (Fig. 6). Thus, the negative feedback loop of inhibition of the miR-200b ~ 200a ~ 429 promoter by ZEB1 and/or SIP1 occurs in mesenchymal breast cancer cells.

Discussion

MicroRNA primary transcripts are products of RNA Polymerase II activity that may be encoded as either independent transcriptional units, or contained within mRNA introns, thereby sharing the same transcript as their protein-coding host genes. Both the paralogous miR-200b ~ 200a ~ 429 and miR-200c ~ 141 clusters are in their own dedicated transcripts, and hence, their expression can be independently controlled without any restriction imposed by the expression requirements of a host gene. We have identified a well-conserved, compact core promoter for miR-200b ~ 200a ~ 429, with all of the elements required for epithelial-specific expression within a 300-bp region upstream of the TSS. We further showed that miR-200b ~ 200a ~ 429 transcription is negatively regulated by the ZEB1 and SIP1 transcriptional repressors, and mediated through paired ZEB-type E-box motifs located within this core 300-bp region. A similar situation has very recently been reported for the miR-200c ~ 141 cluster, with ZEB1 negatively regulating expression through paired E-boxes (24). ZEB1 and SIP1 are in turn negatively regulated by the miR-200 family, generating a double-negative feedback loop. Feedback loops feature in a number of genetic pathways involving microRNAs, where they seem to enhance the functionality and robustness of gene networks (25, 26).

As a general model, the double-negative feedback loop is advantageous in allowing the system to remain reversible. This feature is evident in our previous observations that the switchability between epithelial and mesenchymal states can be achieved solely by manipulation of miR-200 levels (8). In that study, MDCK cells could be triggered to undergo EMT by inhibition of miR-200, whereas the mesenchymal derivative MDCK-Pez cells revert to an epithelial-like phenotype (a MET) by ectopic miR-200 expression (8). Here, we have shown the ectopic expression of the miR-200 targets, ZEB1 and SIP1, is sufficient to induce EMT of MDCK cells, whereas knockdown of both factors was previously found to promote MET-like phenotypic changes in MDCK-Pez cells (8). Similar phenotypic

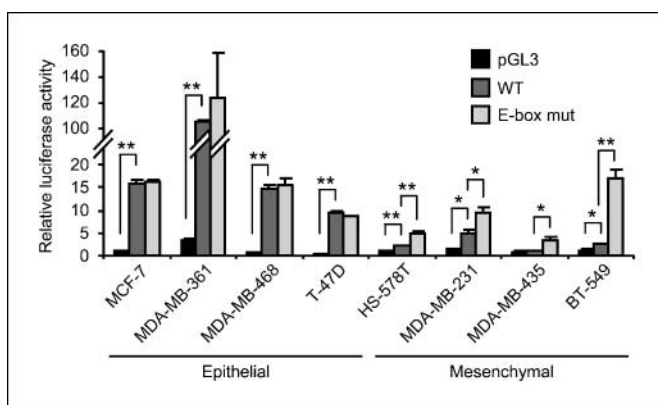


Figure 6. The miR-200b ~ 200a ~ 429 promoter is repressed in an E-box-dependent manner in human mesenchymal breast cancer cells. A panel of human breast cancer cell lines, divided into epithelial or mesenchymal-like phenotypes, was cotransfected with empty, wild-type, or E-box mutant promoter-luciferase plasmids along with Renilla for normalization. Luciferase activity was determined 48 h posttransfection and presented \pm SE. Statistical significance of the wild-type (WT) and E-box mutant (*E-box mut*) relative to wild-type (WT); *, $P < 0.05$; **, $P < 0.01$) was determined using an unpaired two-tailed Student's *t* test.

effects of ZEB1 or SIP1 manipulation have also been observed in a range of human cancer cell lines resulting in a change in their invasive capacity (15, 16, 27, 28). This has bolstered the hypothesis that ZEB1, SIP1, and other EMT-inducing factors can promote tumor metastasis via a reversible process, whereby EMT is required for cells to break away from the primary tumor, invade through the basement membrane, and intravasate into the bloodstream, with a subsequent MET needed for metastases to form at a secondary site (19). In concordance with this proposal, both ZEB1 and SIP1 have been implicated in the progression of multiple human cancers (29–34). This is especially well-characterized in colon cancer, where ZEB1 up-regulation selectively occurs within dedifferentiated cells at the invasive front and correlates with poor patient survival and distant metastasis (33). However, it is likely that several oncogenic events precede the acquirement of invasive properties of a cell and its ability to metastasise (35, 36). For example, mutation of the *APC* gene in colorectal cancer leads to nuclear β -catenin localization and signaling, facilitating subsequent EMT-like processes (37). After initiation of EMT, the presence of a reversible, double-negative feedback system could efficiently facilitate changes in ZEB1 and SIP1 levels endowing cells with the capacity to invade but subsequently to undergo MET to form a metastasis.

Another property of a double-negative feedback loop is that while enabling reversibility, it also allows the maintenance of bistable states, even after cessation of the inducing signal. One could envisage this property being important in processes where irreversible cellular differentiation is required, wherein changes in cell state are buffered from fluxes of signaling that are below a threshold level. As an illustration of this concept, the differentiation of progenitor cells to photoreceptor cells in *Drosophila* was found to be mediated through a double-negative feedback loop involving the transcription factor Yan and miR-7 (38). This feedback loop results in the mutually exclusive expression of Yan and miR-7, whose levels can be reciprocally altered by transient epidermal growth factor receptor signaling. In the case of the miR-200 family and ZEB1-SIP1, we predict that the transition between epithelial and mesenchymal phenotypes is determined by the level of ZEB1 and SIP1, whereby once a critical threshold level for miR-200 promoter repression (or relief of repression) is reached, induction of the double-negative feedback loop is precipitated, leading to cellular reprogramming and generation of a bistable state. Transforming growth factor (TGF)- β signaling is a key inducer of ZEB1 and SIP1 expression and EMT, and we have preliminary evidence suggesting the up-regulation of these factors precedes the loss of mature miR-200 expression after which the double-negative feedback loop would be initiated (data not shown). One consequence of this model is that cells that had been induced to undergo EMT by TGF- β would remain in a mesenchymal state after TGF- β withdrawal, so long as the level of ZEB1-SIP1 had exceeded the threshold for miR-200 promoter repression. This position may be reflected in mesenchymal breast cancer cell lines where ZEB1- and/or SIP1-mediated repression of the miR-200 family seems to be maintained (8). However, TGF- β 1 mRNA levels were recently shown to be significantly higher in mesenchymal than epithelial cell lines of the NCI-60 cancer cell line panel and this correlated with high levels of ZEB1 and SIP1 and low levels of the miR-200 family (9), suggesting the double-negative feedback loop may be stabilized through sustained TGF- β signaling. EMT induced by members of the TGF- β /BMP family are also important during embryonic development in processes such as delamination of the neural crest and palate formation (39, 40). Interestingly, mice

with homozygous deletion in ZEB1 display defects in these processes consistent with a MET having occurred (41). Thus, the double-negative feedback loop between ZEB1-SIP1 and miR-200 may also be important in specifying an epithelial or a mesenchymal state during both embryonic development and tumorigenesis.

In summary, we have identified a feedback mechanism controlling ZEB1-SIP1 and miR-200 family expression, which sheds new light on the regulation of epithelial-mesenchymal transition. Modulation of this pathway may be a method of inhibiting primary tumor invasion through restoring miR-200 expression and preventing EMT. In this regard, manipulation of microRNAs has recently been shown to be readily achievable *in vivo* and holds exciting promise for potential therapeutic applications involving the miR-200 family (42, 43).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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