Regulation of the MicroRNA 200b (miRNA-200b) by Transcriptional Regulators PEA3 and ELK-1 Protein Affects Expression of Pin1 Protein to Control Anoikis*§

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Background: The role of miRNA-200b in the regulation of anoikis is not well understood.
Results: miRNA-200b controls anoikis by targeting Pin1, and its expression is regulated by PEA3 and ELK-1.
Conclusion: Pin1 acts as a functional target gene of miRNA-200b.
Significance: These findings characterize the self-regulation of miRNA-200b and its role in the regulation of anoikis.

MicroRNA (miRNA) 200s regulate E-cadherin by directly targeting ZEB1/ZEB2, which are transcriptional repressors of E-cadherin. Decreased expression of E-cadherin results in cancer cells losing interaction with the extracellular matrix and detaching from the primary tumor. Normally, cells will undergo anoikis after losing interaction with the extracellular matrix. Cancer cells must, therefore, possess the ability to resist anoikis during the process of metastasis. Here we show that miRNA-200b regulates anoikis by directly targeting the 3’ UTR of Pin1 mRNA and regulating Pin1 expression at the translational level. We found that down-regulation of miRNA-200b promotes cancer cells survival during metastasis, and the homeless state of these cells resulted in decreased expression of miRNA-200b in the MCF-7 cell line. We also found that expression of miRNA-200b is down-regulated in human breast cancer during lymph node metastasis, which has a significant negative correlation with Pin1 expression. Two members of the ETS (E-26) family (PEA3 and ELK-1) regulate the expression of miRNA-200b. PEA3 promotes the expression of miRNA-200b, and ELK-1 is a transcriptional repressor of miRNA-200b. In addition, miRNA-200b regulates the activity of PEA3 and ELK-1 via the Pin1-pERK pathway and forms self-regulated feedback loops. This study characterizes the role of miRNA-200b in the regulation of anoikis and demonstrates the regulation of its own expression in the process of metastasis.

Metastasis is the most important feature of malignant tumors. The basic steps of metastasis include local invasion, intravasation, survival in the circulation, extravasation, settlement to new sites, and colonization (1). When cancer cells leave the primary tumor, they must survive in a homeless state. The ability to resist anoikis is required, and the regulation of this ability involves multiple signaling mechanisms.

MicroRNAs (miRNAs)2 are 19–23 nucleotides in length and are small non-coding RNA molecules that can regulate gene expression posttranscriptionally by interacting with the 3’ UTR of target mRNAs. A variety of miRNAs have been shown to be associated with cancer metastasis (2–5). Among them, the miRNA-200s family (including five members that can be grouped into two subfamilies according to their function (miRNA-200a, miRNA-141 and miRNA-200b, miRNA-200c, and miRNA-429)) affect cancer metastasis by regulating the epithelial-mesenchymal transition process as a silencer of ZEB1/2, which are the transcriptional repressors of E-cadherin (6–9). Down-regulation of miRNA-200s decreases E-cadherin expression, causing cancer cells to easily detach from the primary tumor and resulting in cancer cells in a homeless state. These cancer cells must then acquire the ability to resist anoikis. A recent study revealed an important role of the miRNA-200s family in anoikis (10), showing for the first time that miRNA-200c mediated anoikis by targeting TrkB. However, whether the other members of miRNA-200s family are involved in the regulation of anoikis remains unknown.

Peptidyl-prolyl cis/trans isomerase (PPIase/Pin1) specifically binds to phosphorylated serine/threonine-proline (Ser/Thr-Pro) motifs to catalytically regulate the post-phosphorylation conformation of its substrates. The conformational regulation catalyzed by this peptidyl-prolyl cis/trans isomerase has a major effect on key proteins involved in the regulation of cell growth, germ cell development, neuronal differentiation, and survival. Pin1 also plays an important role in tumorigenesis and metastasis (11–17). Recently, it has been reported that Pin1 can affect cancer cell anchorage-independent growth (15, 16, 18). Furthermore, it is critical for the regulation of pERK and pAKT stability and activation, which can suppress anoikis (19–21).

Promoter analysis showed that there are two binding sites for each PEA3 and ELK-1, two members of the ETS family, in the miRNA-200b promoter region. The functions of the ETS family

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2 The abbreviations used are: miRNA, microRNA; FCM, flow cytometry method; SUMO, small ubiquitin-like modifier; ETS, E-26; poly-HEMA, poly(2-hydroxyethyl methacrylate).
are widely involved in cellular differentiation, cell cycle control, cell proliferation, and apoptosis. These proteins function either as transcriptional activators and/or repressors of numerous genes, and the deregulation of the ETS family of proteins is associated with many human cancers (22–26).

In this study, we demonstrate that miRNA-200b can regulate Pin1 expression at the translational level and control anoikis through the Pin1-pERK/pAKT pathway. The homeless state reduces the expression of miRNA-200b, and miRNA-200b regulates its own expression through two feedback loops, miRNA-200b-pELK-1 and miRNA-200b-PEA3 sumoylation. The results suggest that cellular regulation of miRNA-200b can control anoikis in cancer cells and, thereby, play an important role in metastasis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Clinical Specimens**—MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics in a 5% CO2 atmosphere at 37 °C. MDA-MB-231 cells were maintained in L-15 (contains 2 mM L-glutamine (Shanghai, China). siRNA, and ELK-1 siRNA were synthesized by Genepharma.

32 cells were maintained in L-15 (contains 2 mM L-glutamine (Shanghai, China). The ethics committee of the Cancer Institute and Hospital, China approved this study. Informed consent was obtained from each patient.

**Oligonucleotide, siRNA, and Plasmids**—MiRNA-200b, miRNA-200c and miRNA-429 mimics, miRNA-200b inhibitor, PEA3 siRNA, and ELK-1 siRNA were synthesized by GenePharma (Shanghai, China).

The full-length 3′ UTR of Pin1 mRNA was cloned from MCF-7 cDNA reverse-transcribed from total RNA and inserted into the pL50 luciferase plasmid to generate pL50-Pin1-3′ UTR. A mutant construct of the Pin1 3′ UTR, named pL50-Pin1–3′ UTRMut, which carried a substitution of three nucleotides within the binding site of the Pin1 3′ UTR, was made using a site-directed mutagenesis kit (SBS Genetech, Beijing, China).

The promoter of miRNA-200b was cloned from MCF-7 genomic DNA and inserted into the pGL3-basic plasmid to generate the pGL3-miRNA-200b-promoter (PGL3-B-P). Mutants of the PEA3 and ELK-1 binding sites in the miRNA-200b promoter were named PEA3 PUM, PEA3 PDM, PEA3 P2M, ELK-1 EUM, ELK-1 EDM, and ELK-1 E2M. Each contained a substitution of three nucleotides within the two predicted binding sites of PEA3 or ELK-1, which was constructed using a site-directed mutagenesis kit (SBS Genetech).

The plasmid for expression of miRNA-200b was pGCsilencer (GeneChem). The plasmid for expression of Pin1, PEA3, and ELK-1 was PCMV (Origene). The plasmid for expression of SUMO1 with the FLAG tag was provided by Prof. Jinke Cheng (60).

**Transient Transfection**—Plasmid DNA was introduced into cells using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Briefly, 1 × 106 cells were plated in each well of a 6-well plate and incubated overnight. Plasmid DNA and Lipofectamine 2000 were diluted in Opti-MEM, coincubated for 20 min, and then added into each well of the 6-well plate. Cells were incubated with the mixture in regular medium and harvested at indicated time points for specific experiments.

The miRNA mimics (20 nm), miRNA inhibitor (50 nm), and siRNA (20 nm) were transfected using HiperFect (Qiagen, Düsseldorf, Germany) according to the recommendations of the manufacturer. The cells (2 × 104) were plated in each well of a 6-well plate and incubated overnight. Mimics, inhibitors, siRNA, and HiperFect were diluted in Opti-MEM, coincubated for 10 min, and then added to the wells. Cells were incubated with the mixture in regular medium and harvested at indicated time points for specific experiments.

**Total RNA Isolation, RT-PCR, and Quantitative Real-time PCR**—Total RNA was extracted with TRIzol reagent (Invitrogen), and conventional quantitative RT-PCR was done using SYBR Premix Ex Taq (Takara, Dalian, China) according to the recommendations of the manufacturer. Quantification of miRNA-200b/c/429 was performed using stem-loop real-time PCR (61). Sequences and primers for transfection and PCR are shown in [supplemental Table S1](#). For RT-PCR, 2 × TaqPCR StarMix with loading dye (GenStar, Beijing, China) was used, and U6 snRNA served as an internal control.

**FCM Assay**—The cells (1 × 10⁶) were cultured in 60 mm plates and transfected with miRNA.NC or miRNA-200b mimics by using HiperFect (Qiagen). After transfection for 36 h, cells were cultured for 24 h and harvested. For adhesion cultured cells, cells were harvested after transfection for 60 h. The flow cytometry method (FCM) assay was performed using annexin-propidium iodide double staining (BSC, Beijing, China) according to the instructions of the manufacturer.

**Luciferase Reporter Assays**—Cells were cultured in 96-well plates, and cotransfection was performed using Lipofectamine 2000 (Invitrogen). The pRL-CMV Renilla was used as an internal control. Luciferase activity was measured with the Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the instructions of the manufacturer.

**Immunoblotting, Immunoprecipitation, and Antibodies**—For Western blotting, 30 µg of protein extracted from cultured cells or tumor tissues was separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked and blotted with relevant antibodies. Horseradish peroxidase-conjugated secondary antibodies were detected by the LAS400 system (FuJLM).

For immunoprecipitation, protein lysates (100 µg) prepared from cultured cells were used. Immunocomplex pull-down was achieved via overnight incubation of protein lysates with anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO) at 4 °C. After careful washing, loading buffer was added, and the samples were boiled at 100 °C for 5 min. Immunoprecipitated proteins were then subjected to Western blotting as described above.
The following antibodies were used to detect specific proteins: Pin1 (Millipore, Temecula, CA); PEA3, ELK-1, ERK, pERK, AKT, poly(ADP-ribose) polymerase, and pAKT (Santa Cruz Biotechnology, Santa Cruz, CA); pELK-1 (CST, Danvers, MA); cyclin D1 (BGI, Beijing, China); proliferating cell nuclear antigen and caspase 3 (ZSGB-BIO, Beijing, China); and /H9252-actin (Sigma-Aldrich).

Chromatin Immunoprecipitation Assays—MCF-7 cells, grown in DMEM, were cross-linked in 1% formaldehyde for 10 min at 37 °C. DNA from fixed chromatin cells were then subjected to immunoprecipitation using a ChIP assay kit (Millipore) and antibodies against PEA3 and ELK-1 or anti-rabbit IgG. Purified DNA was analyzed by PCR with the primers 5/H11032-GACCGTTT-GTCGTTTCATTA-3/H11032 and 5/H11032-GCCATACCTGCCTGTCTT-3/H11032, which produced a 473-bp fragment of the miRNA-200b promoter between /H11002 and /H11002. Primers 5/H11032-CACCTGTGCAGGTCTGAA-3/H11032 and 5/H11032-ACC-GGCTTCGGAAGGAAT-3/H11032 produced a 187-bp fragment of the miRNA-200b promoter containing the PEA3 and ELK-1 binding sites between /H11001 and /H11001.

FIGURE 1. MiRNA-200b promotes cell anoikis. A, MDA-MB-231 cells grown under anchorage-free conditions after miRNA-200b transfection. MDA-MB-231 cells were suspension-cultured in poly-HEMA-coated dishes for 24 h after miRNA-200b mimic transfection for 36 h (left panel). The histograms show the number of cells calculated using the BIO-RAD TC10 automatic cell counter, and error bars denote mean ± S.E. (right panel). B, Western blot analysis showing the expression of poly(ADP-ribose) polymerase (PARP) and caspase 3. MDA-MB-231 cells were either adhesion-cultured or suspension-cultured after miRNA-200b mimic transfection for 36 h. Cells were harvested at the 12, 24, 36, and 42 h points. C, Flow cytometry showing apoptosis after miRNA-200b mimic transfection. MDA-MB-231 (top panels) and Hs578T (bottom panels) cells were either adhesion-cultured for 60 h after miRNA-200b mimic transfection or suspension-cultured for 24 h after miRNA-200b mimic transfection for 36 h. The histogram shows the apoptotic cell percentage detected by FCM, and error bars denote mean ± S.E. (right panels). Experiments were performed in triplicate, and the histograms in the right panels show the statistical results of FCM experiments (MDA-MB-231 cells, p = 0.6 for adhesion cultures and p < 0.01 for suspension cultures; Hs578T, p = 0.4 for adhesion cultures and p < 0.01 for suspension cultures).

The following antibodies were used to detect specific proteins: Pin1 (Millipore, Temecula, CA); PEA3, ELK-1, ERK, pERK, AKT, poly(ADP-ribose) polymerase, and pAKT (Santa Cruz Biotechnology, Santa Cruz, CA); pELK-1 (CST, Danvers, MA); cyclin D1 (BGI, Beijing, China); proliferating cell nuclear antigen and caspase 3 (ZSGB-BIO, Beijing, China); and /H9252-actin (Sigma-Aldrich).

Chromatin Immunoprecipitation Assays—MCF-7 cells, grown in DMEM, were cross-linked in 1% formaldehyde for 10 min at 37 °C. DNA from fixed chromatin cells were then subjected to immunoprecipitation using a ChIP assay kit (Millipore) and antibodies against PEA3 and ELK-1 or anti-rabbit IgG. Purified DNA was analyzed by PCR with the primers 5/GACCGTGTGTTTCATTA-3/G and 5/GCCATACCTGCTGTCTT-3/G, which produced a 473-bp fragment of the miRNA-200b promoter containing the PEA3 and ELK-1 binding sites between +927 and +687. Primers 5/CACCTGTGCAGGTCTGAA-3/C and 5/ACCGCCTCGGAAAGGAAT-3/C produced a 187-bp fragment of the miRNA-200b promoter containing the PEA3 and ELK-1 binding sites between +105 and +144.
Caspase-Glo 3/7 Assays—Caspase-Glo 3/7 assays (Promega) were performed following the instructions of the manufacturer. Transfections were performed in a 48-well plate with a final concentration of 20 nM miRNA-200b mimic/well using three replicates.

Proliferation Assay—Cell proliferation was measured using a CellTiter 96 AQ One Solution cell proliferation assay (Promega) following the instructions of the manufacturer.

Statistical Analysis—Data are presented as mean ± S.E., and Student’s t test (two-tailed) or analysis of variance was used to compare different groups (p < 0.05 was considered significant) for independent samples. Paired Student’s t test was used to compare paired samples (p < 0.05 was considered significant). The correlation between miRNA-200b and Pin1 expression was analyzed by Spearman’s analysis (correlation is significant at the 0.05 level, two-tailed).

RESULTS

Overexpression of miRNA-200b in MDA-MB-231 Cells Promotes Anoikis—To investigate whether miRNA-200b regulates anoikis, we transfected miRNA-200b mimics into MDA-MB-231 cells and suspended cultured cells in poly-HEMA-coated dishes for 24 h after miRNA-200b transfection for 36 h (27). For comparison, MDA-MB-231 cells were adhesion-cultured for 60 h after miRNA-200b transfection. We found that overexpression of miRNA-200b in suspension-cultured MDA-MB-231 cells increased the number of apoptotic cells and the activity of caspase 3 (Fig. 1, A and B). FCM also showed that overexpression of miRNA-200b in suspension-cultured MDA-MB-231 cells increased the number of apoptotic cells. In contrast, no difference was detected after miR-200b transfection in adhesion-cultured cells. The same phenomenon was also observed in Hs578T cells by FCM (Fig. 1C).

MiRNA-200b Regulates Pin1 Expression at the Translational Level by Targeting Its 3’ UTR—To characterize the molecular mechanism of miRNA-200b in the regulation of anoikis, we used TargetScan and miRbase to predict the target genes of miRNA-200b/c/429. The results showed that Pin1 is one of the target genes and that there is a miRNA-200b/c/429 binding site at nucleotides 111–117 of Pin1–3’UTR. Moreover, when we used TargetScan to search for miRNAs that target Pin1 mRNA, we found that miRNA-200b/c/429 was the only family that could target Pin1 (Fig. 2A). To determine the regulation of miRNA-200b/c/429 on Pin1 expression, we first detected miRNA-200b/c/429 and Pin1 expression in two breast cancer cell lines and found that miRNA-200b/c/429 expression in MCF-7 cells was higher than that in MDA-MB-231 cells, whereas Pin1 expression was the opposite (supplemental Fig. S1A). We then transfected miRNA-200b/c/429 mimics into MDA-MB-231 cells, showing that only miRNA-200b decreased Pin1 expression, whereas miRNA-200c and miRNA-429 did not (supplemental Fig. S1B). To further characterize the effect of miRNA-200b on Pin1, we transfected miRNA-200b mimics into MDA-MB-231 cells and miRNA-200b inhibitor into MCF-7 cells. The results showed that overexpression of miRNA-200b decreased Pin1 expression, whereas knockdown of miRNA-200b increased Pin1 expression. However, expression of Pin1 mRNA did not show any changes (Fig. 2, B and C).

To further examine whether miRNA-200b can regulate Pin1 expression in other cell lines, we transfected miRNA-200b mimics in MDA-MB-453 and Hs578T cells, and the results showed that miRNA-200b can also regulate Pin1 expression in both cell lines (Fig. 2D). Luciferase reporter assays using the 3’UTR of Pin1 indicated that miRNA-200b regulates Pin1 expression by targeting the putative binding site and that several nucleotide substitutions in the core binding site could restore the suppressive effect (Fig. 2E). Taken together, these results suggest that miRNA-200b regulates Pin1 expression at the translational level.

MiRNA-200b Regulates Anoikis through the Pin1-pERK/pAkt Pathway—Because Pin1 can regulate cell anchorage-independent growth and the stability and activation of pERK and pAkt, and because it has been reported that stably expressing miRNA-200b can abolish AKT and ERK1/2 activation (28), we hypothesized that miRNA-200b may control anoikis by regulating Pin1 expression. To test this possibility, we transfected miRNA-200b and/or Pin1 into MDA-MB-231 cells and suspension-cultured the cells in poly-HEMA-coated dishes. The results showed that overexpression of miRNA-200b increased

FIGURE 2. MiR-200b regulates Pin1 expression. A, the human Pin1 3’ UTR and target site predicted by TargetScan and mutation of the Pin1 3’ UTR. B and C, Western blotting showing the expression of the Pin1 protein (top panels) and quantitative real-time PCR showing the expression of Pin1 mRNA (bottom panels) after miRNA-200b mimic (B) or miRNA-200b inhibitor (C) transfection of MDA-MB-231 or MCF-7 cells, performed separately. Cells were harvested after transfection for 48 h. D, Western blotting showing the expression of the Pin1 protein after miRNA-200b mimic transfection in MDA-MB-453 and Hs578T cells. Cells were harvested after transfection for 48 h. E, the luciferase reporter assay shows the suppression of miRNA-200b on Pin1–3’UTR luciferase activity in MCF-7 cells. Error bars indicate the firefly luciferase activities normalized to Renilla luciferase activities. Overexpression of miRNA-200b suppressed p50-Pin1–3’UTR luciferase activity (***, p = 0.001) but had no effect on mutant constructs.
the number of apoptotic cells, whereas coexpression with Pin1 reversed the change (Fig. 3A). Western blotting showed that pERK/pAKT changed correspondingly (Fig. 3B). FCM results also confirmed that coexpression of Pin1 reversed the apoptosis induced by overexpression of miRNA-200b. These results suggest that miRNA-200b regulation of anoikis, at least in part, depends on its regulation of Pin1 (Fig. 3C). To determine whether miRNA-200b controls anoikis through the ERK and/or AKT pathways (29), we treated MDA-MB-231 cells with pERK and/or pAKT inhibitors and then determined the activity of caspase 3/7. MDA-MB-231 cells were exposed to U0126 and/or LY294002 under anoikis conditions after miRNA-200b mimic transfection. Cells were transfected with miRNA-200b mimics and then suspension-cultured on poly-HEMA-coated dishes for 24 h to induce anoikis and an additional 24 h (still on poly-HEMA) with either the pERK inhibitor U0126 (20 μM) or the pAKT inhibitor LY294002 (20 μM) as well as a combined treatment with U0126 (20 μM) and LY294002 (20 μM). The p values were calculated between the miRNA.NC and miRNA-200b groups using Student’s t test (*, p < 0.05 was considered statistically significant; **, p < 0.01).

**FIGURE 3. MiR-200b promotes cell anoikis via regulating Pin1 expression.** A, MDA-MB-231 cells grown under anchorage-free conditions after miRNA-200b and Pin1 transfection. MiRNA-200b mimics and Pin1 were cotransfected in MDA-MB-231 cells. Cells were suspension-cultured for 24 h after cotransfection for 36 h (top panel). The histograms show the number of cells calculated using the BIO-RAD TC10 automatic cell counter, and error bars denote mean ± S.E. (bottom panel). B, Western blot analysis showing the expression of Pin1, pAKT, and pERK after miRNA-200b mimic and/or Pin1 cotransfection. Cells were suspension-cultured for 24 h after cotransfection for 36 h and then harvested. C, FCM showing apoptosis after miRNA-200b mimic and/or Pin1 cotransfection (top panel). The histogram shows the apoptotic cell percentage detected by FCM, and error bars denote mean ± S.E. (bottom panel). The results were analyzed using Student’s t test and show that coexpression with Pin1 can restore cells from anoikis caused by overexpression of miRNA-200b (p < 0.01). D, activity of caspase 3/7 was detected after miRNA-200b mimic transfection. Cells were transfected with miRNA-200b mimics and then suspension-cultured on poly-HEMA-coated dishes for 24 h to induce anoikis and an additional 24 h (still on poly-HEMA) with either the pERK inhibitor U0126 (20 μM) or the pAKT inhibitor LY294002 (20 μM) as well as a combined treatment with U0126 (20 μM) and LY294002 (20 μM). The p values were calculated between the miRNA.NC and miRNA-200b groups using Student’s t test (*, p < 0.05 was considered statistically significant; **, p < 0.01).
pared with those without lymph node metastasis. Statistical analysis revealed that the expression of miRNA-200b and Pin1 in human breast cancer samples had a significant negative correlation (Fig. 4C, \( r = -0.431, p = 0.014 \)), suggesting that the correlation between reduced expression of miRNA-200b and breast cancer metastasis was closely related to the miRNA-200b regulation of Pin1 in these breast cancer specimens.

**Expression of miRNA-200b Is Regulated by PEA3 and ELK-1**

To investigate the transcriptional regulation of miRNA-200b, we performed a promoter analysis and found that one of two binding sites of PEA3 and ELK-1 exists in the promoter of miRNA-200b (30, 31). We then used siRNA to knock down the expression of PEA3 and ELK-1 and found that knockdown of PEA3 decreased and knockdown of ELK-1 increased the expression of miRNA-200b (Fig. 5A). ChIP showed that PEA3 and ELK-1 could bind both of the two predicted binding sites (Fig. 5B). To further explore the functions of PEA3 and ELK-1 in miRNA-200b regulation, a 1729-bp promoter region (−1574 to +155) of miRNA-200b was constructed with the pGL3-basic plasmid (pGL3-B-P) (Fig. 5C). Luciferase reporter assays in MCF-7 cells showed that the interaction of PEA3 with predicted binding sites could promote the activity of the miRNA-200b promoter (\( p < 0.01 \)) and that the interaction of ELK-1 could inhibit the activity of the miRNA-200b promoter (\( p < 0.01 \)) (Fig. 5D). As shown in Fig. 5D, mutation in the binding site of PEA3 between +105 and +111 (PEA3 PDM) significantly reduced relative luciferase activity (\( p < 0.01 \)), which suggested that this region had an important role in PEA3 binding. The binding site between +137 and +144 also had an important role in ELK-1 binding because mutation of this binding site (ELK-1 EDM) resulted in a significantly greater reduction in luciferase activity (\( p < 0.01 \)).

Because pERK can promote the phosphorylation of ELK-1 and sumoylation of PEA3 (32–34), and because Pin1 can isomerize the structure of pERK (35, 36), we hypothesized that overexpression of miRNA-200b may influence phosphorylated ELK-1 and sumoylated PEA3. To test this possibility, miRNA-200b mimics and Pin1 were cotransfected into MDA-MB-231 cells. Western blotting showed that overexpression of miRNA-200b decreased the expression of pELK-1, whereas coexpression with Pin1 can increase the expression of pELK-1 (Fig. 6A). MCF-7 cells were cotransfected with miRNA-200b mimics, Pin1, and FLAG-tagged SUMO1. Immunoprecipitation showed that overexpression of miRNA-200b decreased sumoylated PEA3, whereas coexpression with Pin1 can increase the expression of sumoylated PEA3 (Fig. 6B). These results established a positive feedback loop between miRNA-200b and pELK-1 and a negative feedback loop between miRNA-200b and sumoylated PEA3 through the Pin1-pERK pathway.

**The Homeless State Can Reduce the Expression of miRNA-200b**

We showed that miRNA-200b could regulate anoikis through the Pin1-pERK/pAKT pathway and, therefore, hypothesized that the homeless state could change the expression of miRNA-200b. Suspension-cultured cells in polyHEMA-coated dishes were used to imitate the homeless state. MCF-7 cells were suspension-cultured for 48 h, and, as a control, MCF-7 cells were adhesion-cultured for 48 h. The results showed that the homeless state decreased the expression of miRNA-200b and, subsequently, increased the expression of...
Pin1 and pERK (Fig. 6C). It has been reported that losing cell/matrix adhesion can activate pERK (37), and we found that pELK1 expression was also increased in these cells. Because pERK can regulate ELK1 phosphorylation, we suggested that increased expression of pERK up-regulates pELK1 and further decreases miRNA-200b expression in the homeless state. In addition, because overexpression of miRNA-200b can decrease pERK expression in suspension-cultured MDA-MB-231 cells (Fig. 3B), we speculated that decreased expression of miRNA-200b can regulate pERK expression in the homeless state through the miRNA-200b-Pin1 pathway.

It has been reported that miRNA-200b can regulate cell proliferation. We speculated that the homeless state decreased miRNA-200b expression and resulted in inhibiting proliferation. Western blot analysis shows that proliferating cell nuclear antigen expression is decreased in the homeless state (Fig. 6C). Further, MCF-7 cells were adhesion- or suspension-cultured after miRNA-200b inhibitor transfection for 36 h, and cell pro-

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**FIGURE 5.** Expression of miRNA-200b is regulated by PEA3 and ELK-1. A, the expression level of miRNA-200b after siRNA of PEA3 or ELK-1 transfection. Left panel, quantitative PCR for miRNA-200b expression. Right panel, Western blotting for PEA3 (top right panel) and ELK-1 (bottom right panel) expression after siRNA transfection. Cells were harvested after siRNA transfection for 48 h. B, ChIP assays were performed in MCF-7 cells for both of the putative binding sites of PEA3 and ELK-1 in the miRNA-200b promoter. C, schematic of the −1574 to +155 human miRNA-200b luciferase (Luc) construct. The locations of the putative PEA3 or ELK-1 binding elements and the mutations are indicated. D, luciferase reporter assays showing the stimulation of PEA3 (p = 0.002) (left panel) and suppression of ELK-1 (p < 0.01) (right panel) on the miRNA-200b promoter in MCF-7 cells. Error bars indicate the firefly luciferase activities normalized to Renilla luciferase activities.
liferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt) assay. The results showed that decreased miRNA-200b expression can significantly inhibit cell proliferation in the homeless state (Fig. 6D).

The situation of miRNA-200b expression in human cancer is complex. MiRNA-200s have been reported to be up-regulated, down-regulated or to remain unchanged in human cancers (38–40). In breast cancer, miRNA-200s expression level depends on cell differentiation and cancer subtype (41, 42). It is generally believed that miRNA-200s expression is decreased in breast cancer cells that acquired a metastatic phenotype (7, 31, 43, 44). We detected miRNA-200b expression in 12 cases of human breast cancer and adjacent nontumorous tissues (supplemental Fig. S2A and Table S2), but no significant difference was found between tumorous and nontumorous tissues. Subsequently, miRNA-200b expression was detected in 12 cases of human breast cancer and adjacent nontumorous tissues (supplemental Fig. S2A and Table S2), but no significant difference was found between tumorous and nontumorous tissues. Subsequently, miRNA-200b expression was detected in non-metastatic and metastatic breast cancer cell lines. As shown in supplemental Fig. S2B, miRNA-200b expression in non-metastatic cell lines is obviously higher than in metastatic cell lines.

Taken together, we suggest that, when cancer cells detached from primary tumor, the homeless state results in increasing pERK expression and, thus, decreasing expression of miRNA-200b. This down-regulation of miRNA-200b affects pERK expression and, in turn, controls anoikis through the Pin1-pERK pathway.

DISCUSSION

MiRNA-200s play a key role in the process of epithelial-mesenchymal transition and mesenchymal-epithelial transition via regulating the expression of E-cadherin by directly targeting ZEB1/ZEB2, which are transcriptional repressors of E-cadherin. As an adhesion molecule, E-cadherin mediates cell-cell and cell-matrix interactions. Following reduced expression of E-cadherin, cancer cells are easily detached from the primary tumor to promote metastasis. However, detachment from the primary tumor also results in loss of interaction with the matrix and induction of anoikis, which is believed to act as a barrier to metastasis (45, 46). Resistance to anoikis may allow survival of cancer cells during systemic circulation, thereby facilitating secondary tumor formation in distant organs (47–49). The aim of this study was, therefore, to characterize the possible role of miRNA-200b in the regulation of anoikis.

In this study, we identified Pin1 as a direct target of miRNA-200b. By interacting directly with the 3’ UTR of Pin1 mRNA, miRNA-200b regulates Pin1 expression at the translational level. Pin1 specifically binds to phosphorylated Ser/Thr-Pro motifs to catalytically regulate the post-phosphorylation conformation of pERK and pAKT (19–21). Overexpression of miRNA-200b in suspension-cultured MDA-MB-231 cells reduced the expression of pERK/pAKT and increased the number of anoikis cells, whereas coexpression of Pin1 with miRNA-200b restored the expression of pERK/pAKT and decreased the number of anoikis cells. In addition, FCM showed that overexpression of miRNA-200b in suspension-cultured cells promoted anoikis, whereas coexpression of Pin1 with miRNA-200b restored the occurrence of anoikis. Moreover, the activity of caspase 3/7 increased when cells were exposed to U0126 and LY294002 in suspension culture after miRNA-200b mimic
MiRNA-200b Regulates Anoikis

We subsequently detected the expression levels of miRNA-200b and Pin1 in human breast cancer tissues and found that expression of miRNA-200b was significantly lower in metastatic tissues than in non-metastatic tissues, whereas the expression of Pin1 was the reverse. Moreover, expression of miRNA-200b and Pin1 showed a significant negative correlation in these breast cancer tissues. These results suggest that miRNA-200b regulation of Pin1 may be important for the metastasis of breast cancer.

In conclusion, when cancer cells detach from primary tumors and lost interaction with the matrix, the expression of miRNA-200b was reduced. Reduced miRNA-200b expression resulted in cancer cells acquiring the ability to resist anoikis through the Pin1-pERK/pAKT pathway by regulating Pin1 and/or other expressions. In addition, reduced miRNA-200b expression caused the inhibition of cancer cell proliferation during metastasis. The expression of miRNA-200b was maintained at an appropriate level through two feedback loops, miRNA-200b-pELK-1 and miRNA-200b-PEA3-sumoylation. These findings provide a better understanding of the role of miRNA-200b in the molecular pathogenesis of cancer metastasis, suggesting that miRNA-200b could be a candidate for the treatment and prognosis of breast cancer.

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