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MicroRNAs miR-199a-5p and -3p Target the Brm Subunit of SWI/SNF to Generate a Double-Negative Feedback Loop in a Variety of Human Cancers

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Abstract

The chromatin remodeling complex SWI/SNF is an important epigenetic regulator that includes one Brm or BRG1 molecule as catalytic subunit. Brm and BRG1 do not function identically, so this complex can regulate gene expression either positively or negatively, depending on the promoter to which it is recruited. Notably, Brm attenuation due to posttranscription suppression occurs often in human tumor cells, in which this event contributes to their oncogenic potential. Here, we report that the 3′-untranslated region of *Brm* mRNA has two sites that are efficiently targeted by the microRNAs miR-199a-5p and -3p, revealing a novel mechanism for modulation of Brm-type SWI/SNF activity. Computational mapping of the putative promoter region of *miR-199a-2* (miPPR-199a-2) has defined it as the major contributing genetic locus for miR-199a-5p and-3p production in these tumor cell lines. We validated this predicted region by direct promoter analysis to confirm that Egr1 is a strong positive regulator of the *miR-199a-2* gene. Importantly, we also showed that Egr1, miR-199a-5p, and miR-199a-3p are expressed at high levels in Brm-deficient tumor cell lines but only marginally in Brm-expressing tumor cells. Finally, we also obtained evidence that Brm negatively regulates *Egr1*. Together, our results reveal that miR-199a and Brm form a double-negative feedback loop through Egr1, leading to the generation of these two distinct cell types during carcinogenesis. This mechanism may offer a partial explanation for why miR-199a-5p and -3p have been reported to be either upregulated or down-regulated in a variety of tumors. *Cancer Res; 71(5); 1–10. ©2010 AACR.*

Introduction

The human SWItch/Sucrose Nonfermentable (SWI/SNF) complex is a chromatin-remodeling factor that plays key roles in epigenetic gene regulation (1, 2). The complex itself consists of about 10 protein subunits and contains a single molecule of either Brm or Brahma-related gene 1 (BRG1) as the catalytic subunit responsible for DNA-dependent ATPase activity (3). SWI/SNF can interact with either transcriptional activators such as c-Myc (4), C/EBP-β (5) AP-1 (activator protein 1; ref. 6), Cdx2 (7), p53 (8), and NF-κB (nuclear factor κB; ref. 9) or repressors such as neuron-restrictive silencer factor (NRSF; ref. 10) and methyl CpG binding protein2 (MECP2; ref. 11). These transcriptional regulators recruit the complex to different gene promoters, leading to transcriptional activation or repression (12). In addition, it is now known that the Brm-type and BRG1 type SWI/SNF complexes regulate a set of promoters that do not fully overlap (7, 9, 13, 14).

Accumulating evidence indicates that alterations to SWI/SNF subunits contribute to tumorigenesis. In terms of the Brm catalytic subunit, we and others have reported that it is frequently deficient in various cancer cell lines such as SW13, AZ521, and C33A (15) and also in primary tumors of the lung (16), stomach (14), and prostate (17). We have, however, further found that a functional *Brm* gene is present and actively transcribed in all of the Brm-deficient cell lines that we tested in nuclear run-on transcription assays (15, 18). This indicated that posttranscriptional gene silencing suppresses *Brm* in these human cancer cells. When we exogenously expressed Brm in such deficient cell lines, this resulted in a reduced oncogenic potential, suggesting that Brm has tumor-suppressive properties. This possibility is consistent with our observations that a frequent loss of Brm expression

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in gastric cancers correlates with a less differentiated state (14). However, the molecular mechanisms underlying the posttranscriptional suppression of Brm remained to be elucidated.

MicroRNAs (miRNA) are potent regulators of coding genes at the posttranscriptional level, including human genes (19). These molecules are approximately 22-nucleotide noncoding RNAs that mediate the repression of target transcripts by suppressing their translation or promoting degradation (20). In our current study, we show that *Brm* mRNA is a target of miR-199a-5p and miR-199a-3p, both of which are processed from pre-miR-199a. By promoter analysis of the *miR-199a-2* gene, which was found to be the main contributor to the production of pri-miR-199a in these cell lines, Egr1 was identified as a potent transcriptional activator at this gene locus. We further show from our analysis that the expression patterns of mature miR-199a-5p and -3p, and of the Brm protein, are mutually exclusive in many human cancer cell lines and in some human tumor cells. We finally present evidence that a double-negative feedback mechanism underlies this regulatory network and separates many cancer cell lines into 2 distinct groups.

Materials and Methods

Cell culture

The following human cell lines were used in this study: SW13 [adrenocortical carcinoma; SW13 (vim^-)] was used as a subtype of SW13 because it is strictly deficient in Brm and BRG1; ref. 18]; AZ521 and Kato III (gastric carcinoma); NCI-H522, A549, and NCI-H1299 (non–small cell lung carcinoma); C33A and HeLaS3 (cervical carcinoma); PA-1 (embryonic carcinoma); MDA-MB435 (breast ductal carcinoma); HEK-293FT (embryonic kidney, Invitrogen); KB (oral carcinoma); SW620 and HT29 (colorectal carcinoma); LNCap (prostate carcinoma); and TIG-3 (embryonic lung fibroblasts). All cultures were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. NCI-H522, NCI-H1299, C33A, PA-1, MDA-MB435, KB, SW620, and HT29 cell lines were purchased from the American Type Culture Collection. AZ521, Kato III, A549, HeLaS3, and LNCap cell lines and TIG-3 cell line were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan, and from the Health Science Research Resources Bank, Osaka, Japan, respectively. All the cell lines were passaged for fewer than 3 months after resuscitation.

Lentiviral vector and construction of stable cell lines

Vesicular stomatitis virus-G–pseudotyped lentiviral vectors were produced with the prepackaging cell line HEK-293FT, using the ViraPower Lentiviral Expression System (Invitrogen), in accordance with the manufacturer's instructions. Transductions were then carried out as described previously (21).

RT-PCR

Total RNA was extracted using a mirVana microRNA Isolation kit (Ambion). For the amplification of *Brm*, *BRG1*, *Egr1*, *199a-2/214* transcripts, and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) mRNA, semiquantitative reverse transcriptase PCR (RT-PCR) was carried out using Superscript one-step RT-PCR with Platinum Taq (Invitrogen). Details for quantitative analysis and all primer pairs and PCR conditions are described in Supplementary Information and Supplementary Table S2A, respectively.

Luciferase assays

Following reporter plasmid transfections, dual- or single-luciferase reporter assays (Promega) were carried out according to the manufacturer's instructions. pGL4.74-*Renilla* (Promega) was used as the internal control in the dual-luciferase assay. Luciferase activity was measured using GloMax (Promega).

Results

The 3′-untranslated region of *Brm* mRNA has 2 sites targeted by miR-199a-5p and miR-199-3p, respectively

We computationally screened for miRNA candidates that target *Brm* mRNA, using available algorithms. Among these, 2 candidates with high scores were predicated by PicTar, miR-199a-5p, and miR-199a-3p. Both of these miRNAs are produced from a single molecule, pre-miR-199a, and have been reported to be upregulated in cervical (22) and ovarian (23) cancers but downregulated in liver (24) and oral (25) cancers. We thus focused on these candidates in our analyses and evaluated whether they truly target *Brm* mRNA via the predicted binding sites in the 3′-untranslated region (UTR; Fig. 1A).

*Ikk*β was previously reported as the target genes for miR-199a-5p (26), whereas *fibronectin* and *versican* were identified as targets of miR-199a-3p (27). To identify the distinct mRNA targets of miR-199a-5p and -3p, we utilized our recently developed RNA duplex transfection technique. Using this method, we can selectively load miR-199a-5p or -3p onto an RNA-induced silencing complex (RISC), taking advantage of the phenomenon whereby chemical modification with 1-[3, 5-bis(hydroxymethyl)-phenyl]-4-(dimethylamino) naphthalene (DANap) on the 5′-end of one strand of the duplex supports only the selective loading of the unmodified strand onto RISCs in the transfected cells (28). In our current experiments, MDA-MB435 cells, which express marginal levels of endogenous miR-199a-5p/-3p, were transfected with 5p/DANap-5os and 3p/DANap-3os to express exogenous miR-199a-5p and miR-199a-3p, respectively (Supplementary Fig. S1). Subsequent Western blot analysis revealed a specific reduction in the Ikkβ protein levels in 5p/DANap-5os–transfected cells when compared with mock-transfected controls. In contrast, the fibronectin expression levels were reduced as expected in cells transfected with 3p/DANap-3os (Fig. 1B). Importantly, Brm protein was found to be reduced in cells expressing either 5p/DaNap-5os or 3p/DaNap-3os compared with the mock-transfected cells (Fig. 1B).

To achieve the functional inhibition of specific miRNAs, we previously developed an efficient decoy RNA molecule and designated as TuD RNA (tough decoy RNA; ref. 21). Selective

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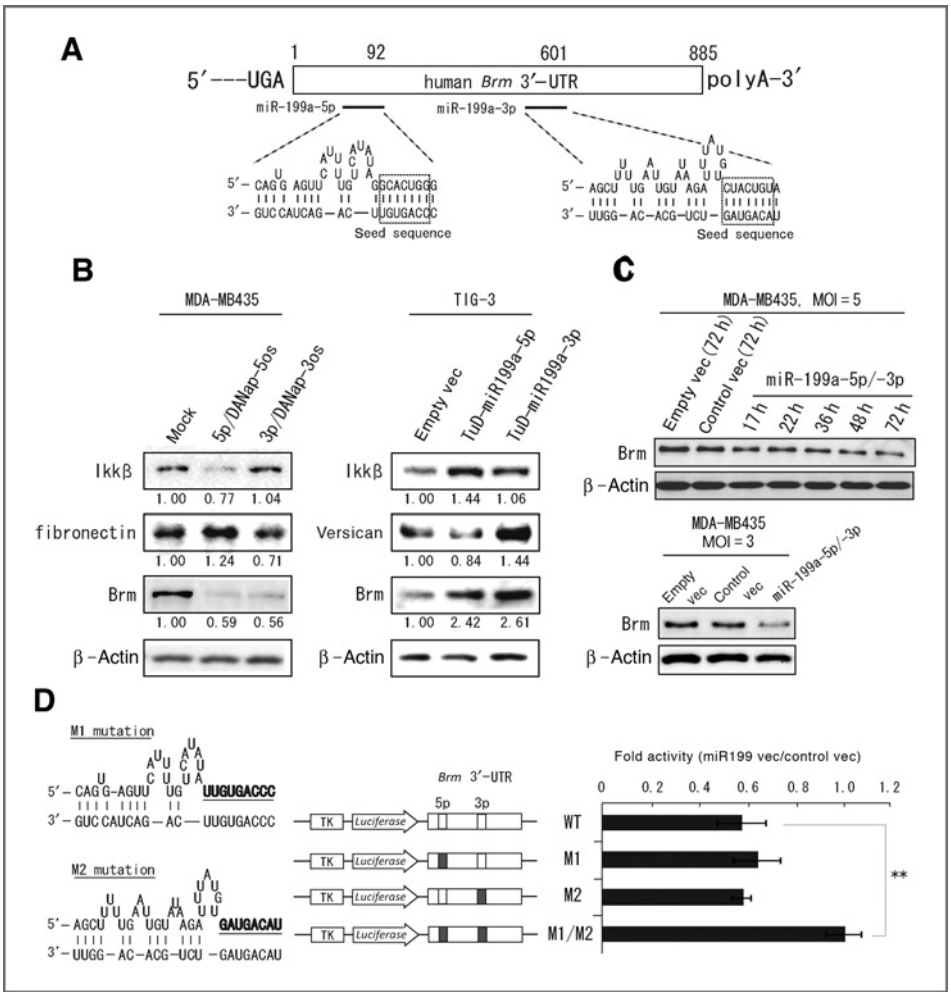

Figure 1. Either miR-199a-5p or miR-199a-3p targets *Brm* mRNA. A, the 3′-UTR region of *Brm* mRNA and putative binding sites for miR-199a-5p and miR-199a-3p. B, detection of target proteins by both high-level expression and suppression of miR-199a-5p and -3p. MDA-MB435 cells were transfected with 30 nmol/L DANap-modified miRNA duplexes. After 48 hours, protein extracts were prepared. TIG-3 cells were transfected with TuD-miR199a-5p, TuD-miR199a-3p, or empty vector, and protein extracts were prepared after 24 hours. The expression of Ikkβ, fibronectin, versican, Brm, and β-actin was analyzed by Western blotting. Each band was quantified by LAS4000, and relative expression levels (compared with mock or empty vector) are shown after normalization to the internal control β-actin as an average of 2 to 3 independent experiments. C, expression of Brm protein in MDA-MB435 cells transduced with lentiviral vectors expressing miR-199a-5p/-3p or control (sh-U3), and an empty vector at a multiplicity of infection of 5 or 3. After transduction, total cell proteins were prepared at the times indicated, and the Brm and β-actin proteins were detected by Western blotting. D, activities of the firefly reporter gene fused to *Brm* 3′-UTR (WT) or its mutants (M1, M2, and M1/M2) measured in the presence or absence of exogenously expressed miR-199a-5p and -3p. M1 carries a mutation in the putative binding site of miR-199a-5p, whereas M2 is mutated in the putative binding site of miR-199a-3p. In the M1/M2 construct, both sites are mutated. HEK-293FT cells were cotransfected with pTK4.12-*Brm* 3′-UTR (WT, M1, M2, or M1/M2; 30 ng) and pGL4.74 (1 ng) with miR-199a-5p/-3p or control vector (sh-lacZ expression vector; 700 ng). Cells were disrupted 24 hours after transfection to enable dual-luciferase measurements. Fold activities indicate the ratio of miR-199a-5p or -3p expression to that of the control vector. **, $P < 0.01$, 1-tailed t test of triplicate. vec, vector.

inhibition of miR-199a-5p and -3p by either TuD-miR199a-5p or TuD-miR199a-3p was confirmed using reporter assays (Supplementary Figs. S2 and S3). In TIG-3 cells, which express high levels of endogenous miR-199a-5p and -3p, TuD-miR199a-5p expression, specifically elevated the expression levels of *Ikk*β, whereas the TuD-miR199a-3p transfection enhanced those of *versican* as expected (Fig. 1B). In addition, both TuD-miR199a-5p and -3p elevated the Brm expression levels. Overall, therefore, the results of these experiments indicated that either miR-199a-5p or -3p negatively regulates Brm production.

When miR-199a-5p/-3p expression lentiviral vector was transduced into MDA-MB435 cells, we observed a clear reduction in the Brm protein expression levels at 2 to 3 days or even 1 month after transduction compared with cells transduced with empty or control vectors (Fig. 1C). We next constructed a luciferase reporter vector covering the entire 3′-UTR region of *Brm* and also mutated reporter constructs in which putative binding sites of either miR-199a-5p (M1) or miR-199a-3p (M2) were singly or doubly (M1/M2) disrupted (Fig. 1D). These reporter vectors were transfected into HEK-293FT cells, which express marginal levels of endogenous miR-199a-5p and -3p.

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Cotransfection of the miR-199a-5p/-3p vector reduced the WT, M1, and M2 reporter luciferase activities to about 60% compared with cotransfection of the control vector. However, the M1/M2 reporter was unaffected in this experiment, indicating that both putative recognition sites need to be disrupted for the efficient release from the suppressive effects of miR-199a-5p and -3p (Fig. 1D). Overall, these results support the contention that Brm is a specific target of both miR-199a-5p and -3p in these cell lines.

Structural characterization of the *miR-199a-2* gene and its promoter

To study the regulatory mechanisms underlying miR-199a-5p/-3p production, we examined the expression status of the 2 human genes, *miR199a-1* (on chromosome 19) and *miR-199a-2* (on chromosome 1), that potentially contribute to pre-miR-199a transcription in cancer cell lines. Using several primer pairs designed to discriminate between pri-*miR-199a-1* and pri-*miR-199a-2*, we carried out a series of RT-PCR experiments with SW13 and C33A cells, both of which express miR-199a-5p and -3p at high levels. The results showed that only *miR-199a-2* transcripts were detectable by these assays (data not shown), and we tentatively concluded that *miR-199a-2* is the major contributor to miR-199a-5p/-3p production in these cells.

In mouse, *miR-199a-2* and *miR-214* have been reported to be produced from a single intron-less transcript, DNM3os (29). We carried out a series of RT-PCR assays, using RNA extracted from SW13 cells and several primer pair combinations that recognize the *miR-199a-2* and *miR-214*–embedded regions, and detected no unspliced transcripts but several transcripts harboring both *miR-199a-2* and *miR-214* which have some splicing site differences (Fig. 2A). These results indicate that, as in mouse, both *miR-199a-2* and *miR-214* are transcribed from the same promoter as a single transcript but that the human transcripts are spliced in several ways.

The putative promoter region of *miR-199a-2* (miPPR-199a-2) was previously predicted using a genome-wide algorithm for *miRNA* gene promoters that was developed in our laboratory and designated as miPPR-199a-2 (30, 31). Using this information, we conducted primer extension experiments to determine the transcriptional start site (TSS) of this gene. We found 2 major TSSs, thymine and neighboring cytosine, just downstream to TATA box in miPPR-199a-2 (Fig. 2A; Supplementary Figs. S4–S6), consistent with our RT-PCR data (Supplementary Fig. S5). From these analyses, we concluded that miPPR-199a-2 is indeed the promoter for *miR-199a-2*/*miR-214* in SW13 cells.

Egr1 occupies the *miR-199a-2*/*miR-214* gene promoter and induces its expression

To elucidate the crucial transcriptional regulators involved in *miR-199a-2* gene expression, we screened for transcriptional factor binding sites in miPPR-199a-2, using online prediction programs, and thereby identified putative binding sites for AP-1, NF-κB, Egr1, and E-box binding proteins (Supplementary Fig. S6). Among these sites, we were interested in 3 consensus regions for Egr1 (#1–#3), a transcription factor whose expression is transiently increased in response to several stimuli (32). Like miR-199a, Egr1 has often been shown to be either upregulated [prostate cancers (33)] or downregulated [breast cancers (34) and gliomas (35)] in different tumor types. We conducted a knockdown of Egr1, using short hairpin RNA (shEgr1), in SW13 cells, which express *Egr1* transcripts at high levels, and found that this caused the reduction of mature miR-199a-5p/-3p and miR-214 levels, eventually leading to an accumulation of *Brm* mRNA (Fig. 2B). This accumulation of Brm would likely be due to a release from the suppressive effects of miR-199a-5p and -3p. When MDA-MB435 cells, which marginally express endogenous *Egr1*, were transfected with an Egr1 expression vector, the expression of miR-199a-5p was elevated (Supplementary Fig. S7A). On the basis of these results, we focused on Egr1 as a strong positive regulator of *miR-199a-2*/*miR-214*.

We constructed a *luciferase* reporter plasmid driven by miPPR-199a-2, which was found to respond to the Egr1 expression vector in a dose-dependent manner in MDA-MB435 cells (Supplementary Fig. S7B). The reporter plasmids and its mutants on the putative Egr1 binding sites were transfected into AZ521 cells, which express *Egr1* at high levels, together with the shEgr1 expression vector or a control hairpin vector (shGFP). The *Egr1* knockdown caused a significant reduction of relative luciferase activity when WT, M1, and M2 reporters were used. In the control vector introduced cells, the luciferase activity was specifically reduced for the M3 and M1/M2/M3 reporters to levels that were similar to those observed in the shEgr1-transfected cells (Fig. 2C). These results collectively indicate that Egr1 binding site #3 is most critical for *miR-199a-2* expression in AZ521 cells. Consistently, when chromatin immunoprecipitation (ChIP) analyses were carried out using SW13 cells (Fig. 2D) and AZ521 cells (Supplementary Fig. S8), Egr1 was shown to specifically occupy the DNA fragment covering site #3 but not the fragments covering sites #1 and #2 of miPPR-199a-2, nor some fragments that do not include Egr1 binding sites. From these results, we conclude that *miR-199a-2*/*miR-214* transcription is activated by Egr1 mainly through its direct binding to the #3 site in miPPR-199a-2 and thereby suppresses Brm protein expression.

Brm negatively regulates the expression of *Egr1* mRNA and of mature miR-199a-5p and -3p

To compare the expression profiles of mature miR-199a-5p, -3p, -214, *pri-miR-199a-2/214* transcripts, and also those of the Brm, BRG1, and Egr1 proteins, we collected both RNAs and proteins from a series of human cancer cell lines. Interestingly, these expression patterns could be classified into 2 cell groups, that is, Brm-deficient cell lines, in which miR-199a-5p, -3p, or Egr1 are expressed at high levels, and Brm-expressing cell lines, in which miR-199a-5p, -3p, and Egr1 are marginally expressed (Fig. 3; Supplementary Figs. S9 and S10). Although these results are consistent with our earlier results (Figs. 1 and 2), we speculated that the distinct cellular categorization obtained here might further reveal the presence of underlying robust regulatory mechanisms to stabilize these expression patterns. We therefore next tested whether Brm can itself affect Egr1 and miR-199a-5p/-3p expression.

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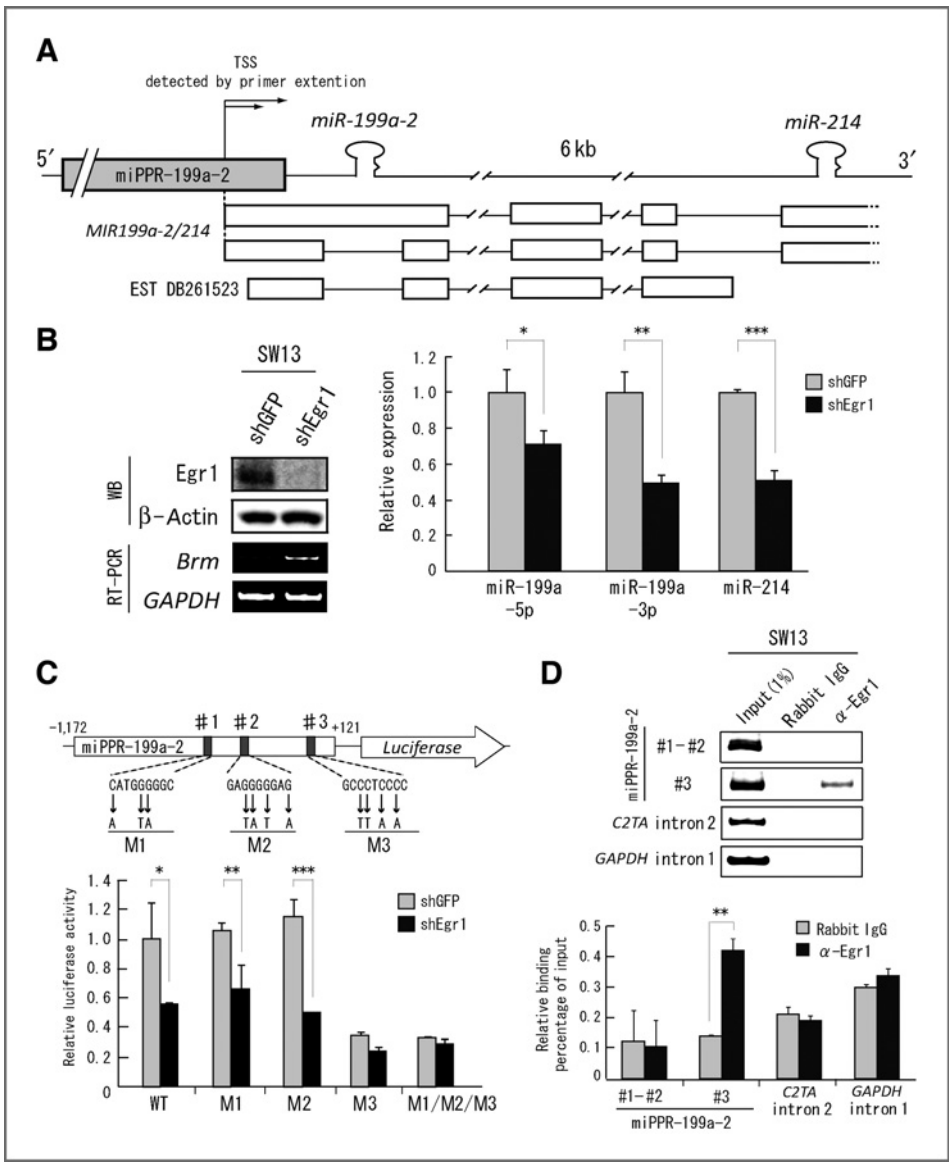

Figure 2. Promoter analysis of *miR-199a-2* gene. A, structure of the *miR-199a-2* locus. MiPPR-199a-2 is located 80 bp upstream of the *miR-199a-2* RNA-embedding region (the length is about 1,300 bp). *miR-214* is located about 6 kb downstream of *miR-199a-2*. DB261523 is one of the expressed sequence tags (EST) that map to the region around human *miR-199a-2* and consists of 4 exons. B, effects of *Egr1* knockdown on the expression levels endogenous of miR-199a-5p, -3p, and -214. SW13 cells were transduced with pLSP-shGFP or pLSP-shEgr1, and total protein and total RNA extracts were prepared. Egr1 and β-actin proteins were then analyzed by Western blotting. The expression of MiR-199a-5p, -3p, 214, and *Brm* was analyzed by RT-PCR. Data are the means ± SD from triplicate experiments. C, AZ521 cells were transfected with 250 ng of pGL4.12-miPPR-199a-2-WT or its mutants and 5 ng of pGL4.74 plus 250 ng of shEgr1 or shGFP expression vector. Luciferase activity was measured after 72 hours. Data are the means ± SD from triplicate experiments. D, the ChIP analysis of the human miPPR-199a-2 region in SW13. DNA isolated by ChIP with Egr1 antibodies (α-Egr1) and nonimmunized rabbit IgG was used. PCR was carried out using primer pairs specific for the #1, #2, or #3 Egr1 binding site and for intron 2 of *C2TA* and intron 1 of GAPDH. The band densities were quantified by LA4000 (bottom). In B–D, *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. WB, Western blotting.

MDA-MB435 cells transfected with a shBrm vector, but not shBRG1, showed elevated expression of *Egr1*, miR-199a-5p, and -3p (Fig. 4A). On the other hand, when SW13 cells (deficient in Brm) were transfected with a Brm expression vector, the expression levels of *Egr1*, miR-199a-5p, and -3p were all reduced (Fig. 4B). These analyses show that Brm negatively regulates *Egr1*, which, in turn, downregulates miR-199a-5p and -3p. We next constructed a reporter plasmid containing the *Egr1*-promoter region fused to the *luciferase*-coding region, and prepared stable MDA-MB435 transfectants of this construct which harbor the exogenous *Egr1* promoter with a native chromatin structure. When these stable transfectants were themselves transiently transfected with a series of shRNA expression vectors, the introduction of shBrm caused a higher luciferase activity than either shBRG1 or shGFP (Fig. 4C). To more precisely map the *Egr1* promoter region suppressed by Brm, MDA-MB435 cells were transfected with 2 truncated reporters and the results indicated that the Brm-responsive region locates mainly between −1,293 and −502 of the *Egr1* promoter (Supplementary Fig. S11). Consistent with these observations, the ChIP analysis in MDA-MB435 cells indicated that Brm, but not BRG1, occupies the region between −1,015 and −643 of the endogenous *Egr1* promoter whereas both Brm and BRG1 occupy the *CD44* promoter (Fig. 4D), as has been reported previously. From these analyses, Brm was shown to specifically occupy the *Egr1* promoter and negatively regulate *Egr1* expression and thus suppress the Egr1 target gene *miR-199a-2/miR-214*.

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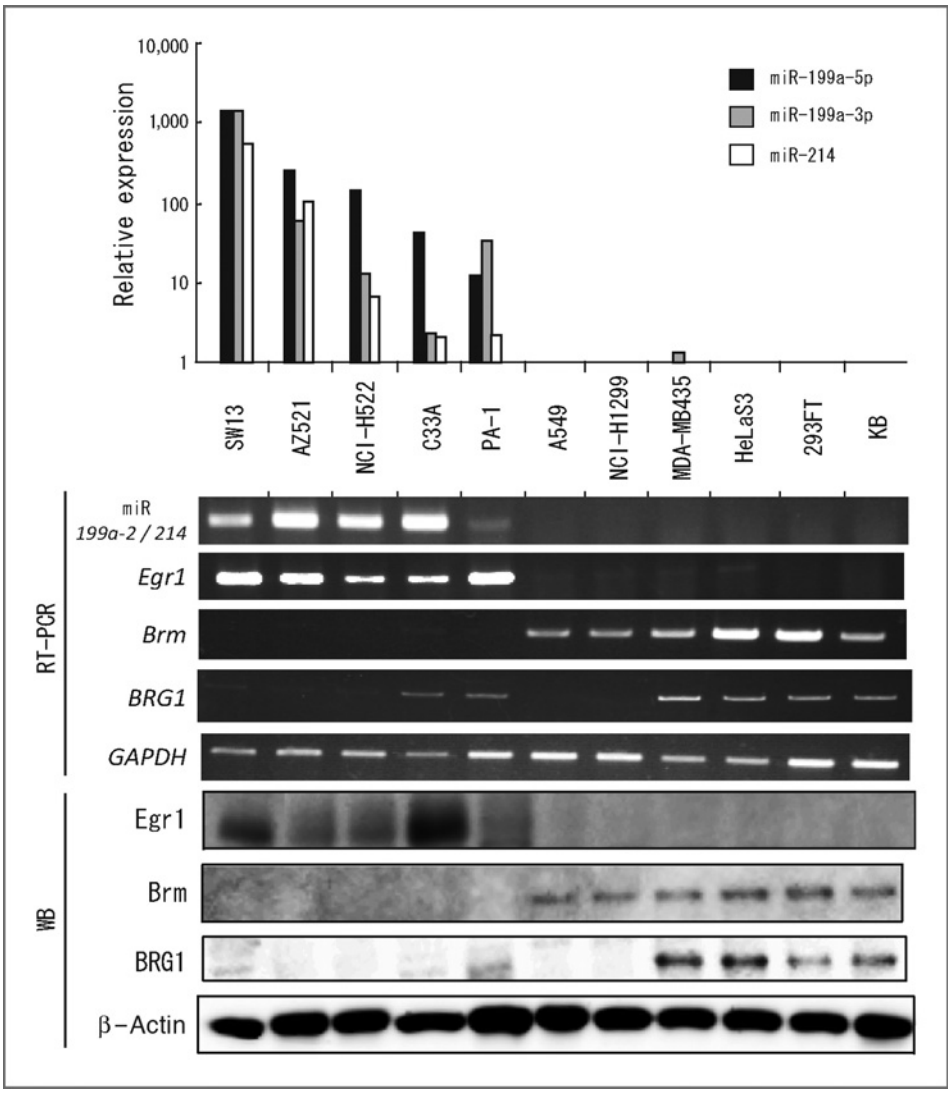

Figure 3. The expression patterns of miR-199a-5p, -3p, and the Brm protein are mutually exclusive in many human cancer cell lines. Expression patterns of mature miR-199a-5p, -3p, miR-214 RNA, and *199a-2/214* transcripts, *Egr1*, *Brm*, and *BRG1* mRNA and Egr1, Brm, and BRG1 proteins in several human cancer cell lines. The relative expression levels of miR-199a-5p, -3p, and miR-214 (average of 2 independent experiments) are normalized to that of 293FT cells. Analysis of the relative protein levels by densitometry is shown in Supplementary Figure S10. WB, Western blotting.

MiR-199a-5p, -3p, and Brm form a double-negative feedback loop through Egr1

Taken together, our current results revealed that the expression patterns shown in Figure 3 reflect the existence of a double-negative feedback loop. High levels of endogenous Egr1 would increase mature miR-199a-5p and -3p expression, which, in turn, represses Brm. Low levels of Brm would then further reinforce the *Egr1* expression level. The converse of these effects in this feedback system would be that a high level of endogenous Brm represses *Egr1* expression and the downstream *miR-199a-2* transcription will also be reduced. This would further enhance Brm production. To test this, we exogenously expressed miR-199a-5p and -3p in MDA-MB435 cells. This caused a reduction in Brm protein expression and, importantly, both the endogenous *Egr1* and pri-*miR-199a-2* expression became detectable (Fig. 5A), supporting our contention (Fig. 5B).

We next examined whether the *miR-199a* and *Brm* double-negative feedback loop we detected in various tumor cell lines operates also *in vivo*. Because invasive squamous cell carcinomas have been reported to express lower levels of miR-199a-5p when compared with normal squamous cells (25), we stained several esophageal squamous cell sections with locked nucleic acid probes against miR-199a-5p or -3p, using a procedure developed previously after testing in cultured cells (Fig. 6; Supplementary Fig. S12; ref. 36). In comparison with cells from a normal epithelium, *in situ* hybridization analysis revealed marginal levels of miR-199a-5p and -3p expression in invasive areas, consistent with a previous report (25), and even in carcinoma *in situ* (Supplementary Table S3). By immunostaining, we also observed lower levels of Egr1 in invasive areas; Brm expression was shown to be equivalent or higher to that in a normal epithelium. We also observed a tendency for the expression levels of Brm to increase with the progression of the cancer (Supplementary Table S3). From these results, we speculate that during the progression toward esophageal carcinoma invasiveness, the *miR-199a* and *Brm* feedback loop observed

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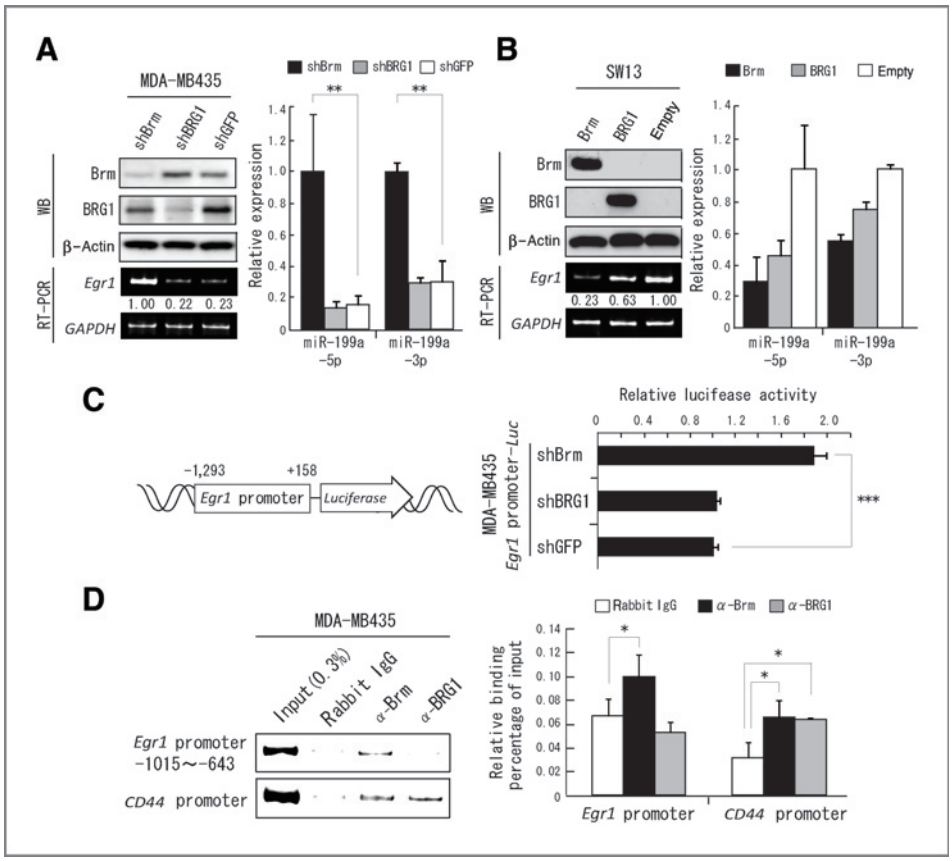

Figure 4. Brm negatively regulates either *Egr1* or *miR199a* gene expression. A, MDA-MB435 cells were transfected with shBrm, shBRG1, or shGFP-expression vectors, and total protein and RNA extracts were prepared from parallel cultures after 72 hours. Brm, BRG1, and β-actin proteins were detected by Western blotting (WB), and the expression of *Egr1*, *GAPDH*, miR-199a-5p, and -3p was analyzed by RT-PCR. B, SW13 cells were transfected with Brm, BRG1, and empty vector (all containing an IRES-GFP fragment). After 48 hours, GFP-positive cells were sorted by flow cytometry and total protein and total RNA extracts were prepared from parallel cultures. Brm, BRG1, β-actin, *Egr1*, *GAPDH*, miR-199a-5p, and -3p expressions were analyzed as described for MDA-MB435 cells. C, a reporter construct for the *Egr1* promoter was integrated into chromosomes by stable transfection (left). MDA-MB435 cells were transfected with pGL4.12-*Egr1* promoter-Luc-CMV-Bla and selected using blasticidin. Stable transfectants were then transfected with shBrm, shBRG1, and shGFP vectors. Firefly luciferase activity was measured after 72 hours. Data are the means ± SD from triplicate experiments (right). D, ChIP analysis of the human *Egr1*-promoter region. DNA templates were obtained from chromatin immunoprecipitated with Brm (α-Brm) and BRG1 (α-BRG1) antibodies and nonimmunized rabbit IgG (rabbit-IgG). PCR was carried out with primer pairs that amplify the region from −1,015 and −643 of the human *Egr1* gene promoter and the *CD44* promoter. In A and B, *Egr1* RT-PCR bands were quantified and shown after normalization to *GAPDH* bands. In A, C, and D, *, $P < 0.05$; ** $P < 0.01$; and ***, < 0.001 (*t* test of triplicate).

in culture will also be gradually formed, leading to a unique pattern of expression of these factors.

Discussion

In this study, we showed that *Brm* mRNA is a specific and functionally relevant target of both miR-199a-5p and miR-199a-3p in tumor cell lines (Fig. 1), which would explain our previous observation that Brm is often silenced in tumor cells at the posttranscription levels. It is noteworthy that these 2 mature miRNAs that are produced from a single pre-miRNA target different sites of the same gene transcript, suggestive of an effective and robust regulatory process. The miPPR-199a-2 region is shown here to be the *bona fide miR-199a-2* promoter that produces the primary transcript harboring the miR-199a-5p, -3p, and miR-214 sequences as a cluster (Fig. 2A). We further identified Egr1 as a potent positive regulator of these miRNAs (Fig. 2C and D, Supplementary Figs. S6 and S8). We also indicated that Brm is a negative regulator of the *Egr1* gene (Fig. 4A–C). Our current data including Figure 5A collectively indicate that the *Brm* and *miR-199a-2* genes form a robust double-negative feedback loop through Egr1.

It is interesting that tumor cell lines originating from the epithelium could be clearly categorized into 2 groups on the basis of their expression patterns; Egr1 and miR-199a-5p and -3p are expressed at high levels in Brm-deficient tumor cell lines, whereas both are only marginally expressed in Brm-expressing tumor cell lines (Fig. 3; Supplementary Figs. S9 and S10). Distinct cell types based on this feedback loop would partially explain why miR-199a-5p and -3p have been reported to be either upregulated or downregulated among the many tumors tested to date. In this regard, it is interesting that 2

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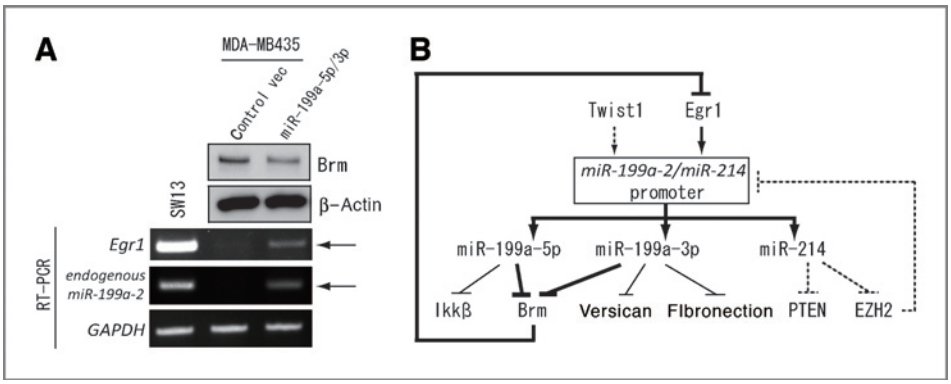

Figure 5. Double-negative feedback loop between *miR-199a* and *Brm* A, MDA-MB435 cells were transfected with miR-199a-5p and -3p expression vectors or a control sh-lacZ expression vector. After 24 hours, total protein and total RNA extracts were prepared. Brm and β-actin proteins were analyzed by Western blotting. *Brm*, endogenous pri-*miR-199a-2*, *Egr1*, and *GAPDH* were analyzed by RT-PCR. SW13 RNA was used as a positive control. B, schematic representation of the proposed double-negative feedback loop formed by *Brm*, *miR-199a*, and *Egr1*. Bold, thin, and broken lines indicate pathways shown in this work, confirmed by this work, and reported by others, respectively. It is noteworthy that Brm and EZH2 are the catalytic subunits of SWI/SNF and Polycomb complexes, respectively, and have strong impacts on epigenetical regulation. Although these 2 complexes are known to serve antagonistic roles in human cells (39), the changes in the activation of miR-199a-2/miR-214 promoter would not disturb the balance between them.

populations of epithelial ovarian cancer (EOC) cells have recently been reported (26, 37). Type I cells are characterized by the low expression of miR-199a-5p and miR-214, whereas type II cells express these miRNAs at high levels. The authors identified Twist1 as the major positive regulator of the *miR-199a/miR-214* gene in these EOC cells (37). It is therefore possible that in some tumor cell lines, Twist1 contributes significantly to the induction of this *miRNA* gene through some of the E-box binding sites in miPPR-199a-2. In our own analysis of Twist1 expression by RT-PCR, however, we found no clear relationship with mature miR-199a-5p and -3p expression in our tumor cell lines (K.S. and H.I., unpublished observation).

We also analyzed esophageal tumor lesions and observed downregulation of miR-199a-5p, -3p, and Egr1, concomitant with the upregulation of Brm in invasive cancers when compared with normal squamous cells (Fig. 6; Supplementary Table S3). These results indicate that a self-reinforcing loop is

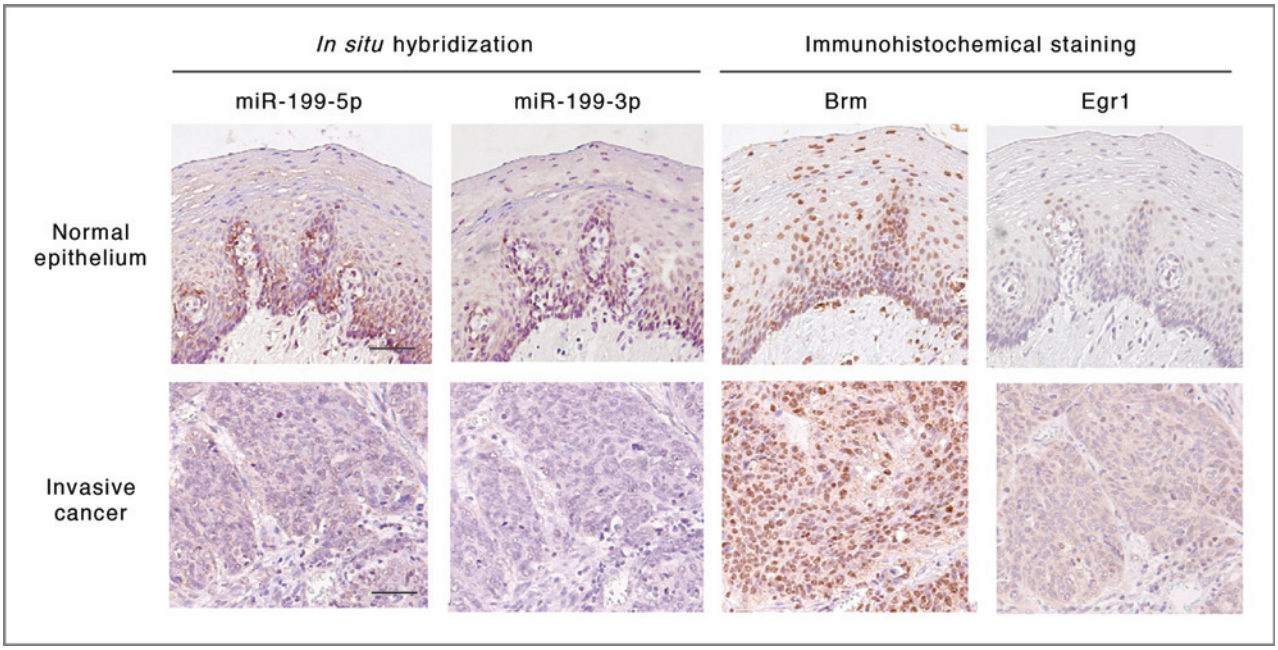

Figure 6. Images from sequential formalin-fixed, paraffin-embedded sections from esophageal invasive carcinoma lesions, in which *in situ* hybridization for miR-199a-5p and -3p and immunostaining for Brm and Egr1 protein were carried out. The specimen from patient 6 in Supplementary Table S3 is shown. MiR-199a-5p, -3p, Brm, and Egr1 were stained brown, and the nuclei were counterstained pale blue with hematoxylin. The sections were evaluated together with hematoxylin and eosin–stained sections from the same lesions by a pathologist. Scale bar, 50 μm.

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operating during the process of carcinogenesis and might further suggest that invasive cancer cells can be stabilized at either of two extreme states through this feedback loop.

Although we speculate that other targets of miR-199a-5p and -3p, and probably of miR-214, may also contribute to the establishment of the 2 distinct cancer cell types, our preliminary experiments showed, however, that the expression levels of Ikkβ (miR-199a-5p target; Fig. 1B), in addition to the reported miR-214 targets PTEN (23) and EZH2 (38), do not correlate well with the miR-199a-5p/-3p expression levels in the series of cell lines we examined (K.S. and H.I., unpublished observations). We believe, however, that it is possible that some of these targets also contribute significantly to the regulation of the *miR-199a-2/-214* gene in some of these lines. Indeed, in stem cell differentiation, miR-214 and its target EZH2 has also been reported to form a double-negative feedback loop (Fig. 5B; ref. 38).

In summary, the miR-199a-5p, and -3p–dependent regulation of Brm by our present analyses indicates a strong linkage between genome-wide epigenetic regulation and miRNA in human cancers and the formation of a robust double-negative feedback loop that seems to switch between 2 types of cancer cell types. Although the molecular factors that act to embed these 2 steady states remain to be elucidated, the fact the histone deacetylase treatment can reverse these states (Supplementary Fig. S13; refs. 15, 18) suggests that these factors may be viable therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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MicroRNAs miR-199a-5p and -3p Target the Brm Subunit of SWI/SNF to Generate a Double-Negative Feedback Loop in a Variety of Human Cancers

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