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Cellular MicroRNAs 200b and 429 Regulate the Epstein-Barr Virus Switch between Latency and Lytic Replication▽

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We previously showed that the cellular proteins ZEB1 and ZEB2/SIP1 both play key roles in regulating the latent-lytic switch of Epstein-Barr Virus (EBV) by repressing *BZLF1* gene expression. We investigated here the effects of cellular microRNA (miRNA) 200 (miR200) family members on the EBV infection status of cells. We show that miR200b and miR429, but not miR200a, can induce EBV-positive cells into lytic replication by downregulating expression of ZEB1 and ZEB2, leading to production of infectious virus. The levels of miR200 family members in EBV-infected cells strongly negatively correlated with the levels of the ZEBs (e.g., −0.89 [$P < 0.001$] for miR429 versus ZEB1) and positively correlated with the degree of EBV lytic gene expression (e.g., 0.73 [$P < 0.01$] for miR429 versus BZLF1). The addition of either miR200b or miR429 to EBV-positive cells led to EBV lytic reactivation in a ZEB-dependent manner; inhibition of these miRNAs led to decreased EBV lytic gene expression. The degree of latent infection by an EBV mutant defective in the primary ZEB-binding site of the EBV BZLF1 promoter was not affected by the addition of these miRNAs. Furthermore, EBV infection of primary blood B cells led to downregulation of these miRNAs and upregulation of ZEB levels. Thus, we conclude that miRNAs 200b and 429 are key regulators via their effects on expression of ZEB1 and ZEB2 of the switch between latent and lytic infection by EBV and, therefore, potential targets for development of new lytic induction therapeutics with which to treat patients with EBV-associated malignancies.

Epstein-Barr virus (EBV) is a human gamma herpesvirus that infects 90% of the world's population. Latent EBV infection is associated with several types of malignancies of epithelial and B-lymphocytic cells, including epithelial nasopharyngeal carcinoma (NPC) (20), posttransplant lymphoproliferative disease (PTLD) (64), Burkitt's lymphoma (BL) (24), Hodgkin's disease (8, 87), and some gastric cancers (78; reviewed in references 74 and 75).

Reactivation of EBV out of latency into lytic replication is necessary for the viral progeny to pass from host to host. It occurs naturally in infected individuals at a low frequency; periodic shedding of the virus into saliva allows for transmission (75). It remains unclear how reactivation occurs *in vivo*. The product of the *BZLF1* gene, known as BZLF1 (also called ZEBRA, Z, Zta, and EB1), is a key player in switching EBV from latency into lytic replication in cells in culture (16, 17, 25, 80; reviewed in references 74 and 75).

BZLF1 is a multifunctional DNA-binding protein belonging to the bZIP member of transcription factors (12). By activating transcription of other viral genes and binding to the viral origin of lytic replication, *oriLyt*, BZLF1 can induce viral genome replication and expression of a cascade of other EBV lytic genes necessary for packaging of the genome into virion particles and exit from the host cell (40, 52, 74, 76, 77). BZLF1 can also interact with several cellular proteins, affecting their activities and cellular localization, further contributing to viral reactivation (61, 74). Because of BZLF1's central role in reactivation of EBV out of latency into lytic replication, factors that regulate its expression have been implicated as potential therapeutics for treating patients with EBV-associated malignancies.

Our laboratory previously reported that the cellular proteins ZEB1 (also known as δEF1, TCF8, AREB6, ZFHEP, NIL-2A, ZFHX1A, and BZP) and ZEB2 (also known as SIP1, SMADIP1, ZFHX1B, and KIAA0569) can both bind to a sequence element, termed ZV, located within the BZLF1 promoter, Zp (22, 47, 48, 92); a second element, ZV′, synergizes with the ZV element for higher-affinity binding of the ZEBs to Zp via their two zinc fingers (Fig. 1A) (X. Yu, P. McCarthy, D. Gorlen, and J. E. Mertz, unpublished data). Both of these ZEB family members bind to target DNA via E-box-binding sequences resembling 5′-CACCTG-3′ (33, 73). Depending on interactions with coactivators and corepressors and associations with histone deacetylases (HDACs) (68, 71, 84), ZEB1 can either activate or repress transcription of its target genes (68, 70, 72); to date, ZEB2 has been reported only to repress transcription (67). We showed that exogenous expression of either ZEB1 or ZEB2 leads to repression of transcription driven from Zp in transient transfection assays (22, 48, 92). Mutation of the ZV element in the context of the B95.8 strain of EBV leads to increased synthesis of lytic viral proteins in B-lymphocytic BJAB cells and spontaneous production of infectious virus in 293 cells (92). Analysis of numerous EBV-positive epithelial and B-lymphocytic cell lines indicated that ZEB1 is fairly ubiquitously expressed, while ZEB2 is expressed only in a small subpopulation of cell lines. Small interfering RNA (siRNA) and short hairpin RNA (shRNA) knockdown of ZEB1 and ZEB2 in EBV-positive cell lines leads to upregulation of EBV

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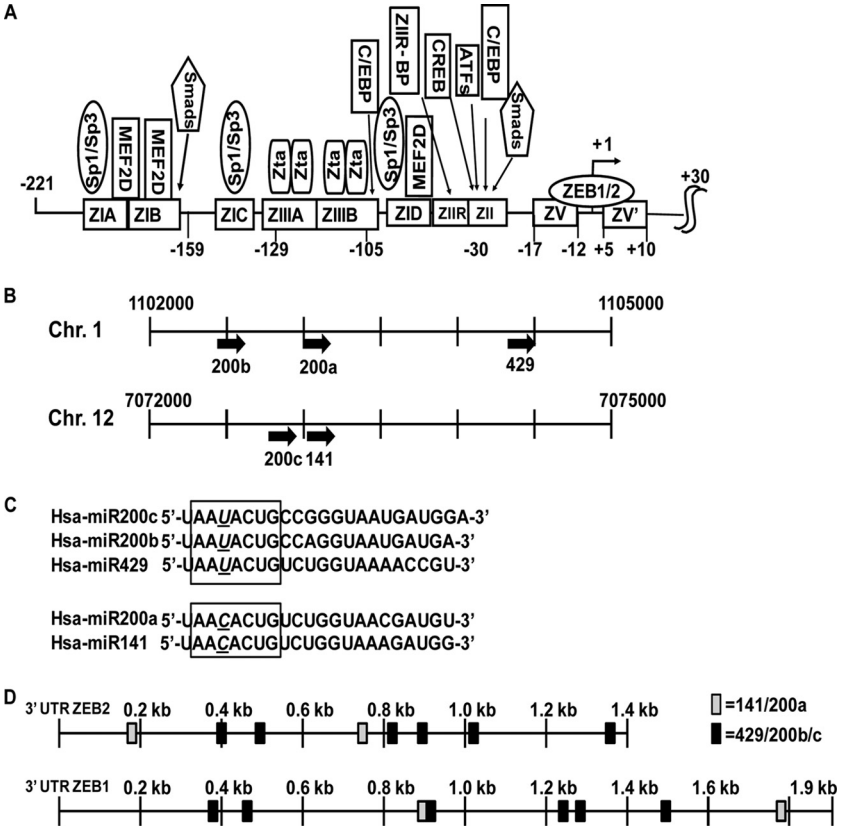

FIG. 1. miR200 family. (A) Schematic diagram of the promoter region of the EBV *BZLF1* gene indicating known *cis*-acting elements and their *trans*-acting factors. (B) Schematic diagram of the miRBase-predicted locations of hsa-miR200a, hsa-miR200b, and hsa-miR429 on chromosome 1 and hsa-miR200c and hsa-miR141 on chromosome 12. (C) Sequences of miRNA200 family members. The miRBase program (50, 51) was used to generate the sequences of the five human miRNA-200 family members, hsa-miR200a, hsa-miR200b, hsa-miR200c, hsa-miR429, and hsa-miR141. Seed sequences for each miRNA are highlighted in the boxed area; polymorphisms in the sequences are underlined. (D) TargetScan (50, 51)-predicted binding sites for miR200 family members in the 3′-UTR regions of ZEB2 and ZEB1 mRNAs. Binding sites for hsa-miR200b, hsa-miR200c, and hsa-miR429 are indicated by black rectangles; binding sites for hsa-miR200a and hsa-miR141 are indicated by gray rectangles.

lytic gene expression, with ZEB2 being the dominant repressor of Zp when present and ZEB1 being the dominant repressor otherwise (22). Thus, we concluded that both ZEB1 and ZEB2 play key roles in the maintenance of EBV latency, doing so in a cell-type-specific manner.

Recently, the 200 family of cellular microRNAs (miR200 family) has been shown to downregulate expression of ZEB1 and ZEB2 (10, 11, 36, 37, 66). MicroRNAs (miRNAs) are small, 21- to 23-nucleotide (nucleotide) noncoding RNAs that are highly conserved structurally and functionally throughout vertebrate species (31; reviewed in reference 5). miRNAs have been implicated not only in normal cellular processes such as embryogenesis (5, 69) and organ development (4, 31) but also in oncogenesis (65). miRNAs originate from large RNAs synthesized in the nucleus that are processed by an enzyme known as Drosha into smaller, 70-nt stem-loop structures known as pre-miRNAs (49). These pre-miRNAs are exported from the nucleus, where they are further processed in the cytoplasm by the enzyme Dicer, resulting in the final 21- to 23-nt mature miRNA (9). Upon incorporation into a complex of proteins and enzymes known as the RNA-induced silencing complex (RISC), miRNAs can bind to their target mRNAs with perfect or near-perfect complementarity, inducing mRNA degradation or translational repression of these mRNAs, respectively (5, 41). Important for targeting miRNAs to the correct mRNAs is a 7- to 8-nt sequence, known as the seed sequence, located near the 5′ end of each miRNA (50, 51).

The miR200 family contains five members: miRNAs 200a, 200b, 200c, 429, and 141 (reviewed in reference 69). These five miRNAs can be grouped by either chromosomal location or seed sequence. When grouped by location, miRNAs 200a, 200b, and 429 are coordinately produced from an RNA synthesized from chromosome 1, while miRNAs 200c and 141 originate from a transcript synthesized from chromosome 12 (Fig. 1B). When grouped by seed sequence, miR200b, miR200c, and miR429 share the same seed sequence, while miR200a and miR141 share a slightly different seed sequence (Fig. 1C). All five of these miRNAs have been shown to target the 3′ untranslated regions (3′-UTRs) of ZEB1 and ZEB2 mRNA (10, 11, 36, 37, 66). Analyses of the 3′-UTRs of ZEB1 and ZEB2 reveal multiple putative binding sites for them (Fig. 1D) (10, 11, 36, 37, 66; reviewed in reference 69). Interestingly, synthesis of both of these pre-miRNA transcripts is negatively regulated by ZEB1 and ZEB2 via ZEB-binding sites present in the promoter regions for these genes (10, 11).

We show here that expression of some miR200 family mem-

bers directly correlates with lytic gene expression in EBV-positive epithelial and B-lymphocytic cell lines. We also show that the addition to these cells of either miR200b or miR429 leads to EBV lytic reactivation, while inhibiting their activities leads to a reduction in EBV lytic gene expression. These effects occur through the ZEBs and the ZEB-binding site in Zp. Furthermore, EBV infection leads to changes in the levels of these miRNAs and ZEBs consistent with their playing physiological roles in establishment of latency. Thus, we conclude that miRNAs 200b and 429 are key effectors of the switch between EBV latency and lytic replication via their ability to downregulate ZEB protein levels in cells.

MATERIALS AND METHODS

Cell lines. (i) EBV-positive B-lymphocytic cell lines. MutuI and MutuIII cells are Burkitt's lymphoma (BL) cell lines in latency types I and III, respectively (35). 721 is a type III latency lymphoblastoid cell line (LCL), Jijoye is a type III BL cell line, and GG68 is a type III latency BL cell line derived from a clonal isolate of the cell line P3HR1. They were obtained from Bill Sugden and originally described in references 43 and 86. Akata, a gift from Kenzo Takada via Shannon Kenney, is an EBV-positive BL cell line in type I latency, originally described in reference 81. BJAB$^{B95.8}$ was obtained by infection of the EBV-negative BL cell line BJAB with the B95.8 strain of EBV present in bacmid p2089 (92). All EBV-positive B-lymphocytic cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin per ml. The media for growing EBV-positive Akata and BJAB$^{B95.8}$ cells also included 500 μg/ml G418 and 300 μg/ml hygromycin, respectively.

(ii) EBV-positive epithelial cell lines. Epithelial gastric carcinoma AGS$^{B95.8}$ cells (a gift from Shannon Kenney) were maintained in F-12 medium supplemented with 10% FBS and 100 U penicillin/streptomycin per ml as previously described (30). Epithelial nasopharyngeal carcinoma (NPC) HONE-1^{Akata} cells (a gift from Lawrence Young via Shannon Kenney; 34) and CNE1^{Akata} and CNE2^{Akata} cells (gifts from Diane Hayward, with permission from Kwok Wai Lo) were maintained in RPMI 1640 plus 10% FBS and penicillin/streptomycin additionally supplemented with 500 μg/ml G418, as described in reference 56. Type II latency gastric carcinoma SNU719 (42) and NPC C666-1 (13) cells were obtained from Bill Sugden. Neuronal 293$^{B95.8}$ and 293^{ZVMT} cells were obtained by infection of 293D cells with the bacmid p2089 and a variant of it containing a 2-bp substitution mutation in the ZV element of the BZLF1 promoter, respectively, as previously described (92); they were maintained in RPMI 1640 plus 10% FBS and 100 U penicillin/streptomycin per ml, additionally supplemented with 100 μg/ml hygromycin (Calbiochem, San Diego, CA).

EBV-negative cell lines. MCF-7 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium plus 10% FBS, 6 ng/ml insulin, 2 mM L-glutamine, 100 μM nonessential amino acids, and 100 U penicillin/streptomycin per ml. 293D cells were maintained in DMEM supplemented with 10% FBS and 100 U penicillin/streptomycin per ml. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

Plasmids. The expression plasmid pcDNA4hismaxCZEB1 contains the human ZEB1 cDNA (provided by Tom Genetta) (33) cloned between the EcoRI and XbaI sites of pcDNA4hismaxC (Invitrogen, Carlsbad, CA) as previously described (22). The expression plasmid pcDNA4hismaxCZEB2 contains the human ZEB2 cDNA (provided by Takahiro Nagase) (62, 63) cloned between the NotI and XbaI sites of pcDNAhismaxC as previously described (22). The luciferase reporter plasmid pZpWT-luc contains the nt −221-to-nt +30 region of the EBV *BZLF1* gene cloned into the pGL3-Basic vector (Promega, Madison, WI). Plasmid pZpZVmt-luc is a derivative of pZpWT-luc containing substitution mutations at nt −13 and nt −12, leading to reduced binding of the ZEBs to Zp (22, 92).

Transfections. The expression plasmids pcDNA4hismaxCZEB1 and pcDNA4hismaxCZEB2 were transiently transfected into CNE1^{Akata} and HONE^{Akata} cells using TransIT-LT1 reagent (Mirus, Madison, WI). Forty-eight hours later, the cells were harvested for whole-cell protein and RNA and assayed for viral lytic proteins and RNAs by immunoblot analysis and reverse transcription quantitative real-time PCR (RT-qPCR), respectively.

Thirty nM concentrations of miRNAs to 200a (PM10991), 200b (PM10492), or 429 (PM10221) or "scrambled" miRNA as a negative control (AM17111; Ambion/Applied Biosystems, Foster City, CA) were transfected into cells in 6-well plates using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the company's instructions. The cells were subsequently incubated for at least 72 h to achieve knockdown of expression of the target genes. When working with 293$^{B95.8}$ cells, the EBV lytic inducers 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 20 ng/ml) and sodium butyrate (3 mM) were added to the medium 48 h prior to harvesting the cells.

Sixty nM concentrations of inhibitors to all five miRNA 200 family members (previously described in reference 66), or inhibitors specific to miRNA 200a (AM10991), 200b (AM10492), or 429 (AM10221), or a negative control (AM17010; Applied Biosystems) were transfected into the cells indicated in Fig. 9 in 6-well plates using Lipofectamine RNAiMAX according to the company's instructions. The cells were incubated for at least 96 h to achieve knockdown of the corresponding miRNAs. When working with CNE1^{Akata} cells, we reactivated the virus by the addition of 20 ng/ml TPA and 3 mM sodium butyrate to the medium 24 h prior to the harvesting of the cells.

Transient transfection assays. 293D cells grown in 24-well plates were transfected using LT1 reagent with 0, 0.5, or 1.0 μg of pcDNA4hismaxCZEB1 or pcDNA4hismaxCZEB2 as indicated, along with 1.0, 0.5, or 0 μg of empty vector pcDNA4hismax to ensure equal amounts of transfected DNA. Twenty-four hours later, the cells were transfected using Lipofectamine 2000 (Invitrogen) with 30 nM miR200b. After another 24 h, the cells were further transfected using LT1 reagent with 0.5 μg of pZpWT-luc or pZpZVmt-luc as indicated in Fig. 11, along with 0.1 μg of pCMXRluc (Stratagene, La Jolla, CA) as an internal control. Cells were harvested 48 h after the third transfection into passive lysis buffer (Promega). Luciferase activity was assayed with a Monolight 3010 luminometer according to the manufacturer's protocol (Promega). Data were normalized both internally to *Renilla* luciferase expression and externally to the controls transfected in parallel as indicated in the figure legends.

Immunoblot analysis. Cells were harvested in small ubiquitin-like modifier (SUMO) buffer and lysed by sonication as previously described (2). To detect BZLF1 and BMRF1, the proteins were resolved by electrophoresis at 120 V for 1 h in SDS gels containing 12% polyacrylamide (ISC Bioexpress, Kaysville, UT) and transferred to a nitrocellulose membrane by electrophoresis at 350 mA for 2 h at 4°C. After incubation in 5% milk in TBST (100 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% Tween 20) for 1 h, the membranes were incubated overnight at 4°C in 5% milk/TBST containing antibody specific to BZLF1 (sc-53904, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA) or BMRF1 (VP-E608, 1:250; Vector Laboratories, Burlingame, CA). Afterward, the blots were washed and incubated for 1 h in 5% milk/TBST containing the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (ThermoScientific, Waltham, MA). The blots were washed with TBST, incubated for 1 min in ECL reagent (Roche, Madison, WI), and developed using an automatic film developer.

To detect ZEB1 and ZEB2, the proteins were resolved by electrophoresis at 120 V for 1.5 h in SDS 3%-to-8% gradient polyacrylamide Tris-acetate gels (Invitrogen) and, subsequently, processed as described above, except the membranes were incubated overnight in the primary rabbit polyclonal antibody ZEB1 (sc-25388, 1:500; Santa Cruz Biotechnology) or ZEB2 (1:2,000 dilution; a gift from Michel Saunders, further purified in our laboratory by X. Yu) followed by incubation with ECL horseradish peroxidase-linked anti-rabbit IgG (GE Healthcare, Piscataway, NJ) as the secondary antibody.

Relative protein levels were determined by densitometry using Adobe Photoshop software, with normalization to the amount of cellular β-actin present in each sample. The amounts of protein present in the experimental samples are indicated in Fig. 6, 8, 9, and 10 relative to the amount in the untreated or negative-control sample processed in parallel.

RT-qPCR. Total RNA from approximately 10^6 cells was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Genomic DNA was removed from the samples using RNase-free DNase (Applied Biosystems). RNA concentration was quantified by Nanodrop Technologies (ThermoFischerScientific, Waltham, MA). cDNA for miRNA analysis was synthesized from 50 ng of total RNA using a TaqMan microRNA RT kit (Applied Biosystems). Detection kits for miRNAs 200a (part number [PN], 4427975; identifier [ID], 000502), 200b (PN, 4427975; ID, 002251), and 429 (PN, 4427975; ID, 001024) and small nucleolar RNA RNU38B (PN, 4427975; ID, 001004) as the control were used to determine the levels of the cDNAs to these miRNAs by qPCR performed with an ABI-7500 (Applied Biosystems, CA). The reaction conditions were as follows for each primer set: 50°C for 2 min, 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min for 40 cycles. Data were internally normalized to RNU38B using the ΔΔCt method and are shown relative to the negative control for each experiment.

For BZLF1, ZEB1, and ZEB2 RNA analysis, cDNA was synthesized from 10 ng of total RNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. BZLF1 cDNA levels were determined by qPCR performed with a MyIQ quantitative real-time PCR detection

system (Bio-Rad) with Po rRNA serving as an internal control. The reaction conditions for each primer set were as follows: 95°C for 10 min, followed by 95°C for 1 min and 60°C for 15 s for 45 cycles. Data were normalized to Po RNA levels present in the same samples using the ΔΔCt method; they are shown relative to the negative control included in each experiment. Primer sets used were as follows: for BZLF1, forward (FWD), 5′-CTGCTCCTGAGAATGCTT-3′, and reverse (REV), 5′-CGGCTTGGTTGGTCTGTT-3′; for Po, FWD, 5′-GACAA TGGCAGCATCTAC-3′, and REV, 5′-GCAGACAGACACTGGTCA-3′; for ZEB1, FWD, 5′-AGCAGTGAAAGAGAAGGG-3′, and REV, 5′-GGTCCTC TTCAGGTGCCT-3′; for ZEB2, FWD, 5′-CAAGAGGCGCAAACAAGC-3′, and REV, 5′-GGTTGGCAATACCGTCAT-3′; and for BZLF1, FWD, 5′-CTG CTCCTGAGAATGCTT3′, and REV, 5′-CGGCTTGGTTGGTCTGTT-3′.

IFS. For immunofluorescence staining (IFS), the cells were fixed by incubation with methanol:acetone (1:1) for 10 min at room temperature, blocked by incubation for 1 h with 20% FBS in phosphate-buffered saline (PBS), and incubated with anti-BMRF1 (VP-E608, 1:45; Vector Laboratories) or anti-gp350 (MAB8183, 1:30; Millipore) monoclonal antibody for 1.5 h. Cells were then washed four times with PBS and incubated for 1 h with Alexa Fluor 568 goat anti-mouse IgG (A11004, 1:100; Invitrogen) as the secondary antibody. Cells were washed five times with PBS, embedded in mounting medium (Vector Laboratories) containing Hoechst 33342 dye, and examined with a fluorescence microscope (Zeiss).

Assay for infectious EBV. CNEI^{Akata} cells in 100-mm dishes were transfected with 30 nM miR200b or negative-control miRNA as described above. After 72 h, we collected the medium in which the cells had been incubating, passed it through a 0.8-mm filter to remove cells, and concentrated the virus 16-fold using an Amicon Ultra-15 filter (molecular weight cutoff, 100,000; Millipore). Relative infectious virus titers in green Raji units (GRUs) were determined essentially as described by Altmann and Hammerschmidt (3). In brief, various amounts of the concentrated medium containing the virus were added to 1×10^5 Raji cells. After incubation at 37°C for 48 h, 3 mM sodium butyrate and 20 ng/ml TPA were added to the cells, and incubation was continued for an additional 24 h prior to counting green fluorescent protein (GFP)-positive cells visualized by UV microscopy.

Infection of primary blood B cells. A stock of infectious virus was produced by transfection of 293$^{B95.8}$ cells grown in 10-cm dishes with 5 μg (each) of plasmids expressing BZLF1 (40) and BARF4/GP110 (3). After incubation at 37°C for 72 h, the medium was harvested from six dishes and concentrated to 1 ml by centrifugation at 22,000 rpm in a Beckman L-70 ultracentrifuge at 8°C for 8 h. The virus titer was determined by a Raji cell assay as described above. Approximately 1×10^5 GRUs/ml was used to infect 1×10^6 cells/ml of primary blood B cells (AllCells, Emeryville, CA). Samples were harvested at the times indicated in Fig. 2 and processed as described above for ZEB and miR200 family RNA levels.

Statistical analysis. Pearson's correlations were calculated using the Mstat program (http://www.mcardle.wisc.edu/mstat).

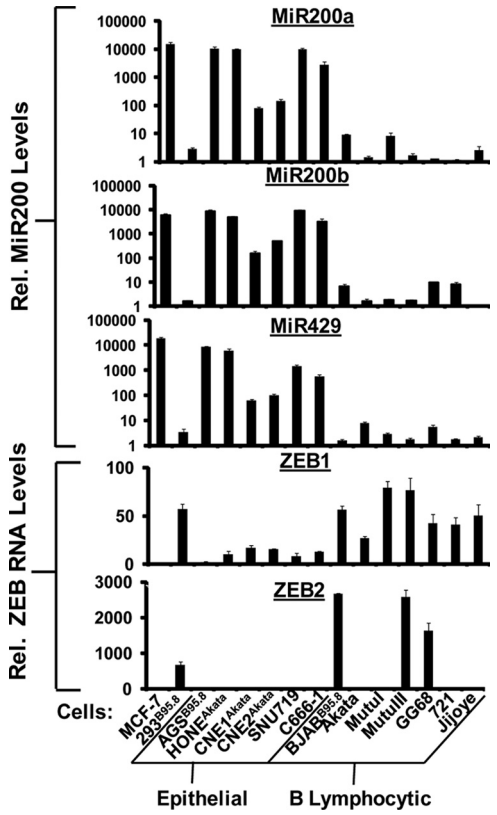

FIG. 2. Correlations between levels of miR200 family members and ZEB1 and ZEB2 RNA. (A) RT-qPCR analysis of levels of miR200a, miR200b, miR429, ZEB1, and ZEB2 RNA in multiple EBV-positive epithelial and B-lymphocytic cell lines and MCF-7 cells. Total RNA was harvested from each indicated cell line, cDNA was synthesized, and the samples were analyzed for relative (Rel.) levels of ZEB1, ZEB2, and the miRNAs 200a, 200b, and 429 as described in Materials and Methods. ZEB1 and ZEB2 RNA levels were normalized to the ribosomal Po level, and miRNA levels were normalized to the RNU38B level present in the same samples.

RESULTS

miR200a, miR200b, and miR429 levels inversely correlate with ZEB levels in EBV-positive cell lines. We previously reported that cellular proteins ZEB1 and ZEB2 play key roles in regulating the latent-to-lytic switch of Epstein-Barr virus; loss of either protein leads to lytic reactivation of latently infected cells in a cell-type-specific manner (22). Because of their central role in the maintenance of EBV latency, we wished to determine mechanisms by which ZEB1 and ZEB2 might, in turn, be regulated by cellular and EBV factors. Recently, cellular miR200 family members have been shown to downregulate expression of ZEB1 and ZEB2. Therefore, we hypothesized that expression of these miRNAs might correlate with lytic gene expression in EBV-positive cell lines.

To test this hypothesis, we first examined the expression levels of miRNAs 200a, 200b, and 429 in multiple EBV-positive epithelial and B-lymphocytic cell lines. We focused on miRNAs 200a, 200b, and 429 because these miRNAs have been reported to regulate expression of both ZEB1 and ZEB2 in several cell types (10, 11, 14, 37) and are all expressed off chromosome 1 from a single polycistronic transcript (Fig. 1B) (37) whose transcription is regulated by ZEB1 and ZEB2 in a double-negative feedback loop (10, 11). In addition, these miRNAs represent the two seed sequence polymorphisms present in the miR200 family members (Fig. 1C). As a control, we also analyzed the RNAs present in MCF-7 cells, an EBV-negative breast carcinoma cell line known to contain high levels of these miRNAs (10, 11, 36, 37, 66) and no ZEB mRNA (Fig. 2, bottom two graphs) (21, 66). RT-qPCR analysis indicated that the levels of these miRNAs ranged over four orders of magnitude among the cell lines examined (Fig. 2, top three graphs). As expected, the levels of all three of these miRNAs within any given cell line were found to be similar (Fig. 2, top three graphs), consistent with their being expressed from a single polycistronic transcript (10, 11, 37). Interestingly, the levels of the miRNAs were very low in all of the B-lymphocytic cell lines and moderate to very high in all of the epithelial cell lines (Fig. 2, top three graphs).

Previous reports involving studies with a variety of EBV-negative cell lines indicated that a negative correlation exists between the levels of the miR200 family members and the

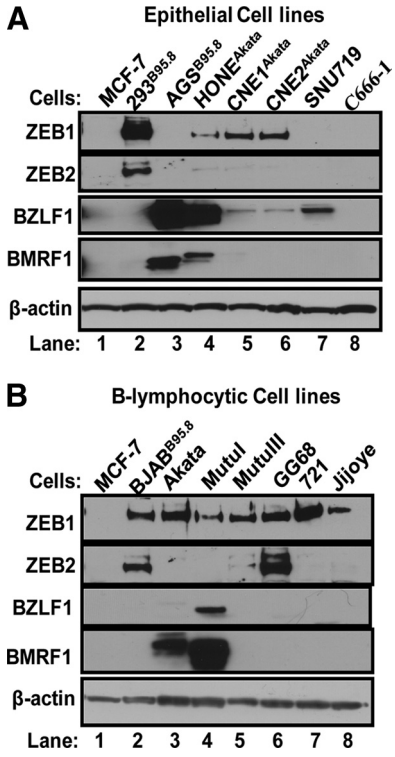

FIG. 3. Immunoblots showing relative levels of ZEB1 and ZEB2 protein and the EBV IE BZLF1 and E BMRF1 proteins present in EBV-positive epithelial (A) and B-lymphocytic (B) cell lines. Thirty μg of whole-cell protein was loaded per lane and analyzed for relative levels of ZEB1, ZEB2, BZLF1, and BMRF1 protein as described in Materials and Methods. β-Actin served as a loading control.

ZEBs, i.e., cell lines with high miRNA levels contain little or no ZEB and *vice versa* (66). We investigated whether this negative correlation applied to our EBV-positive cell lines as well by performing RT-qPCR and immunoblot analysis to determine the cellular RNA and protein levels, respectively, of ZEB1 and ZEB2. Confirming our previous results (22), we found that ZEB1 was expressed to some degree in all of the cell lines examined except for the gastric carcinoma $AGS^{B95.8}$ and SNU719 cell lines, the NPC cell line C666-1, and the negative-control breast carcinoma cell line MCF-7 (Fig. 3A and 3B). An excellent negative correlation was found to exist between the levels of these miR200 family members and ZEB1 RNA, with Pearson's correlations between ZEB1 versus miR200a, miR200b, and miR429 being -0.81 ($P < 0.01$), -0.84 ($P < 0.01$), and -0.89 ($P < 0.001$), respectively (Fig. 4A, panels i to iii). On the other hand, ZEB2 RNA (Fig. 2, bottom panel) and protein (Fig. 3A and B) were observed in only a few of these cell lines. Thus, the negative correlations between the levels of miR200a, miR200b, and miR429 versus ZEB2 RNA were not as striking, with Pearson's correlations being -0.64 ($P < 0.05$), -0.50 ($P < 0.1$), and -0.63 ($P < 0.05$), respectively (Fig. 4B, panels i to iii). We hypothesize that the latter finding is due to ZEB2 being expressed in only a small subset of these cell lines, with some of the ZEB2-negative cell lines containing only low levels of these miRNAs because the presence of ZEB1 is sufficient to repress their synthesis (Fig. 4B, panels i to iii, circled). Overall, we conclude that ZEB levels negatively correlate with miR200a, miR200b, and miR429 levels in EBV-positive cell lines.

miR200a, miR200b, and miR429 levels positively correlate with EBV lytic gene expression. We previously showed that ZEB1 and ZEB2 expression negatively correlates with EBV lytic gene expression (22, 30). Given our finding that the levels of the ZEBs and miR200 family members inversely correlate, we hypothesized that the levels of the miR200 family members positively correlate with EBV lytic protein and RNA levels. To test this hypothesis, we determined by immunoblot and RT-qPCR analyses the relative levels of immediate-early (IE) BZLF1 and early (E) BMRF1 protein (Fig. 3) and BZLF1 RNA (Fig. 5A, bottom panel), respectively, present in our panel of EBV-positive cell lines. We then compared these levels to the levels of miRNAs 200a, 200b, and 429 present in the same samples. As predicted, the findings confirmed our hypothesis, i.e., a positive correlation was found between the levels of these miRNAs and the level of BZLF1 RNA present in the cells, with Pearson's correlations for miRNAs 200a, 200b, and 429 versus BZLF1 being 0.74 ($P < 0.01$), 0.79 ($P < 0.005$), and 0.73 ($P < 0.01$), respectively (Fig. 5B). For example, in $293^{B95.8}$ cells, which express these miRNAs at low levels, we observed low levels of BZLF1 RNA (Fig. 5A) and protein (Fig. 3). On the other hand, we observed very high levels of both the miRNAs and BZLF1 in the gastric carcinoma $AGS^{B95.8}$ and NPC $HONE^{Akata}$ cells (Fig. 3 and 5A). While this paper was undergoing revision, Shinozaki et al. (79) and Lin et al. (54) reported similar findings, including in EBV-positive primary NPCs and lymphoblastoid cell lines, respectively.

There were a few exceptions to this positive correlation. For example, C666-1 cells contained low levels of BZLF1 protein (Fig. 3) and RNA (Fig. 5A) despite expressing these miRNAs at high levels. Other repressors of Zp, e.g., the ZI-binding factor MEF2D (38, 45), that may not be affected by these miRNAs are likely silencing *BZLF1* expression in these cases. Regardless, these findings suggest that these miRNAs probably contribute to EBV lytic replication by inhibiting expression of ZEB1 and ZEB2.

Addition of either miR200b or miR429 induces EBV lytic replication. To directly test this possibility, we next investigated whether the addition of miR200a, miR200b, or miR429 to cells latently infected with EBV can induce reactivation by inhibiting expression of ZEB1 and ZEB2. For the first set of experiments, we chose to use EBV-positive, ZEB1-positive, ZEB2-negative NPC $CNE1^{Akata}$ cells because (i) ZEB1 is known to be a key regulator of the EBV latent-lytic switch in them (22, 92), and (ii) they contain only moderate levels of these miRNAs and very low levels of EBV lytic proteins prior to induction (Fig. 3). Transfection of miR200a, miR200b, or miR429 into $CNE1^{Akata}$ cells increased their levels 300- to 1,200-fold (Fig. 6B), nearly up to the levels naturally present in NPC $HONE^{Akata}$ and gastric carcinoma $AGS^{B95.8}$ cells (Fig. 2, top three panels). These increases were specific to each miRNA. For example, transfection of miR200b increased the level of this miRNA without affecting the level of miR200a or miR429 (Fig. 6B). As expected, increasing the level of miR200b or miR429 led to a reduction of approximately 5-fold in the level of ZEB1 protein present in the cells (Fig. 6A). Concomitantly, IE BZLF1 and E BMRF1 protein levels in-

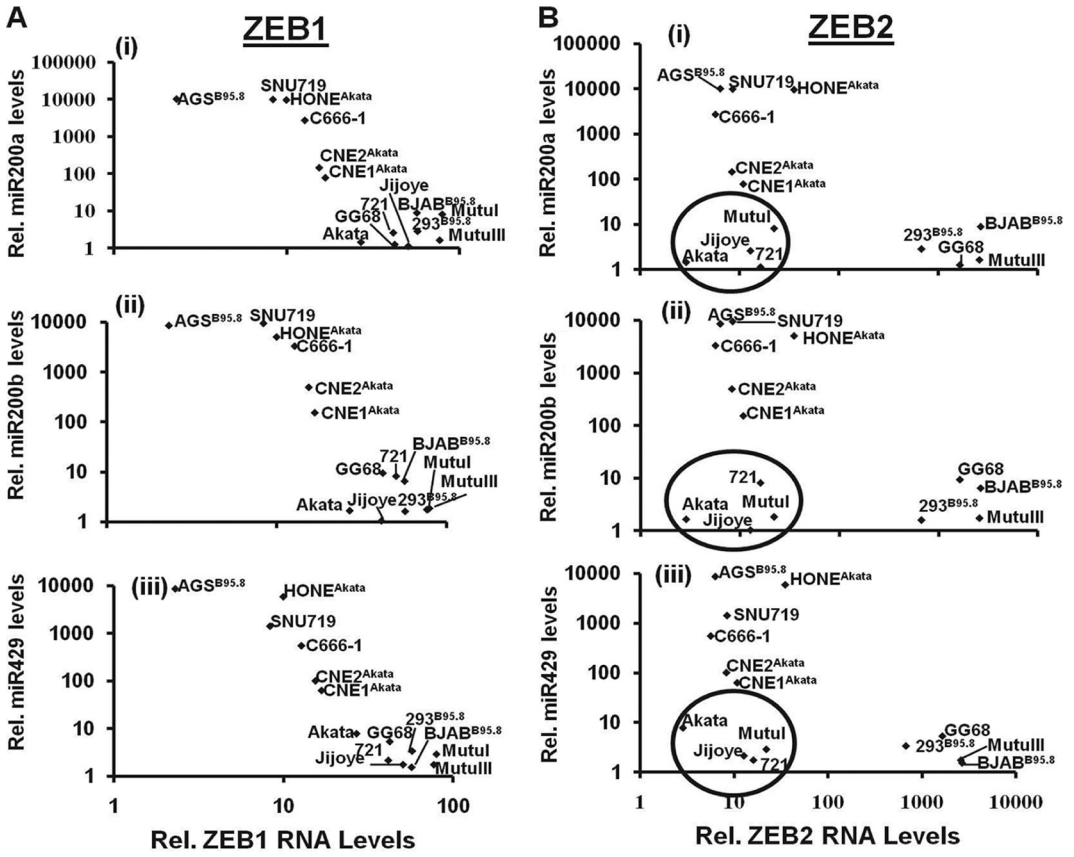

FIG. 4. miR200 family members levels negatively correlate with ZEB1 and ZEB2 levels in EBV-positive cell lines. Scatter plots showing negative correlations between levels of miRNAs 200a (i), 200b (ii), and 429 (iii) and levels of ZEB1 (A) and ZEB2 (B) RNA in EBV-positive cell lines. Data were taken from Fig. 2.

creased significantly following treatment with these miRNAs (Fig. 6A), indicative of induction of EBV out of latency into lytic replication. While transfection with either miR200b, miR429, or all three miRNAs together led to significant EBV reactivation relative to transfection with the control miRNA, transfection with miR200a failed to do so (Fig. 6A, lane 1 versus lanes 2 to 4). The latter finding is consistent with the addition of miR200a knocking down the ZEB1 level only 30%, likely resulting in retention of sufficient ZEB1 to maintain repression of *BZLF1* gene expression and, thus, EBV latent infection.

Addition of miR200b to CNE1^{Akata} cells induces a complete EBV lytic replication cycle. To assess whether the EBV reactivation induced by these miRNAs led to full-blown EBV lytic replication, we also measured by immunofluorescence staining the expression of gp350, an EBV late gene, in the CNE1^{Akata} cells. Approximately 2.6% of the cells contained gp350 at 3 days after addition of miR200b, 11-fold more than we observed in the cells transfected in parallel with the scrambled negative-control miRNA (Fig. 7A). This value is a lower bound of the overall induction level by these miRNAs since (i) some lytically infected cells may have already died and, thus, not have been scored in this assay, and (ii) other cells may not yet have reached this late stage in the EBV lytic cycle due to a delay in the onset of EBV reactivation while the ZEB1 protein level gradually declined following miRNA inhibition of synthesis of new ZEB1 protein. Thus, we conclude that miR200b addition can induce EBV reactivation at least as far as late gene expression.

To assess whether miR200b addition led all the way to production of infectious virus, we harvested the media from the CNE1^{Akata} cells 3 days after transfection with miR200b versus the negative-control miRNA. Using a Raji cell assay, we observed 7-fold more infectious virus present in the medium obtained from the miR200b-treated cells than from the cells transfected in parallel with the negative-control miRNA (Fig. 7B). Thus, we conclude that addition of miR200b can induce production of infectious virus.

miR200b and miR429 can also downregulate ZEB2, leading to lytic cycle induction. We previously showed that ZEB2 can also play a key role in the latent-lytic switch of EBV in some cell lines, including 293$^{B95.8}$ (22). Therefore, we also tested likewise whether transfection of these miRNAs led to EBV lytic reactivation in 293$^{B95.8}$ cells in which ZEB2, not ZEB1, is the major repressor of *BZLF1* gene expression. EBV is in a very tightly latent state in 293$^{B95.8}$ cells; reactivation has been previously reported only for transfection with BZLF1 expression plasmids (e.g., see reference 19; reviewed in references 74 and 75). However, we recently found that knocking down the level of ZEB2 protein along with adding the chemical inducers TPA and sodium butyrate could also lead to induction of EBV lytic protein expression in these cells (22). Therefore, we in-

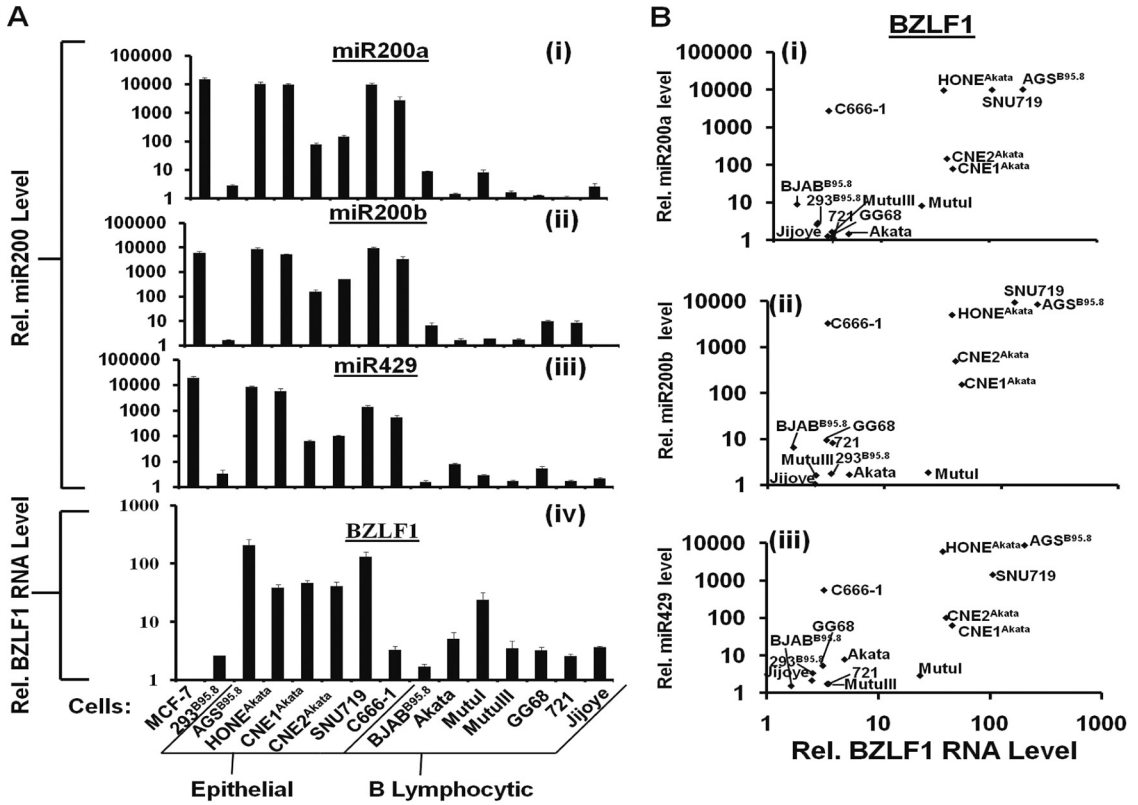

FIG. 5. Correlation between levels of miR200 family members and BZLF1 RNA in EBV-positive cell lines. (A) RT-qPCR analysis of levels of miRNA 200 family members and BZLF1 RNA in multiple EBV-positive epithelial and B-lymphocytic cell lines. Total RNA was harvested, cDNA was synthesized, and samples were analyzed for levels of BZLF1 RNA and miRNA 200a, 200b, and 429 as described in Materials and Methods. BZLF1 RNA levels were normalized to the ribosomal Po level, and miR200 family levels were normalized to the RNU38B level present in the same samples. (B) Scatter plots showing positive correlations between the level of BZLF1 RNA and level of miR200a (i), miR200b (ii), and miR429 (iii) in EBV-positive cell lines. Data were taken from panel A.

cubated the $293^{B95.8}$ cells with TPA plus sodium butyrate after transfecting them with the miRNAs to knock down ZEB2 expression. Similar to the results observed with $CNE1^{Akata}$ cells, transfection of miR200a, miR200b, or miR429 into $293^{B95.8}$ cells led to a large increase in the level of that specific miRNA (Fig. 8B), with miR200b and miR429, but not miR200a, significantly knocking down the level of ZEB2 protein and, consequently, upregulating BZLF1 and BMRF1 protein levels (Fig. 8A, lanes 2 to 4 versus lanes 1 and 5). Likewise, Lin et al. (54) observed a modest level of induction, i.e., 0.3% Zta-positive cells, 14 days after stable addition of miR429 to these cells by retroviral infection even in the absence of these chemical inducers. Therefore, we conclude that high levels of either miR200b or miR429 can lead to induction of EBV lytic replication in $293^{B95.8}$ cells concomitant with reduction in ZEB1 and ZEB2 levels.

miRNA-mediated lytic reactivation occurs via ZEB1 and ZEB2. To eliminate the possibility that these miRNAs were upregulating EBV lytic gene expression through mechanisms other than their direct effects on ZEB1 and ZEB2 levels, we examined likewise 293 cells latently infected with a bacmid containing a variant of the B95.8 strain of EBV possessing a 2-bp substitution mutation in the ZV element of Zp, the promoter of the *BZLF1* gene. We previously reported that ZEB binds to Zp with reduced affinity in these cells relative to 293 cells latently infected with the mutant's parental wild-type EBV bacmid (92). In addition, these cells spontaneously exhibit some EBV lytic gene expression even in the absence of treatment with the inducers TPA and sodium butyrate due to reduced repression of *BZLF1* gene expression by ZEB (92). As observed with the wild-type (WT)-infected cells (Fig. 8A and B), transfection of either miR200b or miR429 led, as expected, to both 80% or more reduction in the levels of ZEB1 and ZEB2 protein and a large increase in the level of the specific transfected miRNA in these cells (Fig. 8C and D). However, transfection of these miRNAs into the 293^{ZVmt} cells, unlike the $293^{B95.8}$ cells, did not lead to a significant increase in EBV lytic gene expression above the background level observed with the negative-control miRNA (Fig. 8C, lanes 1 to 4 versus lane 5). This finding indicates that these miRNAs induce EBV reactivation via a signaling pathway that involves a factor such as ZEB1 or ZEB2 that acts via binding the ZV element of Zp. Therefore, we conclude that much, if not all, of the miRNA-mediated EBV reactivation observed probably occurred through their downregulating ZEB1 and ZEB2 protein levels in these cells.

Inhibition of miR200b and miR429 leads to downregulation of EBV lytic gene expression. We next hypothesized that loss of miR200b and miR429 would lead to upregulation of ZEB protein levels and downregulation of EBV lytic gene expres-

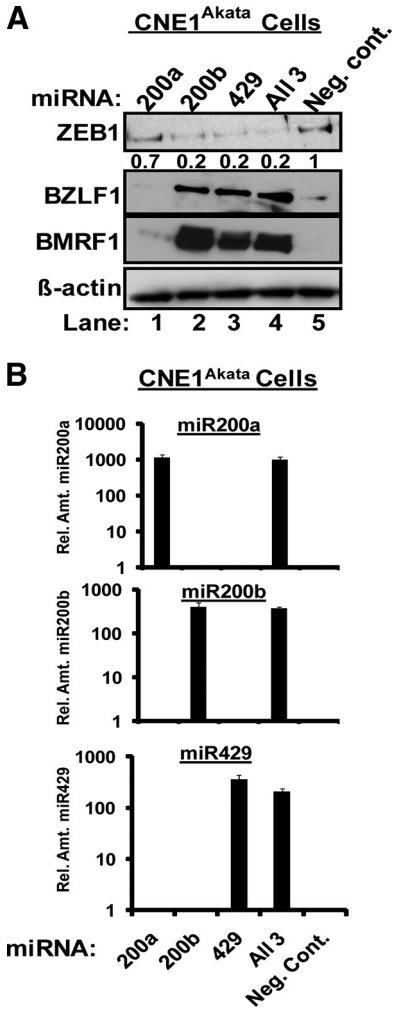

FIG. 6. Transfection into CNE1^{Akata} cells of miR200b or miR429 leads to EBV reactivation. (A) Immunoblot showing levels of the indicated proteins following transfection of CNE1^{Akata} cells with the indicated miRNAs. CNE1 cells latently infected with the Akata strain of EBV were transfected with 30 nM miR200a, miR200b, miR429, all three, or a negative-control miRNA. Seventy-two hours later, cells were harvested for whole-cell protein and RNA. Fifty μg of protein was loaded per well, with β-actin serving as a loading control. Relative protein levels, indicated by the numbers below the blots, were determined by densitometry, with internal normalization to β-actin and external normalization to the negative-control (Neg. cont.) sample processed in parallel. (B) Relative levels of miR200a, miR200b, and miR429 present in the cells from panel A 72 h after transfection. Levels of miR200a, miR200b, and miR429 were determined by RT-qPCR as described in Materials and Methods, with normalization to RNU38B.

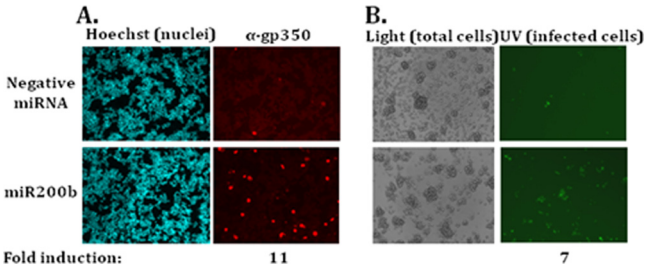

FIG. 7. miR200b activates the full EBV lytic cycle in CNE1^{Akata} cells. (A) Indirect immunofluorescence staining showing the addition of miR200b leads to induction of EBV late gene expression. CNE1^{Akata} cells were transfected with 30 nM of the negative control or miR200b and incubated at 37°C for 72 h prior to processing for gp350 protein as described in Materials and Methods. Shown here are fields of cells observed for all cellular nuclei (Hoechst staining) versus the subset of them containing gp350 protein. (B) Raji cell assay showing increased production of infectious virus in CNE1^{Akata} cells after the addition of miR200b. Media from cells transfected and incubated as described in panel A were concentrated and used to infect Raji cells as described in Materials and Methods. Shown here are fields of Raji cells observed with visible light (Light) for all cells versus UV light (UV) for the EBV-infected, GFP-positive ones.

sion. Modified oligonucleotides containing an O-methyl or "locked" nucleic acid strongly bind complementary single-stranded RNA and DNA molecules (82, 83). Due to the modifications of these oligonucleotides, the binding is more energetically favored than is the binding of miRNAs to RNA and DNA molecules (82, 83). When these modified oligonucleotides duplex with specific miRNAs, the endogenous cellular RNase H degrades these miRNAs, leading to a significant reduction in their levels in cells (23). Therefore, we examined whether the addition to our EBV-positive cells of such inhibitors specific to miR200 family members could lead to down-regulation of EBV lytic gene expression. These experiments were performed with EBV-positive NPC HONE^{Akata} and CNE^{Akata} cells, which naturally contain moderate to high levels of these miRNAs (Fig. 2). In addition, HONE^{Akata} and CNE1^{Akata} cells naturally express EBV lytic genes at moderate to high levels in the absence of inducers or are easily induced into lytic replication (Fig. 3A, lane 4, and Fig. 3A, lane 5, respectively).

HONE^{Akata} cells were transfected with inhibitors to the entire miR200 family (66), miR200a, miR200b, miR429, or an inhibitor not specific to any known sequences in the genome as a negative control. The miR200a and miR429 inhibitors were fairly specific, reducing the levels of miR200a and miR429 to approximately 1/10 and 1/3 to 1/2, respectively, of the levels observed with the control inhibitor, while leaving the levels of the other miRNAs largely unchanged (Fig. 9B). Also as expected, the general 200 inhibitor reduced the levels of all three of these miR200 family members to 1/6 to 1/3 of the levels seen with the control inhibitor. Unfortunately, the miR200b inhibitor behaved likewise, also reducing the levels of all three of these miRNA to $<$1/10 to 1/5 of the levels observed with the control inhibitor. Thus, this miR200b inhibitor was really a second general miR200 family inhibitor.

Immunoblot analysis of protein levels yielded the predicted results. Once again, altering the level of either miR200b or miR429, but not miR200a, led to a significant change in the level of ZEB1 protein present in the cells (Fig. 9A). Concomitant with an increase in the ZEB1 protein level was a decrease in the levels of both BZLF1 and BMRF1 protein compared to the cells treated in parallel with the negative-control miRNA inhibitor (Fig. 9A). Thus, we conclude that miRNA-induced change in the levels of ZEB proteins in either direction leads to a corresponding change in the degree of latency in EBV-infected cells.

To confirm this finding, we examined likewise the effect of the miR200 family inhibitors in the more tightly latently in-

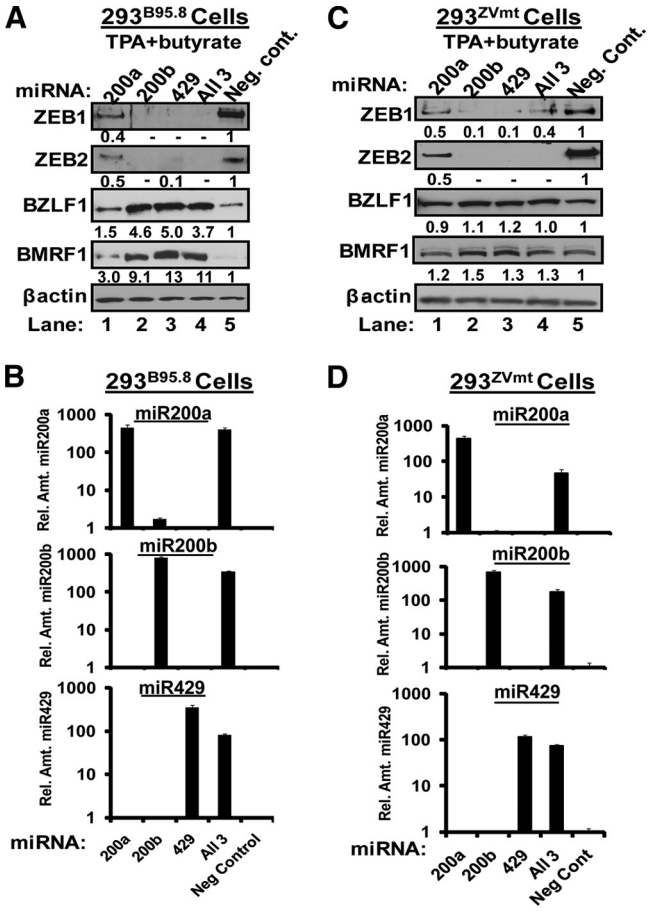

FIG. 8. miRNA-mediated EBV reactivation is mediated via the ZEB-binding element of Zp. (A and C) Immunoblots showing levels of the indicated proteins following transfection of $293^{B95.8\text{-}WT}$ and $293^{B95.8\text{-}ZVmt}$ cells with the indicated miRNAs. 293 cells latently infected with a bacmid containing the B95.8 strain of EBV (A) or a variant of this bacmid with a 2-bp substitution mutation in the ZV element of Zp (C), respectively, were transfected with 30 nM concentrations of the indicated miRNAs. Forty-eight hours later, 20 ng/ml TPA and 3 mM sodium butyrate were added to the medium, and incubation was continued for another 48 h. The cells were then harvested for whole-cell protein and RNA. Thirty μg of protein was loaded per well for analysis of ZEB1 and ZEB2, and 10 μg of protein was loaded per well for analysis of BZLF1 and BMRF1. (B and D) Relative levels (Rel. Amt.) of miR200a, miR200b, and miR429 present in the cells from panels A and C, respectively. Levels of miR200a, miR200b, and miR429 were determined by RT-qPCR as described in Materials and Methods, with normalization to RNU38B.

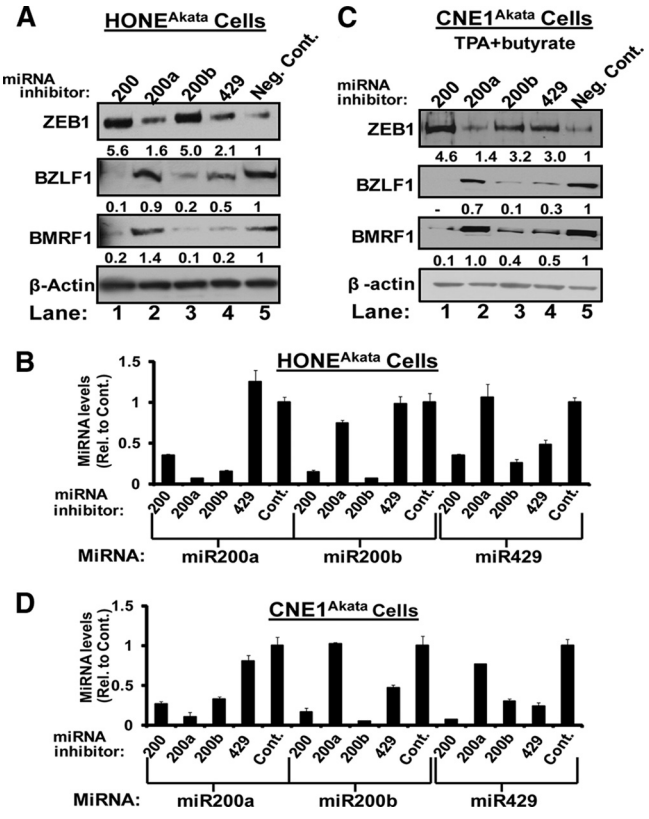

FIG. 9. Inhibition of levels of miR200 family members with modified oligonucleotides reduces EBV lytic gene expression. (A and C) Immunoblots showing levels of the indicated proteins following transfection of $HONE^{Akata}$ (A) and $CNE1^{Akata}$ (C) cells with 60 nM concentrations of the indicated inhibitors of miRNA-200 family members. Seventy-two hours after transfection, 20 ng/ml TPA and 3 mM sodium butyrate were added to the medium containing the $CNE1^{Akata}$ cells, and incubation was continued for an additional 24 h. Whole-cell extracts were prepared for analysis of protein (A and C) and RNA (B and D). Thirty μg of protein was loaded per well for determination of ZEB1 protein levels; 20 μg of protein was loaded per well for determination of BZLF1, BMRF1, and β-actin protein levels. Relative protein levels, shown by the numbers directly below each lane, were calculated by densitometry, with internal normalization to β-actin present in the same samples and external normalization to the protein levels present in the cells processed in parallel that had been transfected with the control miRNA inhibitor. (B and D) Relative levels of miRNAs 200a, 200b, and 429 present in the cells from panels A and C, respectively. Levels of miRNAs 200a, 200b, and 429 were determined by RT-qPCR as described in Materials and Methods, with normalization to RNU38B.

fected $CNE1^{Akata}$ cells. Because $CNE1^{Akata}$ cells naturally exhibit very little EBV lytic gene expression (Fig. 3A), we treated the cells with the chemicals TPA and sodium butyrate to induce EBV lytic replication after knocking down the levels of the miRNAs with the miRNA inhibitors. As with the $HONE^{Akata}$ cells, reduction in the level of either miR200b or miR429, but not miR200a, led to upregulation of the level of ZEB1 and downregulation of the levels of both BZLF1 and BMRF1 compared to the negative control (Fig. 9C). Therefore, we conclude that the level of miR200b or miR429 in cells affects the level of ZEB1 protein which, in turn, affects the level of EBV lytic gene expression via regulating *BZLF1* gene expression.

Inhibition of miR200 family member levels by overexpression of ZEB1 and ZEB2 also leads to downregulation of EBV lytic gene expression. ZEB1 and ZEB2 can repress synthesis of the transcript from which miRNAs 200a, 200b, and 429 are produced by RNA processing via binding an E-box present in the promoter region of this gene (10, 11). Thus, we predicted that exogenous expression of either ZEB1 or ZEB2 in EBV-positive cells would also lead to decreased levels of both the miR200 family members and EBV lytic proteins. To test this prediction, we again utilized $HONE^{Akata}$ and $CNE1^{Akata}$ cells, since they contain moderately high levels of these miRNAs (Fig. 2).

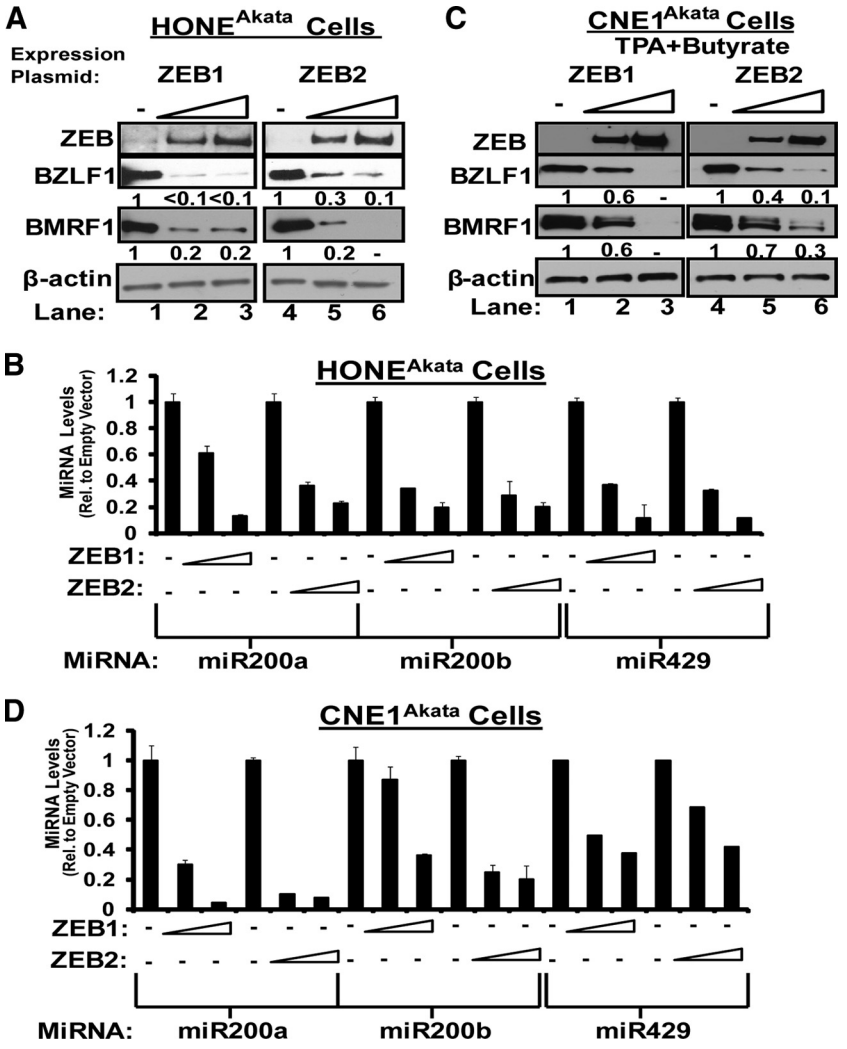

FIG. 10. miR200 family members inhibition via ZEB1 or ZEB2 overexpression decreases EBV lytic gene expression. (A and C) Immunoblots showing relative levels of the indicated proteins in HONE^{Akata} (A) and CNE1^{Akata} (C) cells following transfection with 0, 0.5, or 1.0 μg of an expression plasmid to ZEB1 or ZEB2, as indicated, along with 1.0, 0.5, or 0 μg of their empty vector, pcDNAhismaxC, to apply 1.0 μg total DNA per well in a 6-well plate. Forty-eight hours later, whole-cell extracts were prepared for protein (A and C) and RNA (B and D). The proteins were analyzed for ZEB1, ZEB2, BZLF1, and BMRF1 levels as described in the legend to Fig. 4. (B and D) Relative levels of miRNAs 200a, 200b, and 429 present in the cells from panels A and C, respectively. Levels of miRNAs 200a, 200b, and 429 were determined by RT-qPCR as described in Materials and Methods, with normalization to RNU38B.

When HONE^{Akata} cells were transfected with various amounts of either a ZEB1 or a ZEB2 expression plasmid, we found, as expected, a corresponding downregulation of EBV IE and E gene expression (Fig. 10A); we also observed a corresponding decrease in the levels of miR200a, miR200b, and miR429 (Fig. 10B). Similar results were obtained when we overexpressed ZEB1 or ZEB2 in CNE1^{Akata} cells that had been induced by incubation with TPA and sodium butyrate (Fig. 10C and D). Thus, we conclude that a high level of either ZEB1 or ZEB2 does, indeed, lead to repression of both EBV reactivation and synthesis of miRNAs 200a, 200b, and 429 in EBV-positive cells.

To demonstrate that these effects occur independently of any EBV-encoded proteins or RNAs, we also performed transient transfection assays with a Zp-driven luciferase reporter in an EBV-negative cell line. Transfection into 293D cells of miR200b prior to transfection with the reporter pZpWT-luc led to a nearly 6-fold upregulation of luciferase activity relative to the cells transfected in parallel with the negative-control miRNA (Fig. 11). Cotransfection of ZEB1 or ZEB2 expression plasmids along with the pZpWT-luc reporter largely negated the miR200b-mediated upregulation of transcription from Zp (Fig. 11, black bars). In comparison, transfection of neither miR200b nor the ZEB expression plasmids significantly affected luciferase activity in the cells transfected in parallel with the pZpZVmt-luc reporter (Fig. 11, white bars), a variant of pZpWT-luc containing a 2-bp substitution mutation in the ZV element of Zp, the main binding site for the ZEBs (22, 48, 92). The level of luciferase activity observed with the pZpZVmt-luc reporter, 4-fold-higher than that with pZpWT-luc, is due to the endogenous ZEB proteins repressing the latter in 293D cells (48). Thus, the responses observed with the WT reporter con-

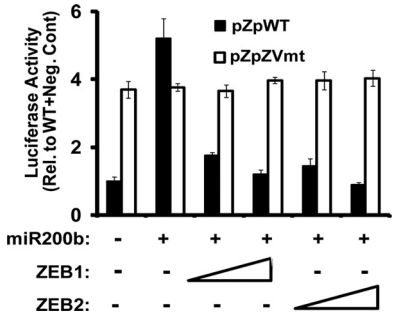

FIG. 11. Overexpression of either ZEB1 or ZEB2 inhibits induction of transcription from Zp by miR200b. 293D cells were transfected with 30 nM miR200b or negative-control miRNA (indicated by "-"). Twenty-four hours later, the cells in 12-well plates were cotransfected with a total of 2.0 μg DNA per well consisting of (i) 0.5 μg of the reporter plasmid pZpWT-luc (black bars) or pZpZVmt-luc (white bars), (ii) 0, 0.25, or 0.5 μg of a plasmid expressing ZEB1 or ZEB2, as indicated, along with 1.0, 0.75, or 0.5 μg of their empty vector pcDNAhismaxC, respectively, and (iii) 0.5 μg of pCMXRluc as an internal control. Forty-eight hours later, the cells were harvested. Firefly luciferase activity was determined, with internal normalization to *Renilla* luciferase and external normalization to the value obtained with the cells transfected in parallel with pZpWT-luc and the negative-control miRNA in the absence of a ZEB expression plasmid. Data shown are from a representative experiment of assays performed in triplicate on three separate occasions.

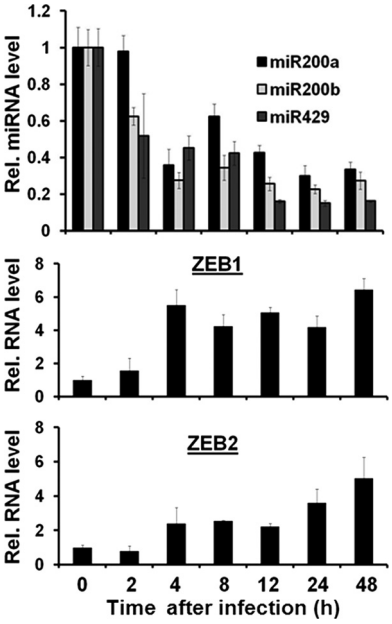

FIG. 12. EBV infection of primary blood B cells leads to upregulation of ZEB and downregulation of miR200 family member levels. Primary blood B cells (1×10^6 cells/ml) were infected with 1×10^5 GRU/ml of EBV prepared as described in Materials and Methods and incubated at 37°C for the times indicated. Relative miRNA, ZEB1, and ZEB2 RNA levels were determined by RT-qPCR as described in Materials and Methods. Approximately 65% of them scored as GFP positive (i.e., EBV infected) by 48 h after infection. Data were normalized first to RNU38B for miRNAs and ribosomal Po for ZEB RNAs present in the same samples and then to the levels present at time zero.

struct were specifically mediated by the ZEBs through the ZV element of Zp. Therefore, we conclude that ZEB1 and ZEB2 directly downregulate expression of both these miR200 family members and *BZLF1* gene expression, doing so independently of the presence of any EBV RNAs or proteins.

Effect of EBV infection on levels of miR200 family members and the ZEBs. One interesting question is whether primary infection by EBV alters the balance between the levels of the ZEBs and miR200 family members, possibly in a cell-type-dependent manner, thereby controlling whether the result is latency or lytic replication. To begin to answer this question, we infected primary blood B cells with EBV and measured by RT-qPCR the relative levels of these RNAs over the subsequent 48 h. Interestingly, the levels of these miRNAs significantly dropped, while the level of ZEB1 mRNA significantly rose within 4 h (Fig. 12). Shinozaki et al. (79) likewise reported downregulation of miRNAs 200a and 200b and upregulation of ZEB1 and ZEB2 following EBV infection of gastric carcinoma cell lines, in some cases by as much as three orders of magnitude by the time the EBV-infected cells were selectively grown out. Our finding of only a 4- to 6-fold change in the levels of the miRNAs and ZEBs within 48 h was likely due, in part, to the presence in our samples of significant numbers of uninfected cells. Remaining unclear is which EBV-encoded protein(s) or RNA(s) is responsible for this phenomenon. Regardless, these findings suggest that downregulation of the miR200 family members, along with concomitant upregulation of the ZEBs, contributes to the establishment and maintenance of EBV latency in both epithelial and B cells.

DISCUSSION

We identified here the cellular microRNAs 200b and 429 as inducers of EBV reactivation into lytic replication, doing so through downregulating expression of the cellular repressor proteins ZEB1 and ZEB2, which play a central role in silencing expression of the latent-lytic switch *BZLF1* gene during EBV latency. First, we showed that the levels of the cellular miRNAs 200a, 200b, and 429 negatively correlate with the levels of ZEB1 and ZEB2 (Fig. 2 and 4) and positively correlate with the level of BZLF1 (Fig. 3 and 5) in EBV-positive epithelial and B-lymphocytic cell lines. Next, we demonstrated that the addition of miRNAs 200b and 429, but not 200a, led to both downregulation of ZEB protein levels and upregulation of EBV lytic gene expression (Fig. 6 and 8). By 3 days after the addition of miR200b, EBV late gene products were readily detectable in 3% of $CNE1^{Akata}$ cells; production of infectious virus had also significantly increased (Fig. 7). Conversely, degradation of these microRNAs by the addition of specific modified oligonucleotides (Fig. 9) or inhibition of their synthesis by overexpression of ZEB1 or ZEB2 (Fig. 10) led to downregulation of EBV lytic gene expression. We further showed that this effect of these miRNAs on EBV lytic gene expression was specifically mediated through the ZEB-binding ZV element of the BZLF1 promoter both in the presence (Fig. 8) and absence (Fig. 11) of the EBV genome. Last, we found that EBV infection (Fig. 12) led to significant alterations in the levels of miR200 family members and the ZEBs in the directions consistent with the expected physiological responses. Thus, we conclude that some miR200 family members can play an important physiological role in regulating the switch between

latent and lytic infection by EBV, doing so, at least in large part, via their regulation of ZEB1 and ZEB2 levels in cells.

Inverse correlation between levels of ZEBs and miR200 family members in EBV-positive cells. We previously reported that ZEB1 and ZEB2 are potent repressors of EBV lytic replication, maintaining the virus in a latent state through silencing expression of the *BZLF1* gene (22, 47, 48, 92). We sought to identify factors that regulate expression of ZEB1 and ZEB2 because of their central role in regulating the EBV life cycle. Several groups have reported that an inverse correlation exists between the levels of miR200 family members and the ZEBs via a double-negative feedback loop (11, 14, 36, 37, 44, 66). Our data reported here (Fig. 2 and 4) confirm this conclusion for ZEB1, extending it to numerous EBV-positive cell lines. Given the findings of Park and colleagues that ZEB2 and miR200 family members exist in a near-perfect negative correlation (66), we expected a negative correlation to apply to ZEB2 and miRNAs in our EBV-positive cell lines as well. However, we found that this negative correlation was weaker for ZEB2 (Fig. 4B) due to the absence of ZEB2, but not ZEB1, from most of the EBV-positive cell lines examined by us (Fig. 3). Nonetheless, in agreement with the findings of others (10, 36, 66), we did observe that overexpression of miR200 family members led to potent downregulation of ZEB2 (Fig. 8A). Given these findings, we speculate that yet-to-be-identified factors (e.g., promoter methylation) in addition to miR200 family members must be significantly contributing to the regulation of ZEB2 expression.

miR200 family member expression and EBV lytic replication. We demonstrated here that high-level expression of miR200 family members positively correlated with EBV lytic gene expression, including in the type II latency SNU719 cell line derived from an EBV-positive gastric carcinoma (Fig. 3 and 5). While this paper was under revision, Lin et al. (54) likewise reported that the levels of these miRNAs were very low in a variety of highly latent EBV-positive BL B-cell lines, including type I latency Rael cells, yet quite high in NPC CNE1 and gastric carcinoma AGS cells and EBV-positive derivatives of them known to be readily inducible, or even highly lytic (30). Shinozaki et al. (79) also just reported that miR200a and miR200b levels are significantly lower in EBV-positive primary gastric carcinomas that were presumably in a type II latency form of infection than they were in either EBV-negative primary gastric cancers or normal gastric mucosa, cells in which EBV infection is usually lytic. Thus, natural endogenous levels of miR200 family members probably play an important, physiological role in determining whether EBV infection of a cell leads to latency or lytic replication.

However, there were a few exceptions to this strong correlation. The NPC C666-1 cell line expressed high levels of these miRNAs, yet a low level of BZLF1 RNA (Fig. 5A). Likewise, we found that knockdown of ZEB protein levels by itself was insufficient to induce *BZLF1* gene expression in 293 cells within 4 days; chemical inducers were needed as well (22) (Fig. 8). Similarly, Lin et al. (54) did manage to achieve some EBV reactivation in 293 cells by stable addition of miR429; however, only 0.3% of their cells stained positive for Zta protein after 14 days versus 3% of CNE1 cells stained positive for gp350 by us at 3 days after the transient addition of miR200b (Fig. 7A). Conversely, the BL MutuI cell line expressed low levels of these miRNAs, yet a fairly high level of BZLF1 RNA (Fig. 5A). Consistent with the latter finding, Lin et al. (54) observed at most a few-fold increase relative to their control in EBV IE and E lytic gene expression in MutuI cells after high-level stable expression of miR429 for 14 days. We speculate that these exceptional cases arise because regulation of Zp is rather complex, with multiple transcriptional activators and repressors in addition to the ZEBs affecting overall control of *BZLF1* gene expression (reviewed in references 74 and 75). Other negative regulatory factors, including MEF2D (38, 45) and the yet-to-be-identified ZIIR-binding protein (55), and positive regulatory factors, including ATF-1 (85), C/EBPα (90), and c-jun (30, 85), also contribute to silencing and activating *BZLF1* gene expression, respectively. Nevertheless, a very strong correlation was found between the levels of these miR200 family members and lytic gene expression (Fig. 5B), confirming a central role for the ZEBs in controlling the EBV latent-to-lytic switch.

Interestingly, the addition of either miR200b or miR429, but not miR200a, led to significant downregulation of the ZEBs and upregulation of lytic gene expression (Fig. 6 and 8). These findings are fully consistent with those of others that miRNAs 200b, 200c, and 429, with the seed sequence 5′-AAUACUG-3′, have significant effects on ZEB expression, while miRNAs 200a and 141, with the seed sequence 5′-AACACUG-3′, have more modest effects, particularly on downstream molecular targets of the ZEBs (18, 44, 46, 66). Using the miRBase and TargetScan programs (51) to look for binding sites for miR200 family members, we (Fig. 1D) and others (14, 36, 37, 44, 66) have identified only two binding sites for miRNAs 200a and 141, yet five or more binding sites for miRNAs 200b, 200c, and 429 in the 3′-UTRs of both ZEB1 and ZEB2. Thus, we conclude that miRNAs 200b and 429, but not 200a, are important regulators of the EBV switch between latency and lytic replication, doing so indirectly via their effects on expression of ZEB1 and ZEB2.

Role of miR200 family members in regulating the EBV latent-lytic switch. Based upon our findings presented here, together with those of others (10, 11, 36, 37, 54, 66, 79, 91), we propose a model for how the double-negative feedback loop that exists between the ZEBs and these miRNAs regulates whether EBV infection is latent or lytic. miRNAs 200b, 429 and, presumably, 200c and the ZEBs inhibit each other's synthesis (Fig. 13A). Whether these miRNAs or the ZEBs are highly expressed in a particular cell determines whether infection by EBV is largely latent or lytic. In cells containing high levels of these miR200 family members, the absence of significant levels of the ZEBs enables *BZLF1* gene expression, leading to EBV lytic replication (Fig. 13B). In cells with low levels of the miR200 family members, ZEB1 or ZEB2 levels are high; EBV infection leads to establishment and maintenance of latency because ZEB1 or ZEB2 is available to efficiently repress *BZLF1* gene expression (Fig. 13C).

In addition to these miRNAs, other cellular factors, including NF-κB (15, 67) and Snail (6, 39), can affect the levels of the ZEBs. Our finding that many EBV-positive cell lines contain ZEB1, but not ZEB2 (Fig. 4), clearly indicates that expression of these two genes can be differentially regulated by yet-to-be-determined factors.

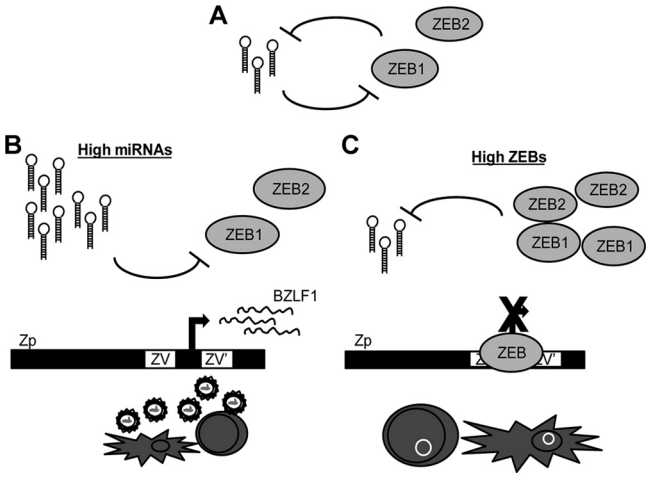

FIG. 13. Model for roles of ZEBs and miR200 family members in infection by EBV. (A) Schematic representation of the double-negative feedback loop between the ZEBs and the miR200 family members. (B) When these miRNAs are present at high levels, ZEB1 and ZEB2 protein synthesis is inhibited. In EBV-positive cells, this leads to lytic replication. (C) When either ZEB1 or ZEB2 is present at a high level, it inhibits expression of both miR200 family members and the *BZLF1* gene, leading to EBV latency.

miRNAs and carcinogenesis: potential for use in EBV lytic induction therapy. Induction of latent EBV into lytic replication in the presence of the nucleoside analog ganciclovir (GCV) leads to the specific killing of EBV-positive cells since the virus-encoded protein kinase, BGLF4, expressed during reactivation converts ganciclovir to its phosphorylated cytotoxic form (26–28, 57–60, 89). Reactivation of EBV in the presence of GCV has been termed lytic induction therapy; it holds promise as a potential therapy for treating patients with EBV-associated malignancies (26–29, 57–59). Thus, factors that reactivate EBV out of latency into lytic replication are potential candidates for use in lytic induction therapy. Our findings presented here are among the first to show that specific cellular miRNAs, i.e., miR200b and miR429, can be used to induce EBV lytic gene expression. Therefore, these miRNAs, delivered either directly to EBV-infected cells or expressed in appropriately engineered retroviruses or lentiviruses, might serve as a novel "drug," together with acyclovir or GCV, for lytic induction therapy.

One caveat of EBV lytic induction therapy is that only a small subset of the tumor cells may actually be dependent upon EBV for their survival in some cases, i.e., cells that had originally been EBV positive may have lost the virus while remaining cancerous. Many of these EBV-negative cells might still be killed from the bystander effect of GCV (32, 53). However, some of the cancerous cells might survive lytic induction. Interestingly, high ZEB levels also correlate quite well with the metastatic capabilities of many types of epithelial cell cancers and poor patient outcome (7, 65, 88). Furthermore, as tumors progress, miR200 family members levels drop (65). Conversely, high levels of miR200 family members correlate well with a low degree of tumorigenicity and good patient outcome (1, 88). These observations are likely due, in part, to the ZEBs being repressors of E-cadherin expression (reviewed in reference 67), a protein usually lost during transformation of epithelial cells into metastatic cancerous ones. Therefore, the use of miR200 family members to treat ZEB-positive malignancies may lead to decreased aggressiveness of the tumors and increased long-term survival of the patients. With respect to ZEB-positive, EBV-associated malignancies, we predict that miR200-based therapeutics may prove to be especially beneficial due to their both inducing EBV reactivation and knocking down ZEB levels, leading to reexpression of E-cadherin and other genes associated with a less tumorigenic phenotype in the tumor cells not killed by EBV lytic replication. Indeed, the addition of miR200 family members to EBV-positive epithelial NPC C666-1 and CNE1^{Akata} cells has recently been shown to lead to decreased cell motility and growth (91).

In conclusion, we speculate that some form of these miR200 family members may function quite well as one of the components of a cocktail of drugs used in treating patients with some EBV-associated malignancies by lytic induction therapy, given that they represent natural cellular products for upregulating EBV lytic gene expression. Other than the ZEBs, little is currently known regarding how expression of these miRNAs is regulated. Thus, in addition to miRNAs 200b and 429 themselves, compounds that upregulate their expression may also prove useful as a new class of drugs for lytic induction therapy for EBV-associated malignancies and other cancers in which the ZEBs play major roles in maintaining the metastatic phenotype.

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