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Differential Expression of the miR-200 Family MicroRNAs in Epithelial and B Cells and Regulation of Epstein-Barr Virus Reactivation by the miR-200 Family Member miR-429▽

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The miR-200 microRNA family is important for maintaining the epithelial phenotype, partially through suppressing ZEB1 and ZEB2. Since ZEB1 inhibits Epstein-Barr virus (EBV) reactivation, we hypothesized that expression of miR-200 family members in epithelial cells may partly account for higher levels of EBV reactivation in this tissue (relative to nonplasma B cells). Here we show that, whereas miR-200 family members are expressed in epithelial cells, their expression is low in latently infected B cells. Furthermore, the miR-200 family member miR-429 shows elevated expression in plasma cell lines and is induced by B-cell-receptor activation in Akata cells. Lastly, expression of miR-429 can break latency.

Epstein-Barr virus (EBV) is an important human pathogen that is involved in a variety of malignant and nonmalignant diseases (16). EBV utilizes the following two distinct stages in its life cycle: latency and a viral replication stage (lytic reactivation). Expression of the viral lytic gene product Zta or Rta initializes the entire cascade of lytic gene expression, leading to production of infectious virus. The switch mechanism controlling the transition from latency to the lytic replication phase is tightly regulated to maintain a homeostatic viral life cycle. A number of cellular and viral factors that regulate the Zta and Rta promoters have been identified. Kraus et al. (10) have shown that the epithelial-to-mesenchymal transition (EMT) regulator ZEB1 can bind to an inhibitory element in the Zta promoter (Zp) named ZV. The binding of ZEB1 to ZV inhibits the reactivation process by blocking the expression of Zta (7, 10, 20). Furthermore, recombinant EBV with a disrupted ZV element displays a higher reactivation rate and higher yields of infectious virions than wild-type virus (20).

MicroRNAs (miRNAs) are involved in regulating a wide spectrum of cellular processes as well as the life cycles of viruses. Herpesviruses encode their own sets of microRNAs that modulate host signaling pathways in ways that promote their virus infection cycle (3–5). Viruses also utilize host cell miRNAs to modulate the cellular environment in ways that similarly support their infection cycle (18).

The miR-200 family of microRNAs function in various biological and pathological processes, and there is accumulating evidence that they can function as potent tumor suppressors (15). This family has five members that are located in two distinct genomic loci/clusters (Fig. 1A). One cluster is located on chromosome 1 and carries miR-200a, miR-200b, and miR-429. The second cluster is located on chromosome 12 and carries miR-200c and miR-141. Members of each cluster are largely coregulated as a result of being processed from the same primary transcript. The miR-200 family can also be divided into two presumed functional groups based on their seed sequences (miR-429, miR-200b, and miR-200c have identical seed sequences, which differ by 1 base from the miR-200a and miR-141 seed sequences). Although a 1-base difference in seed sequence is expected to confer largely distinct targeting specificity, Park et al. (14) have shown that these subgroups have highly overlapping targetomes, suggesting that they have evolutionarily conserved functions.

Expression of the miR-200 family miRNAs in EBV-trophic cell lines. The level of miR-200 family expression is commonly altered in both physiological processes (e.g., EMT and embryogenesis) and pathological processes (e.g., tumorigenesis). As a direct target of the miR-200 family, ZEB1 shows an expression pattern inverse to that of the miR-200 family (14). Considering the fact that ZEB1 serves as a master regulator for EBV reactivation, we tested the expression of miR-200 family miRNAs in tissue types that are naturally permissive for EBV infection. Total RNA was extracted from EBV-negative and EBV-positive (type I or type III latency) B-cell lines (Burkitt's lymphomas and lymphoblastoid cell lines) and epithelial cells using a modified TRIzol method (2) and an miRNeasy kit (Qiagen). Quantitative reverse transcription-PCR (RT-PCR) analysis of the miR-200b–miR-200a–miR-429 primary transcript revealed little expression in each B-cell line tested (Fig. 1A and B). In contrast, expression of this cluster was readily detected in all EBV-positive and EBV-negative epithelial cell lines (Fig. 1B). We then examined expression of the mature/processed form of a representative miR-200 family member from this locus, miR-429, using a TaqMan miRNA assay (Applied Biosystems). Again, while expression was observed in all epithelial cell lines, little or no expression was detected in any of these B-cell lines (Fig. 1C). Lastly, expression of a member of the second miR-200 family locus, miR-200c, was similarly found to be substantially lower in each B-cell line than in epithelial cells (Fig. 1D).

Induction of EBV lytic reactivation in miR-429-transduced EBV-293 cells. To investigate the influence of miR-200 family members on reactivation, we introduced a control or a miR-

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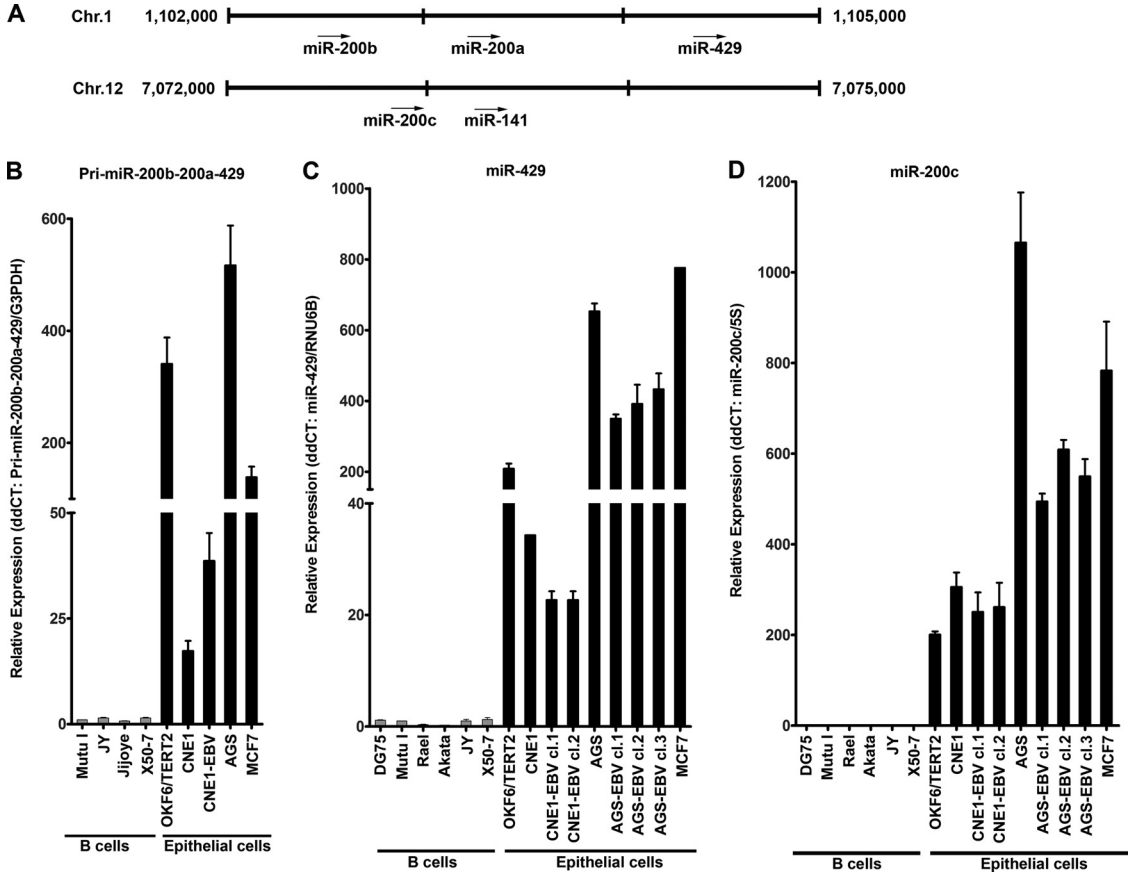

FIG. 1. Expression of miR-200 family miRNAs in EBV-trophic cell lines. (A) miR-200 family genomic loci. miR-200b, miR-200a, and miR-429 are transcribed as a single polycistronic transcript from chromosome (Chr.) 1. miR-200c and miR-141 are carried by a single transcript from chromosome 12. Chromosomal coordinates are based on the 2009 version of the public human genome assembly (GRCh37). (B) Quantitative RT-PCR of the miR-200b–miR-200a–miR-429 primary transcript (Pri). Primer set for the miR-200b–miR-200a–miR-429 primary transcript: 5′-AGTGGGGCTCACTCTCCAC-3′ and 5′-AGGAGGAGGAGGAGGAGAAA-3′ (1). Primer set for glyceraldehyde 3-phosphate dehydrogenase (G3PDH): 5′-GCCAAGGTCATCCATGACAACTTTGG-3′ and 5′-GCCTGCTTCACCACCTTCTTGATGTC-3′. Expression of the miR-200b–miR-200a–miR-429 primary transcript was determined by the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$). Mutu I, EBV-positive type I latency Burkitt's lymphoma cell line; JY, Jijoye, and X50-7, EBV-positive type III latency B-cell lines; OKF6/TERT2, EBV-negative telomerase immortalized human keratinocyte cell line; CNE1 and CNE1-EBV, EBV-negative and -positive nasopharyngeal carcinoma cell lines, respectively; AGS, EBV-negative human gastric adenocarcinoma cell line; MCF7, EBV-negative human breast cancer cell line. (C) Mature miR-429 expression was quantified using a TaqMan microRNA assay (Applied Biosystems). RNU6B was analyzed as a reference. The expression of miR-429 was determined by the comparative C_T method ($2^{-\Delta\Delta CT}$). DG75, EBV-negative Burkitt's lymphoma cell line; Rael and Akata, EBV-positive type I latency Burkitt's lymphoma cell lines; AGS-EBV, AGS cells infected with a recombinant EBV. Other cells used are as described in the legend to panel B. (D) Mature miR-200c expression was assessed using a mirVana (quantitative RT-PCR [qRT-PCR]) microRNA detection kit (AM1558; Ambion) with the mirVana qRT-PCR miR-200c primer set (AM30096; Ambion) and the mirVana qRT-PCR 5S primer set (AM30302; Ambion), according to the manufacturer's protocol. The expression of miR-200c was determined by the comparative C_T method ($2^{-\Delta\Delta CT}$). Cell lines are defined in the legends to panels B and C.

429-expressing pMSCV-puro-GFP-miR (2) retrovirus (in triplicate) into EBV-infected 293 (EBV-293) fibroblastic cells. A higher level of mature miR-429 was detected in all miR-429-transduced cell cultures than in control-transduced cultures (Fig. 2A). Consistent with previous studies showing that miR-200 microRNAs can function as master regulators of EMT, miR-429-transduced EBV-293 cells lost their spindle-shaped cell morphology and acquired clustered growth characteristics with cobblestone-shaped appearance (Fig. 2B). Western blot analysis of ZEB1 expression (anti-ZEB1 polyclonal antibody, sc-25388; Santa Cruz) showed the expected reduction in miR-429-transduced cells (Fig. 2C) (anti-actin polyclonal antibody, sc-1615; Santa Cruz). This indicated to us that the level of miR-429 expressed in cells transduced with the miR-429 retrovirus was sufficient to elicit an expected change in phenotype and the expected suppression of a miR-429 target.

We then assessed the number of Zta-positive cells in miR-429 and control-transduced cultures by immunofluorescence, following staining with an anti-Zta antibody (11-007; Argene) and a goat anti-mouse Alexa Fluor 594 (A11032; Invitrogen) secondary antibody. A greater number of Zta-positive cells were observed in all three miR-429-transduced cell cultures than in control-transduced cultures (Fig. 2D), indicating that this miR-200 family member can shift the latent/lytic balance toward the lytic cycle in EBV-293 cells.

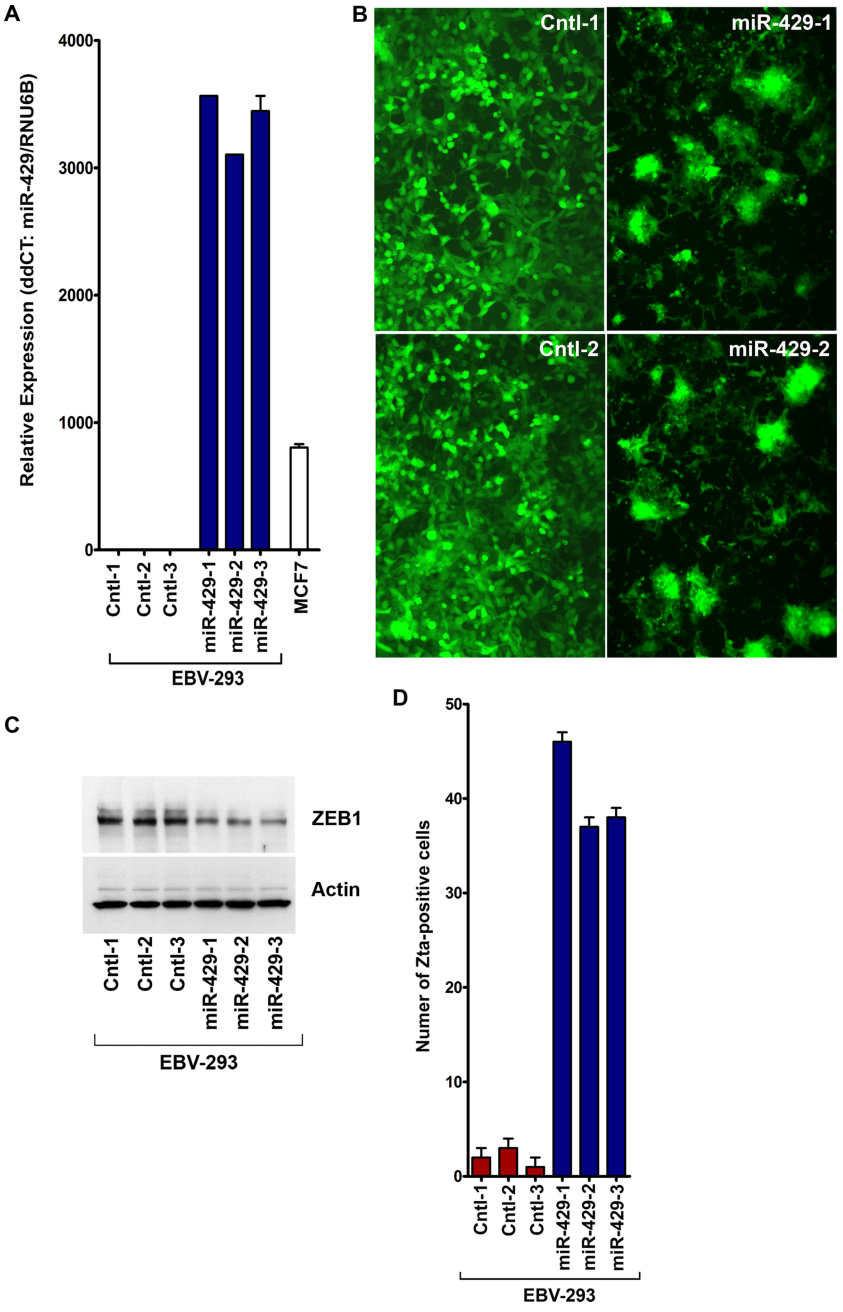

FIG. 2. Induction of EBV lytic reactivation in miR-429-transduced EBV-293 cells. (A) A 441-bp region spanning the miR-429 hairpin sequence (chromosome 1: positions 1,104,265 to 1,104,705 of the GRCH37 assembly) was isolated from JY genomic DNA by PCR and cloned into the plasmid pMSCV-puro-GFP-miR (2). Retroviral infection was conducted as described previously (19), and cells were selected with puromycin for 14 days prior to analysis. Mature miR-429 expression was analyzed by quantitative RT-PCR, and values were determined by the comparative C_T method ($2^{-\Delta\Delta CT}$), using RNU6B as a control (Cntl). (B) Two separate miR-429-transduced EBV-293 cell cultures show morphological changes relative to two separate control-transduced EBV-293 cell cultures. (C) Western blot analysis of ZEB1 expression in retrovirally transduced EBV-293 cells. (D) Immunofluorescence assay of Zta in retrovirally transduced EBV-293 cells. The assay was performed as previously described (13). The number of Zta-positive cells on coverslips containing 12,000 total cells was counted for each culture. The averages and standard errors from the results of triplicate coverslips are shown for each cell culture.

Induction of EBV lytic reactivation in miR-429-transduced B-cell lines. Next, we examined whether miR-429 could disrupt latency in B cells. Mutu I cells transduced with the miR-429 retrovirus showed higher levels of mature miR-429 expression in all three infections than cells transduced with the control retrovirus, with a concordant reduction in ZEB1 levels (Fig. 3A). Western blot analysis of the immediate-early and early genes Zta (11-007; Argene), Rta (11-008; Argene), and BMRF1 (EBV-018-48180; Capricorn) showed increased expression for all three of these viral genes in miR-429-trans-

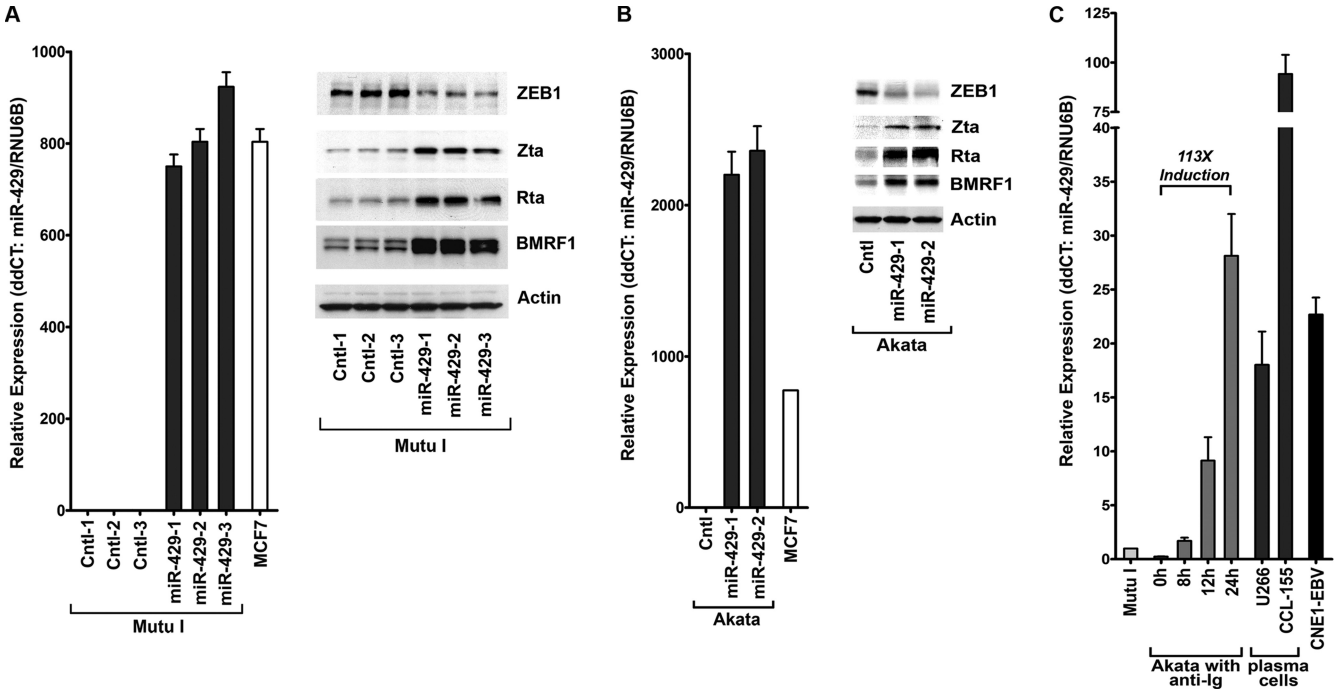

FIG. 3. Induction of EBV reactivation in miR-429-transduced B-cell lines. (A) Generation of retrovirally transduced Mutu I cell lines and analysis of mature miR-429 expression were performed as described in the legend to Fig. 2A. ZEB1 and actin were detected using polyclonal antibodies, as in Fig. 2C. (B) Retrovirally transduced Akata cell lines were generated in the same manner as the Mutu I cell lines, and analysis of mature miR-429 expression was performed as described in the legend to Fig. 2A. ZEB1 and actin were detected using polyclonal antibodies, as in Fig. 2C. (C) Mature miR-429 expression was quantified using a TaqMan microRNA assay (Applied Biosystems). RNU6B was analyzed as a reference. The expression of miR-429 was determined by the comparative C_T method ($2^{-\Delta\Delta CT}$). Akata cells were treated with anti-human Ig (I5260; Sigma), and cells were harvested at 0 h, 8 h, 12 h, and 24 h posttreatment. Human plasma cell lines U266 and CCL-155 were obtained from the ATCC.

duced cells (Fig. 3A) (14 days after selection). Similarly, introduction of a miR-429 retrovirus into another EBV-positive B-cell line, Akata, induced Zta, Rta, and BMRF1 expression (Fig. 3B). Together, these results demonstrate that miR-429 can also induce EBV reactivation in B cells.

miR-429 is induced by B-cell receptor activation and shows increased expression in plasma cell lines. *In vivo*, EBV reactivation in B cells is thought to occur through B-cell differentiation, and high lytic activity is observed in tonsillar plasma cells (11). Synchronous EBV reactivation can be achieved in tissue culture through activation of the B-cell receptor (BCR) (17). BCR activation in Akata cells by treatment with anti-Ig (I5260; Sigma) resulted in a 113-fold increase in mature miR-429 levels (Fig. 3C). Analysis of miR-429 expression in two plasma cell lines showed elevated levels relative to the expression level observed in Mutu I or unstimulated Akata cells (Fig. 3C). Together, these data suggest that in addition to playing a possible role in supporting lytic replication in epithelial cells, the miR-200 family may support BCR-mediated reactivation in B cells and reactivation in plasma cells.

miR-429 induces the Zta promoter. To investigate whether miR-429 can induce reactivation in part through the Zta promoter (Zp), we carried out a reporter experiment in 293 cells infected with a Zta knockout EBV [EBV(Zk/o)/293 cells] (6). Plates (10 cm) of EBV(Zk/o)/293 cells were cotransfected with 5 μg of a control or a miR-429 expression vector plus 2 μg of a luciferase reporter plasmid containing Zp sequences (positions −221 to +13 [9], which contain the previously identified ZEB1 binding site [10]). In this setting, miR-429 induced Zp activity by 1.8 fold (Fig. 4A). In the presence of 0.5 μg of a cotransfected Zta expression vector (to facilitate an activated state of the Zta promoter [8, 12]), miR-429 induced Zp activity by 2.4 fold (Fig. 4A). We then mutated the ZV-ZEB1 binding element (using a site-directed mutagenesis kit [200519; Stratagene]) in the Zp reporter plasmid and tested whether this abrogates responsiveness to miR-429. A statistically significant reduction in responsiveness to miR-429 was observed, but it nevertheless remained responsive to miR-429 (Fig. 4B and C). The mutation that we introduced in our construct was shown previously to largely abrogate ZEB1 binding to this element (7, 10), so it is unlikely that the remaining response is due to residual binding of ZEB1 to this element. These results indicate that miR-429 induces the Zta promoter through the ZV-ZEB1 binding site but that it is likely that additional elements/factors help facilitate induction of the Zta promoter.

We propose that the miR-200 family, which is a critical microRNA determinant of epithelial cell phenotype, may be a contributing effector of EBV's propensity to exhibit a bias toward the lytic phase in epithelial cells. On the other end of the spectrum, we observe little if any miR-200 family expression in any nonplasma B-cell line tested, consistent with the generally more latent bias observed in this setting. The finding that miR-429 expression is elevated in plasma cells and following BCR engagement suggests that miR-200 family members

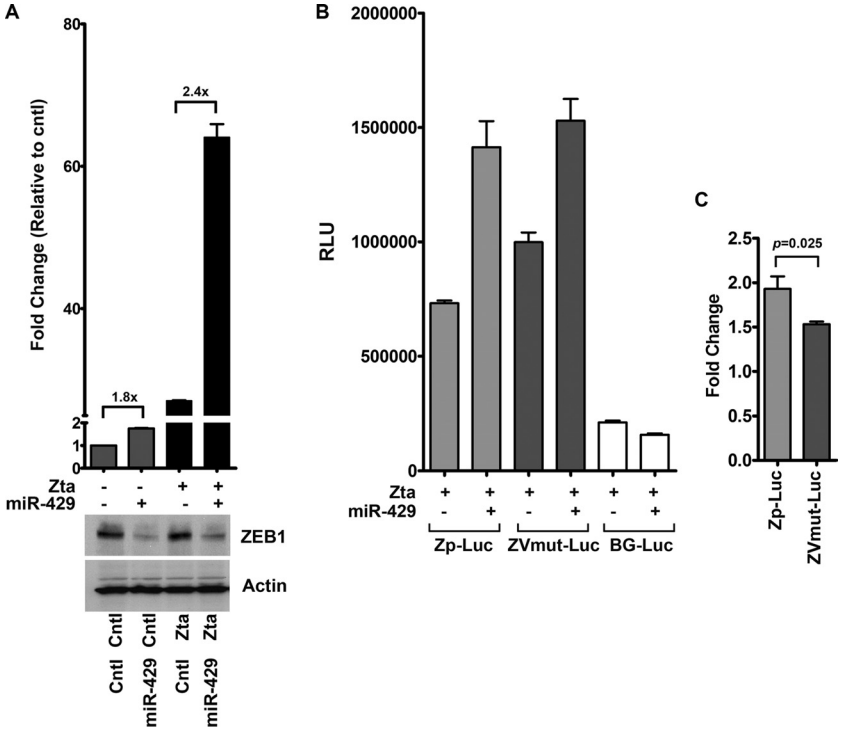

FIG. 4. miR-429 induces the Zta promoter. (A) 293 cells stably infected with a Zta knockout EB virus [EBV(Zk/o)/293] (6) were transfected using a modified calcium phosphate precipitation method (13). Cells were cotransfected with either a miR-429 expression vector (pMSCV-puro-GFP-miR-429) or a control vector (pMSCV-puro-GFP-miRcntl) plus either a Zta expression vector [BS(SVp/e)-Zta] or its control vector [BS(SVp/e)]. Cells were harvested 72 h after transfection, reporter activity was analyzed by a luciferase assay, and protein levels were assessed by Western blot analysis. Fold changes are relative to cells transfected with Zta control vector plus miR-429 control vector. (B) EBV(Zk/o)/293 cells were transfected with the indicated plasmids. Zp-Luc is a luciferase reporter plasmid containing Zp sequences (positions −221 to +13). ZVmut-Luc is a luciferase reporter plasmid bearing a defective ZV-ZEB1 binding site that was generated by changing the A at position −12 (relative to the Zp transcriptional initiation site) into a C. BG-Luc, which contains a minimal beta-globin promoter, was used as a control reporter construct. RLU, relative light units. (C) Fold changes are relative to Zta-induced reporter activity in the absence of miR-429. Statistical significance of the difference in fold change between the Zp-Luc group and the ZVmut-Luc group was analyzed by Student's *t* test.

may also be involved in supporting reactivation in B-cell settings where viral reactivation occurs. It is also notable that histone deacetylase (HDAC) inhibitors, which are known to induce EBV reactivation, have been shown to induce miR-200 microRNAs (21). It is conceivable that HDAC inhibitors may induce reactivation in part through their influence on this pathway.

Lastly, while the ZV-ZEB1 binding site in the Zp may mediate the influence of miR-429 on the Zp, induction of reactivation is likely to be more complex. There may be other uncharacterized ZEB1 binding sites within the Zp, and there may be other Zp binding factors that are influenced by miR-429. Furthermore, ZEB1 has been shown to bind to the Rta promoter (10, 20), raising the possibility that the respective binding site(s) may similarly be responsive to miR-200 family members and thereby contribute to miR-429-mediated reactivation.

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