Clustered MicroRNAs of the Epstein-Barr Virus Cooperatively Downregulate an Epithelial Cell-Specific Metastasis Suppressor

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ABSTRACT

The Epstein-Barr virus (EBV) encodes its own microRNAs (miRNAs); however, their biological roles remain elusive. The commonly used EBV B95-8 strain lacks a 12-kb genomic region, known as BamHI A rightward transcripts (BART) locus, where a number of BART miRNAs are encoded. Here, bacterial artificial chromosome (BAC) technology was used to generate an EBV B95-8 strain in which the 12-kb region was fully restored at its native locus [BART(+) virus]. Epithelial cells were stably infected with either the parental B95-8 virus or the BART(+) virus, and BART miRNA expression was successfully reconstituted in the BART(+) virus-infected cells. Microarray analyses of cellular gene expression identified N-myc downstream regulated gene 1 (*NDRG1*) as a putative target of BART miRNAs. The *NDRG1* protein was barely expressed in B cells, highly expressed in epithelial cells, including primary epithelial cells, and strongly downregulated in the BART(+) virus-infected epithelial cells of various origins. Although *in vitro* reporter assays identified BART22 as being responsible for the *NDRG1* downregulation, EBV genetic analyses revealed that BART22 was not solely responsible; rather, the entire BART miRNA cluster 2 was responsible for the downregulation. Immunohistochemical analyses revealed that the expression level of the *NDRG1* protein was downregulated significantly in EBV-positive nasopharyngeal carcinoma specimens. Considering that *NDRG1* encodes an epithelial differentiation marker and a suppressor of metastasis, these data implicate a causative relationship between BART miRNA expression and epithelial carcinogenesis *in vivo*.

IMPORTANCE

EBV-related epithelial cancers, such as nasopharyngeal carcinomas and EBV-positive gastric cancers, encompass more than 80% of EBV-related malignancies. Although it is known that they express high levels of virally encoded BART miRNAs, how these miRNAs contribute to EBV-mediated epithelial carcinogenesis remains unknown. Although a number of screenings have been performed to identify targets of viral miRNAs, many targets likely have not been identified, especially in case of epithelial cell infection. This is the first study to use EBV genetics to perform unbiased screens of cellular genes that are differentially expressed in viral miRNA-positive and -negative epithelial cells. The result indicates that multiple EBV-encoded miRNAs cooperatively downregulate *NDRG1*, an epithelial differentiation marker and suppressor of metastasis. The experimental system described in this study should be useful for further clarifying the mechanism of EBV-mediated epithelial carcinogenesis.

The Epstein-Barr virus (EBV) is a common herpesvirus that is widespread in all human populations. Primary EBV infections in adolescence often manifest as infectious mononucleosis (1). EBV infection is also associated with several types of lymphomas and epithelial malignancies. The B95-8 strain of EBV, an infectious mononucleosis-derived isolate, is biologically indistinguishable from other isolates of EBV in that it efficiently produces progeny virus and transforms peripheral B-lymphocytes *in vitro* (2). The marmoset lymphoblastoid B95-8 cell line is one of the most widely used EBV-producing cell lines. Although the B95-8 strain was assumed to be a prototype EBV, restriction mapping and DNA sequencing analyses revealed that its genome contains a deletion of approximately 12 kb (3–5). This deleted region is apparently dispensable for progeny virus production and B-cell transformation; hence, its importance has been underestimated.

The recent discovery of EBV-encoded microRNA (miRNA) genes within the 12-kb region has dramatically changed the situation. The initial discovery of five EBV miRNAs was followed by the subsequent identification of a number of additional miRNAs (6–9); to date, 44 mature miRNAs have been identified, of which 4 are encoded at the *BHRF1* locus, and 40 are encoded at the BART

locus. A complete list of EBV miRNAs, including their mature and precursor sequences, is available at miRBase (www.mirbase.org). BART miRNAs are of particular interest because 17 of their 22 pre-miRNAs are located within the B95-8 deleted region. When studies were conducted to identify genes that were uniquely expressed in EBV-infected epithelial cells, this region was found to be actively transcribed in nasopharyngeal carcinoma (NPC) cells (10, 11). The transcripts were named as complementary-strand

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transcripts (10), BARF0 (12), or BARTs (13). Whether the transcripts are translated to one or more proteins remains enigmatic; however, it is now clear that they serve as primary transcripts that are processed to generate mature BART miRNAs. BART miRNAs are derived from BART introns prior to splicing (14). Similar to the high expression levels of BART RNAs in epithelial cells, BART miRNAs are also expressed at high levels in NPC cells (9, 15) and in gastric carcinoma cells (16). BART miRNAs are also expressed at high levels in NK/T lymphoma-derived cell lines (17). On the other hand, few viral proteins are expressed in EBV-infected epithelial cells, suggesting that BART miRNAs contribute to epithelial tumorigenesis (18–20).

Many target proteins of BART miRNAs have been identified to date. Those that are encoded by EBV itself include BALF5 (21), LMP1 (22), LMP2A (23), and BHRF1 (23, 24). Previously identified cellular targets include Bim (25), CAPRIN2 (24), CASP3 (26), DAZAP2 (27), DICER1 (28), E-cadherin (29), IPO7 (26, 30), and PUMA (31). Many other candidate target genes have been identified by miRNA targetome studies (24, 27, 30, 32); the biological significances of these miRNA-target interactions have yet to be clarified. BART miRNA targets in epithelial cells have not been explored extensively; thus, many targets have probably not been identified.

The high expression levels of BART miRNAs in epithelial cells suggest that the B95-8 strain of EBV, which lacks many of the BART pre-miRNA genes, is phenotypically different from wild-type EBV. Thanks to the recent development of bacterial artificial chromosome (BAC) systems that enable the manipulation of EBV genomes in *Escherichia coli* (33), it has become feasible to restore the deleted region of the B95-8 strain using the equivalent DNA fragment of an EBV strain that retains the region. Here, we used BAC technology to seamlessly restore the missing 12-kb region at its native locus in a recombinant EBV B95-8 strain. We identified small but significant differences in the gene expression patterns of epithelial cells harboring recombinant viruses with or without the 12-kb deletion. We present genetic evidence here that multiple BART miRNAs cooperatively downregulate an epithelial cell-specific metastatic suppressor protein.

MATERIALS AND METHODS

Cell culture. B95-8 is a lymphoblastoid cell line obtained by infection of marmoset monkey peripheral blood leukocytes with EBV (2). HEK293 cells are neuro-endocrine cells obtained by transformation of embryonic kidney cells with adenovirus (34), and they have been used as EBV-producer cells (35). B95-8 cells, HEK293 cells, and Burkitt's lymphoma-derived Akata cells (36) were maintained in RPMI medium (Sigma-Aldrich Fine Chemicals, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). AdAH cells (37, 38) were maintained in Dulbecco medium Eagle medium (Sigma) supplemented with 10% FBS. C666-1 cells (a gift from Kowk-Wai Lo) (39) was maintained in RPMI medium supplemented with L-glutamine (Life Technologies) and 10% FBS. PC-3 cells (JCRB cell bank) were maintained in F-12K medium (Life Technologies) supplemented with 7% FBS.

Cloning of EBV genome as BAC clones. An experimental strategy to clone B95-8 strain EBV genome into a BAC vector is essentially the same as previously described methods (35, 40), except that a targeting vector with different right and left homology arms was used to facilitate a subsequent reconstitution of the B95-8 deleted region. A targeting vector with enhanced green fluorescent protein (EGFP) and hygromycin resistance genes (40), flanked by a right homology arm from the NheI site (nucleotide [nt] 143210) to the MluI site (nt 149732) of B95-8 strain EBV genome (V01555) and a left homology arm (from the BssHII site [nt 146953] to

the NheI site [nt 154909]) was constructed. B95-8 cells were transfected with a NheI-linearized targeting vector, selected by hygromycin, and hygromycin-positive cell clones were obtained. Episomal fractions (41) of 14 hygromycin-resistant, EGFP-positive cell clones were used to transform electrocompetent DH10B cells. Bacterial colonies were obtained from 6 of 14 clones, and DNA miniprep of 6 bacterial clones revealed that 2 of 6 clones were homologously recombined clones.

B95-8 cells harboring homologously recombined EBV-BAC clones were induced to productive replication by transducing BZLF1 (a switch gene of productive replication), and culture supernatants containing mixture viruses of parental B95-8 strain EBV and a targeted virus were used to establish lymphoblastoid cell lines (LCLs). EBV-BAC clones were subsequently rescued from the established LCLs, and an EBV-BAC clone with fewer (five copies) BamHI W repeats was selected. The estimated size of the obtained EBV-BAC clone, referred to as B95-8 EBV-BAC here, was ~169.5 kb.

Recombinogenic engineering to restore the B95-8 deleted region. Counter selection BAC modification kit (Gene Bridges, Dresden, Germany), a rapid homologous recombination system in *Escherichia coli*, was used to obtain a BART-restored EBV-BAC clone. A double-stranded DNA fragment containing kanamycin resistance and streptomycin sensitivity genes (rpsLneo) was amplified using primers listed in Table S1 in the supplemental material. A PCR product with 50-bp sequences homologous to subgenomic regions flanking the deletion junction (nt 152012) was obtained. The PCR product was inserted into the B95-8 deletion junction of the B95-8 EBV-BAC to obtain the intermediate clone by using neo as a positive selection marker. A DNA fragment of Akata strain EBV (36) was then used to restore the B95-8 deleted region. A part of BamHI B1 fragment (the MluI-BamHI subfragment, corresponding to nt 137443 to 146422 of EBV-wt) and an entire BamHI W11I fragment (corresponding to nt 146422 to 148949 of EBV-wt), both derived from Akata strain EBV, were tandemly cloned into pMBL19 vector (42) to obtain pMBL19 B/B1ΔW11I. The rpsLneo gene of the intermediate clone was replaced with a BbvCI fragment of pMBL19 B/B1ΔW11I using rpsL as a negative selection marker. Bacterial colonies that were resistant to chloramphenicol and streptomycin were screened to obtain BAC clones that lost the rpsLneo cassette. As a result, the B95-8 deleted region was seamlessly restored in the BART(+) EBV-BAC. The sequence file of BART(+) EBV-BAC has been assembled from sequences of EBV B95-8 genome (V01555) (3) and the Family of Repeats (AJ278309) (43), EBV Akata sequence spanning the B95-8 deleted region (unpublished), and the transgenes (BAC vector and EGFP and hygromycin resistance genes) as shown in the supplemental material.

To generate a BART-deleted revertant, the rpsLneo PCR product described above was used to replace the 12-kb region of the BART(+) EBV-BAC with rpsLneo by using neo as a positive selection marker. A DNA fragment spanning nt 151912 to 152118 of B95-8 strain EBV was PCR amplified using the primers listed in Table S1 in the supplemental material. The obtained PCR product was subsequently used to replace the rpsLneo cassette by using rpsL as a negative selection marker.

To generate recombinant viruses with specific BART miRNA(s) deleted, rpsLneo was PCR amplified using the primers listed in Table S1 in the supplemental material, and a pre-miRNA gene of BART22 was first replaced by the rpsLneo cassette to obtain an EBV-BAC clone, BART22-rpsLneo. A double-stranded 120-bp PCR product, consisting of 60-bp upstream and 60-bp downstream sequences flanking the BART22 pre-miRNA sequence, was obtained by using oligonucleotides listed in Table S1 in the supplemental material. The rpsLneo of cassette of BART22-rpsLneo was replaced by the 120-bp PCR product to obtain a BAC clone of BART22Δ with no selective marker left behind. A similar experimental strategy was used to obtain EBV-BAC clones of BART8-11Δ and BART21-14Δ by using BART22-rpsLneo as a starting material. Primers used to make these recombinant genomes and to verify the deletions are listed in Table S1 in the supplemental material.

Recombinant virus production and infection. EBV-BAC DNAs were prepared from each 200-ml bacterial culture by using a Nucleobond BAC100 kit (Macherey-Nagel, Duren, Germany). HEK293 cells were transfected with BAC clone DNAs (1 μ g of each) using Lipofectamine 2000 (Invitrogen). At 2 days posttransfection, the transfected cells were replated to 10-cm collagen-coated dishes in medium containing 150 μ g of hygromycin per ml. Hygromycin-resistant colonies with bright EGFP fluorescence were grown, and cell clones that were highly competent for entering lytic replication after BZLF1 transfection were selected. Recombinant virus production was performed as described previously (40).

A retroviral vector pCLMFG-CR2, encoding EBV receptor CR2 (CD21), was constructed by inserting a PCR-amplified CR2 gene into pCLMFG-MCS vector, and VSVG-pseudotyped retroviral vector was produced as described previously (44). AdAH and PC-3 cells were infected with the retroviral vector expressing CR2 and were subsequently infected with either the recombinant viruses. Pools of stably infected AdAH and PC-3 cells were obtained by hygromycin selection. Lymphoblastoid cell lines (LCLs) were established by infecting peripheral blood mononuclear cells with the recombinant viruses as described previously (40).

miRNA expression analyses. Total RNAs were isolated from various cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For Northern blot analyses, aliquots (5 μ g) of RNAs were electrophoresed through 15% polyacrylamide-urea gels, and separated RNAs were transferred onto Hybond N+ membrane (Amersham). Radiolabeled oligonucleotide probes specific for EBV miRNAs were prepared by end labeling of synthetic oligonucleotides with [γ - ^{32} P]ATP (Perkin-Elmer Life & Analytical Sciences). Sequences of oligonucleotide probes are listed in Table S2 in the supplemental material. Membranes were prehybridized in ULTRAhyb-Oligo buffer (Ambion) at 37°C for 30 min, followed by hybridization with radiolabeled oligonucleotide probes at 37°C overnight. Membranes were washed with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.05% sodium dodecyl sulfate (SDS) for 30 min at room temperature and then twice with 0.1 $\times$ SSC containing 0.1% SDS for 15 min at room temperature. Signals were detected by means of BAS2500 analyzer (Fuji Film Inc.). For relative quantification of BART miRNAs in the infected cells, aliquots (100 ng) of RNAs were subjected to TaqMan small RNA assay (Applied Biosystems) (45) according to the manufacturer's instructions.

Cellular gene expression analyses. Total RNAs of two independent B95.8v-infected and two independent BART(+)-v-infected HEK293 cell clones (or two independent pools of the infected AdAH cells) were processed for Microarray analysis using 3D-Gene human Oligo Chip 25k (Toray, Tokyo, Japan). cDNA synthesis, antisense RNA (aRNA) amplification, Cy5 labeling of aRNA, and hybridization to oligonucleotide tips were performed according to the supplier's protocol (3D-Gene; Toray). Hybridization signals were scanned using 3D Gene Scanner 3000 (Toray). The raw data for each spot were normalized by substitution with the mean intensity of the background signal. The detected signals for each gene were normalized by a global normalization method (the median of the detected signal intensity was adjusted to 25).

Quantitative reverse transcription-PCR (RT-PCR) analyses were performed by using the One-Step SYBR PrimeScript RT-PCR Kit II (TaKaRa Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Primer pairs used for NDRG1 and GAPDH cDNA amplification are listed in Table S3 in the supplemental material.

Western analyses. Aliquots of cells (4×10^6 cells each) were pelleted, resuspended in 150 μ l of phosphate-buffered saline (without magnesium and calcium) and 300 μ l of 1.5 $\times$ lysis buffer (187.5 mM Tris-HCl [pH 6.8], 4.5% SDS, 9% urea, and 15% glycerol), and sonicated to obtain whole-cell extracts. Protein concentrations of the extracts were determined by using a DC protein assay kit (Bio-Rad). The equal aliquots of the extracts were electrophoresed through or SDS–10% polyacrylamide gels (for NDRG1, GAPDH, LMP1, and LMP2A) or SDS–7% polyacrylamide gels (for EBNA) and subjected to Western blot analyses. Expressions of

NDRG1 and GAPDH were detected by using either anti-NDRG1 rabbit monoclonal antibody D8G9 (Cell Signaling, catalog no. 9485) or anti-GAPDH antibody 14C10 (Cell Signaling, catalog no. 2118) as primary antibodies and a horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) as a secondary antibody. Expression of EBNA was detected with human serum reactive to six EBNA as a primary antibody and a horseradish peroxidase-conjugated anti-human IgG as a secondary antibody. The expression of LMP1 was detected using monoclonal antibody (MAb) S12 (specific to LMP1) and peroxidase-conjugated anti-mouse IgG, whereas the expression of LMP2A was detected using rat MAb 15F9 (Abcam) and peroxidase-conjugated anti-rat IgG.

Reporter assay and miRNA mimic transfections. Primer sequences used for making various NDRG1-related constructs are listed in Table S3 in the supplemental material. The 3' untranslated region (UTR) of the NDRG1 gene was amplified by using genomic DNA of HEK293 cells as a template. The PCR product was digested by SpeI and cloned between SpeI-PmeI sites of pMIR-report (Ambion) to obtain pMIR-luc-NDRG1. NDRG1 cDNA was PCR amplified using pINCY-NDRG1 (Incyte cDNA Collection, clone LIFESEQ7593133; Thermo Scientific) as a template. The EcoRI-BamHI-digested PCR product was cloned into EcoRI-BglII-digested pSG5 vector (Stratagene) to make pSG5-NDRG1. A PCR-based mutagenesis protocol (46) was used to simultaneously introduce PstI sites to disrupt three BART22 seed-matched sequences within NDRG1 the 3' UTR of pMIR-luc-NDRG1 (see Table S3 in the supplemental material).

Synthesized mirVana miRNA mimics of BART miRNAs were purchased from Applied Biosystems. For each well of 48-well dishes, the B95.8v-infected HEK293 cells (5×10^4 cells) were cotransfected with pMIR-luc-NDRG1 (2.5 ng), pRL-TK (1 ng), and 6 pmol of miRNA mimics (6 pmol/300 μ l = 20 nM) by using Lipofectamine RNAiMAX (Invitrogen) according to an RT protocol. Triplicate transfections were performed for each sample. At 48 h posttransfection, cells were lysed by 1 $\times$ passive lysis buffer (Promega) using 65 μ l of per each well, and 20- μ l aliquots were assayed by using a dual-luciferase reporter assay system (Promega) and a MicroBeta² LumijET (Perkin-Elmer).

Immunohistochemistry and EBER-ISH of NPC biopsy specimens. Biopsy specimens were obtained from NPC patients who had been diagnosed at the Division of Otolaryngology at Kanazawa University Hospital, as well as its branch hospitals, according to protocols approved by the institutional review board. The data were analyzed anonymously. Paraffin-embedded specimens were used for the immunohistochemical analysis of NDRG1 (using anti-NDRG1 antibody; Cell Signaling, catalog no. 9485), as well as for EBER-ISH, as previously described (47).

RESULTS

Restoration of the 12-kb deleted region of the EBV B95-8 strain.

The 12-kb genomic region that is deleted in the EBV B95-8 strain spans nt 139724 to 151554 of the EBV-wt sequence (NC_007605.1) (48). In the B95-8 strain (GenBank V01555), the deletion is located at nt 152012 (Fig. 1A). Notably, 17 of the 22 BART pre-miRNA genes are encoded within the deleted region (Fig. 1B).

The entire genomic sequence of the EBV B95-8 strain was cloned into a targeting vector containing a BAC vector sequence, a hygromycin resistance gene, and an enhanced green fluorescent protein expression cassette (Fig. 1A). The insertion sites of the transgenes differed from those of the previously described EBV-BAC clones 2089 (35) and 172-kb BAC (40) (Fig. 1A). Different insertion sites were chosen to facilitate the subsequent restoration of the 12-kb B95-8 deleted region. A B95-8 EBV-BAC clone with 5 copies of BamHI W repeat ($\sim$ 169.5 kb in size) was obtained (Fig. 1C). The clone with five BamHI W repeats was intentionally chosen (see Materials and Methods for details), as it should be small enough to accommodate a 12-kb additional sequence of the BART miRNA region later.

FIG 1 Experimental strategy used to restore the deleted BART miRNA cluster in the EBV B95-8 strain. (A) Schematic map of the deletion junction of the EBV B95-8 strain. The deletion junction is indicated with nucleotide no. of the B95-8 genome (V01555). The left (lt) and right (rt) homologous arms of the targeting vector and the restriction enzymes used to construct the vector are indicated. The viral open reading frames (horizontal arrows), BART transcription start sites (P1 and P2), and positions of the BART pre-miRNA genes (numbered vertical lines) are also shown. (B) Schematic map of the 12-kb region of EBV-wt that is missing in the EBV B95-8 strain. The numbered vertical lines indicate the positions of the pre-miRNA genes; those that are deleted in the EBV B95-8 strain are shown in gray. The horizontal arrows indicate the positions of viral open reading frames. (C) Schematic illustrations of the B95-8 EBV-BAC and the BART(+) EBV-BAC. The latter was obtained from the former via the intermediate clone with positive and negative selection marker genes (rpsLneo). A DNA fragment of EBV Akata strain was used to restore the missing 12-kb region of B95-8 EBV-BAC. (D) Restriction enzyme mapping and Southern blot analyses of the B95-8 and the BART(+) EBV-BAC clones. The intermediate clone was also included to the analysis. The DNAs were digested with BamHI or EcoRI and analyzed by agarose gel electrophoresis followed by ethidium bromide staining (left panel). The BamHI and EcoRI fragments spanning the deletion junction are indicated by arrowheads, and their calculated sizes (see Table S4 in the supplemental material for the details) are indicated at the bottom. The gel was subsequently processed for Southern blot analysis (right panel) using the BamHI W111 fragment of the EBV Akata strain as a probe. Note the 12-kb increase in the size of the EcoRI fragment spanning the deletion junction in the BART(+) EBV-BAC clone.

FIG 2 Expression of BART miRNAs in the cells infected with the recombinant viruses. (A) The expression levels of 13 BART miRNAs (four from cluster 1 and eight from cluster 2) in the B95.8v-infected and the BART(+v)-infected HEK293 cells, AdAH cells, and LCLs were examined by Northern blotting.

The obtained BAC clone was further modified using *E. coli*-mediated recombinogenic engineering. First, tandemly connected positive and negative selection marker genes were inserted to the B95-8 deletion junction to obtain an intermediate clone (Fig. 1C). Subsequently, the DNA fragment of the EBV Akata strain (36), which is a type 1 EBV, like the B95-8 strain, was used to replace the marker genes of the intermediate clone. The BART-restored EBV-BAC clone, designated BART(+) EBV-BAC (Fig. 1C), was ~181.5 kb in size, which is within the size limits for efficient packaging into virions (49). Restriction enzyme mapping and Southern blot analyses of the repaired region confirmed the expected 12-kb increase in the sizes of bands spanning the repaired region (Fig. 1D and see Table S4 in the supplemental material).

Establishment of cell lines infected with the B95-8 or the BART-restored virus. To generate virus-producing cells, the newly obtained B95-8 and BART(+) EBV-BAC clones were stably transfected into HEK293 cells, and multiple HEK293-derived cell clones capable of producing recombinant viruses [here referred to as B95.8v and BART(+v)] were obtained. The integrity of the EBV genomes in the stably transfected HEK293 cell clones was confirmed by recovering the BAC clones from the cells and subjecting them to restriction enzyme analyses (data not shown). The obtained virus-producing HEK293 cells are here referred to as “B95.8v-infected” and “BART(+v)-infected” HEK293 cells. Frozen virus stocks of B95.8v and BART(+v) with up to 10^{4.5} CFU/ml transforming titers were obtained and used for infection experiments.

For epithelial cell infection, AdAH cells were chosen as recipient cells. The AdAH cells had long been assumed as human adenoid epithelium-derived cells (38). However, they are likely to be contaminated with human cervical carcinoma-derived HeLa cells (37), as they were actually positive for human papillomavirus type 18 DNA and E6 E7 mRNA expression (data not shown). AdAH cells were transduced with the EBV receptor CR2 (CD21) and then infected with the viruses. Pools of stably infected AdAH cells were obtained by hygromycin selection. Multiple LCLs were also established by infecting peripheral B lymphocytes with the viruses.

Total RNAs were extracted from the infected HEK293 and AdAH cells, as well as from the LCLs, and the expression levels of 13 BART miRNAs were examined by Northern blotting (Fig. 2A). The total RNA of the EBV-positive NPC cell line C666-1 (39) was used as a positive control. BART3-3p and BART2-5p, which are encoded upstream and downstream of the B95-8 deleted region, respectively, were expressed in all of the cell types. Notably, BART2-5p expression was enhanced slightly in the B95.8v-infected cells, presumably because the BART2-5p gene was located near the BART transcription start site due to the 12-kb deletion (Fig. 1B).

As expected, all of the BART miRNAs that are encoded within the B95-8 deleted region (16, 17-5p, 6-5p, 8-5p, 9-5p, 9-3p, 22, 10-3p, 11-5p, 11-3p, and 19-3p) were expressed only in the cells that were infected with the BART-restored virus (Fig. 2A). BART9-5p and BART20-5p were hardly expressed in all of the

Total RNA extracted from the NPC-derived C666-1 cell line was used as a control. The expression of U6 snRNA was used as a loading control. (B) TaqMan small RNA assay was used to determine relative expression levels of BART17-5p and BART22 in the BART(+v)-infected HEK293 cells, AdAH cells, and LCLs. The values of miRNA expression levels were normalized by those of hsa-miR-16 expression levels among different cells. The average miRNA expression levels in the LCLs were adjusted to 1.

FIG 3 Immunoblot analyses of viral latent gene expression in cells infected with the recombinant viruses. (A) Whole-cell extracts of the B95.8v-infected and the BART(+)-v-infected HEK293 cells, LCLs, and AdAH cells were analyzed by immunoblotting using EBV-immune human serum (top panel), a monoclonal anti-LMP1 antibody (middle panel), or an anti-GAPDH antibody (bottom panel) as a control. (B) LMP1 and LMP2A protein levels of two additional lines of the B95.8v-infected and the BART(+)-v-infected LCLs were examined by Western blotting analyses. (C) The AdAH cells infected with the recombinant viruses were processed for immunofluorescence to detect the expression of EBNA. The EBNA staining of C666-1 is shown as a positive control.

examined cells (Fig. 2A and data not shown). Notably, the expression levels of the restored miRNAs were comparable to those of the endogenous miRNAs in the C666-1 cells, which express high levels of viral miRNAs (15).

Previous studies demonstrated that relative BART miRNA expression levels in LCLs were far less than those in epithelial cells (50), which was not very obvious in our Northern blot data. Thus, two of the BART miRNAs (BART17-5p and BART22) were chosen and subjected to TaqMan small RNA assay to determine miRNA expression levels in the BART(+)-v-infected epithelial cells relative to those in the BART(+)-v-infected LCLs. The results revealed that relative BART17-5p expression levels in the infected epithelial cells were comparable to those in the LCLs, whereas BART22 expression levels in BART(+)-v-infected epithelial cells were approximately five times (HEK293 cells) and two times (AdAH cells) more than those in the BART(+)-v-infected LCLs (Fig. 2B). These results were in good concordance with the Northern blot data, indicating that estimation of miRNA expression levels by Northern blotting data is reliable.

Whole-cell extracts were prepared from various B95.8v-infected and BART(+)-v-infected cells (HEK293, LCLs, and AdAH cells), and the expression levels of viral latent proteins were examined by immunoblotting. The Epstein-Barr virus nuclear antigen 1 (EBNA1), EBNA2, and EBNA3 proteins were expressed at comparable levels in B95.8v-infected and BART(+)-v-infected HEK293 cells and LCLs (Fig. 3A). Latent membrane protein 1 (LMP1) and LMP2A were expressed in the LCLs, but their expression levels were highly variable among different LCL cell clones (Fig. 3A and B). Thus, the effect of BART miRNA expression on LMP1 and LMP2A expression was obscure in this experimental setting. Although only traces of EBNA1 and LMP1 were detected by Western blotting, the infected AdAH cells were nearly 100% EBNA-positive (Fig. 3C), arguing against the possibility that only minor populations of AdAH cells were infected. These results in-

dicate that the absence or presence of BART miRNAs did not significantly affect viral latent protein expression.

NDRG1 is downregulated in the cells infected with the BART-restored EBV. Next, microarray analyses were performed to identify differences between the expression levels of host genes in the B95.8v-infected and the BART(+)-v-infected HEK293 cells (see Dataset S1 in the supplemental material). Two independent B95.8v-infected and two independent BART(+)-v-infected HEK293 cell clones, each of which were derived from a single colony after hygromycin selection, were chosen based on their good virus producing abilities. Representative scatter plots of the global gene expression profiles of the B95.8v-infected and the BART(+)-v-infected HEK293 cells are shown in Fig. 4A. We focused on cellular genes that were downregulated in the BART(+)-v-infected HEK293 cells, since these genes are more likely to be direct targets of the EBV BART miRNAs. The analysis identified 19 genes that were expressed at moderate to high levels in the B95.8v-infected HEK293 cells and downregulated (0.75-fold or less) in the BART(+)-v-infected HEK293 cells (see Table S5 in the supplemental material). We then used the DIANA-microT program (51) to search for possible direct target genes of each BART miRNA. Of the downregulated genes shown in Table S5 in the supplemental material, *NDRG1*, *NOM1*, and *ANGEL2* were identified as possible direct targets of BART miRNA (see Dataset S2 in the supplemental material). *NDRG1* (N-myc downstream regulated gene 1) was chosen for further analyses, because the gene had previously been identified as a BART miRNA target by a photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation analysis (32). *NDRG1* mRNA expression was downregulated in the BART(+)-v-infected AdAH cells as well (Fig. 4A and see Table S6 in the supplemental material). The downregulation of *NDRG1* expression in the BART(+)-v-infected HEK293 cells was confirmed by quantitative RT-PCR (Fig. 4B).

Whole-cell extracts were prepared from B95.8v-infected and

FIG 4 Downregulation of NDRG1 mRNA and protein in epithelial cells infected with the BART-restored virus. (A) Representative scatter plots of the global gene expression profiles of the B95.8v-infected and the BART(+)-v-infected HEK293 and AdAH cells. The black dots (indicated by arrows) represent *NDRG1*, and the top and bottom diagonal lines indicate 2-fold differences between the expression levels in the two samples. The signal intensities of selected genes, including *NDRG1*, are shown in Tables S5 and S6 in the supplemental material. (B) Quantitative RT-PCR analyses of the expression levels of *NDRG1* mRNAs in the B95.8v-infected and the BART(+)-v-infected HEK293 cells (two independent clones of each). The values were normalized by using those of *GAPDH* mRNA. The data are represented as the means $\pm$ the standard deviations (SD) of $n = 3$ replicates. (C) Immunoblot analyses of NDRG1 and GAPDH protein levels in whole-cell extracts of the B95.8v-infected cells, the BART(+)-v-infected cells, and the revertant virus-infected cells (HEK293 and AdAH cells, two independent clones of each).

BART(+)-v-infected HEK293 and AdAH cells, and the expression levels of the NDRG1 protein were examined by immunoblotting. In agreement with the mRNA levels, NDRG1 protein expression was downregulated in the BART(+)-v-infected HEK293 cells (Fig. 4C). To confirm that the 12-kb region of EBV that is deleted in the B95-8 strain is responsible for downregulating NDRG1 expression, this region was removed from the BART(+)-EBV-BAC clone to generate a BART-deleted revertant. An immunoblot analysis revealed that HEK293 stably infected with the revertant virus exhibited high levels of NDRG1 expression that were similar to those in the B95-8-infected cells (Fig. 4C). In the AdAH cells, NDRG1 was detected as a double band, and transfection of exogenous *NDRG1* cDNA produced a protein band that comigrated with the smaller band (data not shown). Both the larger and the smaller NDRG1 protein bands were downregulated in the BART(+)-v-infected AdAH cells, and the downregulation disappeared in the revertant virus-infected cells (Fig. 4C).

These results indicate that downregulation of NDRG1 expression in the BART(+)-v-infected cells occurs at both the mRNA and protein levels and that the 12-kb region of the EBV genome encompassing the BART miRNAs is responsible for the downregulation.

NDRG1 is expressed at a high level in epithelial cells but not B cells. It was previously reported that NDRG1 is expressed at a high level in epithelial cells (52); therefore, the expression levels of NDRG1 in various epithelium-derived cell lines were examined by

immunoblotting. Substantial levels of NDRG1 expression were detected in AdAH cells, primary human bronchial epithelium-derived cells (HBEC1), human colon cancer-derived epithelial cell line Caco-2, human prostate cancer-derived epithelial cell line PC-3, and primary human prostate epithelium PrEC (Fig. 5). Consistent with the notion of its downregulation by EBV infec-

FIG 5 NDRG1 is expressed at a high level in epithelial cells but not B cells. Immunoblot analyses of NDRG1 and GAPDH protein levels in whole-cell extracts of various epithelial cells (AdAH cells, primary human bronchial epithelial HBEC1 cells, colon cancer-derived Caco-2 cells, prostate cancer-derived PC-3 cells, primary prostate epithelial PrEC cells [purchased from Lonza], and C666-1 cells) and B cells (Akata, P3HR-1, Daudi, and LCLs). The presence (+) or absence (–) of EBV infection in each cell line is indicated. The recombinant viruses used to establish the LCLs are indicated.

tion, the expression level of NDRG1 in C666-1 cells, which have abundant levels of BART miRNAs, was relatively low (Fig. 5). Although substantial levels of NDRG1 protein expression were detected in various epithelium-derived cells, Burkitt's lymphoma-derived cell lines (Akata, P3HR-1, and Daudi) and the LCLs barely expressed the NDRG1 protein (Fig. 5). These results indicate that, among EBV host cells, epithelial cells but not B cells express high levels of NDRG1 protein.

Screening of viral miRNAs putatively responsible for downregulating NDRG1 expression. Next, we investigated the possibility that BART miRNAs mediate the downregulation of NDRG1 expression directly in EBV-infected epithelial cells. Because miRNAs bind to specific sequences located within the 3' UTR of a target mRNA (53), a reporter construct containing the 3' UTR of *NDRG1* located between a luciferase open reading frame and a polyadenylation signal was constructed (Fig. 6A). The abilities of BART miRNA mimics corresponding to the 17 miRNAs encoded in the 12-kb deleted region of the EBV B95-8 strain (BART5, -16, -17, -6, -21, -18, -7, -8, -9, -22, -10, -11, -12, -19, -20, -13, and -14; indicated in gray in Fig. 1B) to downregulate luciferase gene expression in the B95-8v-infected HEK293 cells were determined. Some BART miRNA mimics upregulated the reporter, presumably due to their indirect effect on cytomegalovirus (CMV) promoter activity of the reporter gene. Thus, we focused on miRNA mimics that downregulated the reporter gene. The BART22 mimic suppressed the luciferase activity most strongly (Fig. 6A). This result agrees with that of a previous photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation analysis, which demonstrated that the *NDRG1* mRNA coprecipitates with BART17-5p and BART22 (32), both of which were expressed at high levels in the BART(+)-v-infected HEK293 cells (Fig. 2A). BART9-5p, BART11-5p, and BART11-3p were also strong suppressors in the reporter assay (Fig. 6A). However, BART9-5p was hardly expressed in the infected cells (Fig. 2A), which had been demonstrated by deep sequencing (50, 54). BART11-5p and BART11-3p were found to suppress a reporter gene that lacked the *NDRG1* 3' UTR (data not shown), suggesting they influence the expression of transcription factors that regulate CMV promoter of the reporter gene.

Although four putative BART17-5p binding sites and three putative BART22 were identified in the 3' UTR of *NDRG1* using the miRNA target prediction program DIANA microT (51) (Fig. 6B), only the BART22 mimic downregulated the reporter gene expression (Fig. 6A and C). Notably, this mimic failed to downregulate luciferase gene expression when the three putative binding sites in the 3' UTR of *NDRG1* were mutated (Fig. 6C). These results indicate that the downregulation of luciferase activity by

the BART22 mimic was specifically dependent on the presence of the BART22 binding sites within the 3' UTR of *NDRG1*. Transfection of B95.8v-infected HEK293 cells with the BART22 mimic also downregulated NDRG1 protein expression, while transfection of these cells with the BART17-5p mimic had no effect (Fig. 6D). This result supports the concept that BART22 is responsible for downregulating NDRG1 expression in EBV-infected epithelial cells.

Clustered EBV miRNAs cooperatively downregulate NDRG1 expression. The effects of BART miRNAs on NDRG1 expression were then verified by generating recombinant viruses containing specific deletions of BART miRNA genes. A BAC engineering technique was used to obtain modified EBV-BAC clones that specifically lacked the pre-miRNAs of BART22 only (BART22Δ), BART8 through BART11 (BART8-11Δ), or BART21 through BART14 (BART21-14Δ) (Fig. 7A). The genome of the BART21-14Δ virus, which had a 3.3-kb deletion, lacked the 13 pre-miRNA genes of cluster 2 BART miRNAs (18) but retained the three open reading frames (LF1, LF2, and LF3 in Fig. 1B). Restriction enzyme mapping of these BAC clone DNAs demonstrated that they did exhibit identical digestion pattern except for the fragments corresponding to the modified region (Fig. 7B, and see Table S7 in the supplemental material), and the result was verified by Southern blotting analyses using a specific probe for detecting the region (Fig. 7B). These modified BAC clones were stably transfected into HEK293 cells, and stably infected cell clones were established. Northern blot analyses demonstrated a specific loss of BART22 expression in the BART22Δv-infected cells (Fig. 7C). Immunoblot analyses indicated that NDRG1 was expressed at low levels in the BART22Δv-infected HEK293 cells, indicating that BART22 is not solely responsible for the downregulation of this protein (Fig. 7D). Similarly, NDRG1 was expressed at low levels in the BART8-11Δv-infected cells. However, NDRG1 expression levels were significantly higher in the BART21-14Δv-infected cells (Fig. 7D). No significant difference of viral latent gene expression was observed among the cells harboring different recombinant viruses; they expressed comparable levels of EBNA1, EBNA2, EBNA3s, and LMP1 (Fig. 7D). These results imply that clustered viral miRNAs cooperatively downregulate NDRG1 expression.

Metastatic prostate cancer-derived PC-3 cells, which expressed high levels of NDRG1 (Fig. 5), were then used as recipient cells of EBV infection to verify the results described above. PC-3 cells were transduced with CR2, infected with various recombinant EBVs, and pools of stably infected cells were established. Again, NDRG1 protein level was low in the BART(+)-v-infected PC-3 cells, but the protein level recovered in the BART21-14Δv-infected cells (Fig. 7E). Only traces of EBNA1 protein, but no other viral latent

FIG 6 *NDRG1* is a target of the BART22 miRNA. (A) The upper panel shows a schematic illustration of the reporter construct containing the 3' UTR of *NDRG1* between the luciferase gene (under the control of a cytomegalovirus immediate-early promoter, CMVp) and a simian virus 40 polyadenylation signal [poly(A)]. The lower panel shows the abilities of a series of BART miRNA mimics (indicated in gray in Fig. 1B) to downregulate luciferase activity in the B95.8v-infected HEK293 cells. The activity of firefly luciferase was normalized to that of *Renilla* luciferase (control), and the average values obtained by transfecting the control mimic were adjusted to 1. The data are represented as the means $\pm$ the SD of $n = 3$ independent experiments. (B) The predicted BART17-5p and BART22 target sequences in the 3' UTR of *NDRG1*. The miRNA sequences are shown in green. The mutations introduced into the BART22 binding sites are indicated in blue. (C) BART17-5p mimic and BART22 mimic were tested for their abilities to downregulate the luciferase gene expression from the reporter construct shown in panel A or from a reporter construct containing mutations in the BART22 bindings sites in the *NDRG1* 3' UTR. The B95.8v-infected HEK293 cells were used for the assay. The activity of firefly luciferase was normalized to that of *Renilla* luciferase (control), and the average values obtained by transfecting the control mimic were adjusted to 1. The data are represented as the means $\pm$ the SD of $n = 3$ independent experiments. (D) Immunoblot analyses of NDRG1 and GAPDH (control) protein levels in the B95.8v-infected HEK293 cells transfected with synthetic miRNA mimics (as indicated). The whole-cell extracts were prepared at 48 h posttransfection.

FIG 7 The EBV BART miRNA cluster 2 is responsible for the downregulation of NDRG1 expression. (A) Schematic illustrations of the BART miRNA cluster 2 deletion variants. The positions of the pre-miRNA genes are indicated by numbered vertical lines. The gaps in the horizontal lines indicate the deleted regions. The BglII sites flanking the region are indicated with nucleotide no. of EBV-wt. (B) The indicated EBV-BAC clone DNAs were digested with BglII and analyzed by agarose gel electrophoresis (left panel), and the gel was subsequently processed for Southern blot analysis (right panel). A DNA fragment of BART21-14Δ was PCR-amplified (arrows in panel A) and used as a probe. The bands representing the BART miRNA cluster 2 region (arrowheads in left) were detected by Southern blotting. The calculated sizes (see Table S7 in the supplemental material for the details) of the detected bands are indicated. (C) BART miRNA expression pattern of various recombinant viruses. Total RNAs of C666-1 cells and HEK293 cells infected with the indicated recombinant viruses were subjected to Northern blot analyses. Note that BART22Δ virus did not express BART22 miRNA but did express flanking miRNAs (BART8-5p and BART11-3p). (D) Protein levels of NDRG1, EBNA3s, LMP1, and GAPDH in HEK293 cells infected with the indicated recombinant viruses (two independent cell clones for each virus). (E) Protein levels of NDRG1, EBNA1, and GAPDH in PC-3 cells infected with the indicated recombinant viruses.

proteins, were expressed in these cells (Fig. 7E and data not shown). The result reinforces the argument that the BART miRNA cluster 2 is responsible for the NDRG1 downregulation.

NDRG1 protein expression is downregulated in EBV-positive NPC tissues. The results thus far obtained indicate that NDRG1 was downregulated by BART miRNAs in EBV-infected epithelial cell cultures. We then examined whether NDRG1 was

downregulated in EBV-positive epithelial tumors *in vivo*. The expression level of the NDRG1 protein in NPC biopsy specimens (Table 1) was examined by immunohistochemical staining. *In situ* hybridization (ISH) of EBV-encoded small RNA (EBER) was used to verify the presence of EBV infection. The results revealed that nine of the 10 EBER-ISH-negative NPC specimens were positive for NDRG1 expression (Table 1 and Fig. 8A). In contrast, six of the

TABLE 1 Clinicopathological features of the patients and the expression of NDRG1 in biopsy specimens

NPC type	No. of cases	Mean age (yr ± SD) ^a	No. of patients						
			WHO classification ^b			Stages ^c		NDRG1 ^d	
			I	II	III	I, II	III, IV	–	+
EBER-ISH positive	9	54.0 ± 10.4	0	7	2	2	7	6	3
EBER-ISH negative	10	59.1 ± 8.0	1	8	1	3	7	1	9

^a *P* = 0.244 (Student *t* test).
^b *P* = 0.509 (χ^2 test).
^c *P* = 0.708 (χ^2 test).
^d *P* = 0.02 (Student *t* test).

nine EBER-ISH-positive NPC specimens were negative for NDRG1 expression (Table 1 and Fig. 8B). The difference between the NDRG1 expression levels in the EBER-ISH-positive and EBER-ISH-negative NPC specimens was statistically significant (*P* = 0.02; Table 1). These results indicate that downregulation of NDRG1 expression is common in EBER-ISH-positive NPC tissues and reinforce the biological significance of NDRG1 downregulation during NPC tumorigenesis.

DISCUSSION

It is well known that the commonly used EBV B95-8 strain lacks a 12-kb genomic region that is actively transcribed in EBV-positive epithelial malignancies. Recent studies have indicated that the transcripts encoded by this region are primary miRNAs that are processed to mature miRNAs (known as BART miRNAs). A few previous studies have successfully used recombinant EBV technology to investigate the roles of BART miRNAs (55, 56); however, to our knowledge, the specific roles of BART miRNAs in EBV-infected epithelial cells have not yet been analyzed using recombinant viruses.

Here, we established epithelial cells that were latently infected with the EBV B95-8 virus lacking the actively transcribed 12-kb region, or the BART(+) virus in which this region was restored using BAC technology. The 12-kb region encodes 17 of the 22 BART miRNAs and three open reading frames (LF1, LF2, and LF3). Physiological levels of BART miRNA expression were reconstituted in the BART(+)v-infected epithelial cells. In our system, the BART miRNA genes were seamlessly restored at their native loci and expressed under the control of their native promoters (57). This experimental strategy likely contributes to achieve appropriate expression levels of BART miRNAs.

Via unbiased screening under physiological conditions, in combination with miRNA target prediction, NDRG1 was identified as a direct target of BART miRNAs in EBV-infected epithelial cells. We found that a recombinant EBV lacking BART miRNA cluster 2 (BART21-14Δv) failed to downregulate NDRG1 in two different types of cells (HEK293 and PC-3 cells). Since BART miRNA cluster 2 is located within the intron of BART mRNA, open reading frames of putative BART-encoded protein(s) are preserved in the BART21-14Δ recombinant virus. Furthermore, the BART21-14Δ virus retains intact LF1, LF2, and LF3 open reading frames as well. Therefore, although we cannot completely exclude the possibility that altered expression of putative viral protein(s) encoded by BART mRNA somehow contribute to this NDRG1 dysregulation, it is highly likely that BART miRNAs *per se* are responsible for the NDRG1 downregulation. Transfection assay using BART miRNA mimics identified the BART22 miRNA mimic as the strongest suppressor of NDRG1 expression at both mRNA and protein levels. This is in good agreement with the previous report that NDRG1 mRNA was in complex with BART17-5p and BART22 in primary effusion lymphoma cell lines (32). It is important to note that BART22 is one of the highly expressed BART miRNAs in EBV-infected epithelial cells (9, 54, 58). However, a recombinant EBV lacking the BART22 pre-miRNA gene (BART22Δv) was still able to downregulate NDRG1 expression. This apparent inconsistency can be interpreted that

FIG 8 NDRG1 protein expression is downregulated in EBER-ISH-positive NPC biopsy specimens. (A and B) ISH analyses of EBER expression (left panels) and immunohistochemical analyses of NDRG1 expression (right panels) in NPC biopsy specimens. The magnifications are indicated. (A) Representative images of EBER-ISH-negative specimens. (B) Representative images of EBER-ISH-positive specimens.

miRNA-mediated gene regulation is highly redundant; other viral miRNAs likely can compensate for the loss of BART22. BART miRNAs may also bind to 5' UTR and/or the coding region of NDRG1 mRNA to downregulate protein expression. BART miRNA-mediated downregulation of NDRG1 may not be restricted to direct interaction between BART miRNAs and NDRG1 mRNA. Furthermore, the use of miRNA target prediction programs revealed that NDRG1 may be targeted by several cellular miRNAs, including miR-182, a well-known metastasis-regulatory miRNA (59). Further investigations of the cooperative effects of viral and cellular miRNAs are warranted.

NDRG1 expression increases in response to cell differentiation signals (52, 60). Our results indicate that NDRG1 is expressed at high levels in primary epithelial cells (Fig. 5). Therefore, a possible scenario is that when EBV infects cells in epithelial lineages *in vivo*, BART miRNA targets NDRG1 to avoid growth arrest and terminal differentiation. Alternatively, EBV may preferentially establish latent infection in undifferentiated epithelial cells with little NDRG1 expression, assuming that NDRG1 expression is disadvantageous for the proliferation of the infected cells. NDRG1 has also been characterized as a metastasis suppressor (61), although its antimetastatic potential was mostly demonstrated by overexpressing NDRG1 in tumor cells. Due to its pleiotropic functions of NDRG1 protein (60), it is currently difficult to envision how NDRG1 downregulation contributes to epithelial carcinogenesis. A recent study indicated that NDRG1 interacts with the Wnt receptor LRP6 and blocks Wnt signaling (62). Thus, NDRG1 downregulation may contribute to the activation of Wnt signaling pathway, which is common in gastrointestinal epithelium-derived malignancies (63). For note, other regulators of Wnt signaling pathway, such as CARPIN2 (24) and DAZAP2 (27), were also identified as BART miRNA targets (64).

For unknown reasons, most of the previously identified BART miRNA targets were not identified under the experimental conditions used here; NDRG1 (32) and API5 (30) are the two exceptions (See Dataset S1 and Table S5 in the supplemental material). API5 protein levels were also examined in the established cells, but downregulation of API5 in the BART(+)v-infected cells was not as apparent as that of NDRG1 at the protein level (data not shown).

Thus far, we have found no obvious phenotypic differences between the B95.8v-infected and BART(+)v-infected epithelial cells in their morphologies and proliferation rates. This can be interpreted that one should instead use primary epithelial cells as recipient cells. Alternatively, it may be that BART miRNAs should cooperate with viral oncoproteins to induce phenotypic changes in infected epithelial cells. Importantly, it is known that the viral major oncoprotein LMP1 encoded by NPC-derived EBV is functionally different from that encoded by the B95-8 strain EBV (65); therefore, it may be critical to examine the roles of BART miRNAs in the presence of NPC-derived LMP1 protein. The EBV-BAC technology described in the present study should be useful for further clarifying the mechanism of EBV-mediated epithelial carcinogenesis.

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