Activation of DNA Methyltransferase 1 by EBV LMP1 Involves c-Jun NH_2-Terminal Kinase Signaling

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Abstract

EBV latent membrane protein 1 (LMP1) activates cellular DNA methyltransferases, resulting in hypermethylation and silencing of E-cadherin. However, the underlying mechanism remains to be elucidated. In this study, we show that LMP1 directly induces the *dnmt1* promoter activity through its COOH-terminal activation region-2 YYD domain. Using (*i*) LMP1 mutants, (*ii*) dominant negative mutants c-jun NH_2-terminal kinase (JNK)-DN, p38-DN, and constitutive active mutant IκB, as well as (*iii*) dsRNAs targeting c-Jun, JNK, and tumor necrosis factor receptor–associated death domain protein, and (*iv*) signal transduction inhibitors, we show that LMP1-mediated DNA methyltransferase-1 (DNMT1) activation involves JNK but not nuclear factor κB and p38/mitogen-activated protein kinase signaling. In addition, LMP1 is unable to activate *dnmt1*-P1 promoter with activator protein-1 (AP-1) site mutation. Chromatin immunoprecipitation assay results also confirm that LMP1 activates P1 promoter via the JNK-AP-1 pathway. Furthermore, chromatin immunoprecipitation assay data in LMP1-inducible cells disclose that LMP1 induces formation of a transcriptional repression complex, composed of DNMT1 and histone deacetylase, which locates on E-cadherin gene promoter. Treatment with JNK inhibitor, SP600125, prevents the formation of this repression complex. Statistical analyses of the immunohistochemical staining of 32 nasopharyngeal carcinoma (NPC) biopsies show LMP1 expression (18 of 32, 56.25%), DNMT1 expression (31 of 32, 97%), and phospho-c-Jun (27 of 32, 84.38%), suggesting that overexpression of these proteins is observed in NPC tumor. Overall, these results support a mechanistic link between JNK-AP-1 signaling and DNA methylation induced by the EBV oncogene product LMP1. (Cancer Res 2006; 66(24): 11668-76)

Introduction

EBV is closely associated with human malignancies, including nasopharyngeal carcinoma (NPC; ref. 1), Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma (2), and invasive breast cancer (3). NPC is a human squamous cell cancer prevalent in southeastern China and Taiwan, which comprises ~40% of the head and neck cancers and is notorious for its highly metastatic nature (4). In NPC, EBV infection is predominantly latent and viral gene expression is restricted. One of the viral genes, latent membrane protein 1 (*LMP1*), expressed in ~70% of NPC (5), has the ability to transform rodent cells (6) and renders cell growth in soft agar (7). Human epithelial cells expressing LMP1 display significantly higher invasive capacity, correlating with a decreased expression of a cell-surface adhesion molecule, E-cadherin (8). An earlier study shows that E-cadherin repression is the result of LMP1-induced hypermethylation of the E-cadherin gene promoter through activation of cellular DNA methyltransferases (9). This finding strongly suggests that LMP1 down-regulates the expression of critical genes using cellular DNA methylation machinery.

DNA methylation plays an important role in regulating various cellular and developmental processes. However, aberrant methylation patterns within specific CpG islands are a hallmark of human cancers; hypermethylation of CpG islands at promoter region acts as a strong suppressor in transcription (10, 11). Three active mammalian DNA methyltransferases (DNMT), specifically DNMT1, DNMT3a, and DNMT3b, have been identified. These enzymes modify chromatin structure by adding a methyl group to cytosine of CpG dinucleotides. Methylation of CpG islands located within promoter and proximal exon regions of a gene leads to recruitment of additional protein factors, such as methyl-CpG binding proteins and transcriptional repressors. This, in turn, alters the chromatin structure of the region, making it inaccessible for transcription factors, and results in gene silencing. Transcriptional regulatory region of *dnmt1* containing four promoters (P1, P2, P3, and P4) were identified (12). Among these, P1 is the major promoter located within a CG-rich region whereas P2 to P4 are minor and CG-poor promoters (12).

Structural and functional analyses of LMP1 provide indications about the role of LMP1 in cellular signaling (13). This 63-kDa integral membrane protein is composed of a short NH_2-terminal domain, six transmembrane domains, and a 200-amino-acid COOH-terminal domain. Most LMP1-mediated signals are restricted to the COOH-terminal activation region (CTAR), which is further subdivided into two major domains, CTAR1 and CTAR2. CTAR1 associates with tumor necrosis factor (TNF) receptor–associated factor proteins (TRAF), whereas CTAR2 interacts with TNF receptor–associated death domain protein (TRADD); both CTAR1 and CTAR2 mediate nuclear factor κB (NF-κB) and p38/mitogen-activated protein kinase (MAPK) pathways (14, 15). CTAR2 of LMP1, specifically the last three amino acids (YYD), is the key region triggering LMP1-mediated activator protein-1

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(AP-1) activity via the c-Jun NH_2-terminal kinase (JNK) pathway (16). Mutation or deletion of these regions often abolishes LMP1-regulated signaling.

In this study, we show how LMP1 directs *dnmt1* activation at molecular level. We also provide clinical evidence revealing expression correlation among LMP1, DNMT1, and phospho-c-Jun in NPC.

Materials and Methods

Cell culture and reagents. The NPCTW02 cell line was provided by Dr. C.T. Lin (National Taiwan University, Taipei, Taiwan, Republic of China). LMP1-expressing NPC cell clones were established by cotransfection with LMP1 expression plasmid pT7E (17) and pSV2-neo (Promega, Madison, WI), followed by selection with 800 μg/mL and maintenance with 500 μg/mL G418. Doxycycline-inducible LMP1-expressing cells were generated by cotransfection of pTRE-LMP1 and pTK-hygro (Clontech, BD, Mountain View, CA) into 293 Tet-on cells (Clontech, BD), followed by selection with G418 (800 μg/mL) and hygromycin (500 μg/mL). LMP1 expression was induced by the addition of doxycycline (5 μg/mL) in culture medium. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. NP69 and NP69LMP1 were normal human nasopharyngeal epithelial cells described previously (18). NP69 cell was cultured in defined keratinocyte-SFM medium (Invitrogen, Carlsbad, CA). For inhibitor studies, MAPK/extracellular signal–regulated kinase (ERK) kinase (MEK)-1 inhibitor, PD98059 (10 μmol/L), JNK inhibitor, SP600125 (5 μmol/L), and NF-κB inhibitor, BAY 11-7082 (5 μmol/L), were used to block specific signaling pathways.

Plasmids construction. Please refer to Supplementary data.

DNA transfection and luciferase reporter assay. Please refer to Supplementary data.

siRNA transfection. NP69LMP cells (5×10^5; six-well plate) were transfected with 50 nmol/L dsRNA duplex and 50-μg dsRNA transfection reagent *Trans*IT-TKO (Mirus Bio, Madison, WI) according to manufacturer's protocol. A pool of three 21-bp RNA duplex, si-TRADD (SMARTpool), was purchased from Dharmacon (Lafayette, CO.); si-JNK, si-Jun, si-LMP, and negative control siRNA were synthesized by Research Biolabs, Ayer Rajah Industrial Estate (Singapore). Cells were harvested after 72 hours and then RNA and protein were extracted. Western blotting was done to confirm that the transfected RNA duplex had the ability to knock down the corresponding gene expression. Real-time reverse transcription-PCR (RT-PCR) was conducted to measure the endogenous *dnmt1* transcript, which was normalized by internal controls proliferating cell nuclear antigen (*pcna*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*).

Western blotting. Western blotting was done according to a previous report (9). Extracts were isolated from NPCTW02 cells transiently transfected with pT7E or pUC18 control plasmid, or from LMP1 293 Tet-on cells without or with doxycycline, in the presence or absence of SP600125. Proteins of interest were detected with specific antibodies and the enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, NJ) according to manufacturer's instructions. The DNMT1-specific antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or IMEGNEX (San Diego, CA) and antibodies specific for c-JNK, p-c-Jun, c-Jun, and tubulin were obtained from Santa Cruz Biotechnology.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done as previously described (19). Please refer to Supplementary data.

Real-time RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen). The mRNA (1 μg), purified by the oligo(dT) column, was used for synthesis of first-strand cDNA with an oligo(dT) primer using either TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) or Transcriptor reverse transcription kit (Roche Diagnostics, Basel, Switzerland). Specific primers were used to detect the *dnmt1* gene and the internal controls *gapdh* and *pcna* (9). Quantitative RT-PCR was done on a LightCycler instrument (Roche Diagnostics) according to manufacturer's instructions with FastStart DNA Master SYBR Green I (Roche Diagnostics). Results of *dnmt1* were normalized to *gapdh* or *pcna* data.

Recombinant adenovirus infection. Recombinant adenovirus containing LMP1 gene (rAdLMP1) and the control virus rAdLacZ (9), at a multiplicity of infection of 100, were used to infect the NPCTW02 cells. Cells were harvested 12 hours postinfection for *dnmt1* promoter chromatin immunoprecipitation assay with anti-c-Jun antibody (Santa Cruz Biotechnology).

JNK kinase assay. 293 Tet-on LMP1 cells were lysed in kinase lysis buffer [20 mmol/L Tris (pH 7.6), 0.5% Triton X-100, 250 mmol/L NaCl, 3 mmol/L EGTA, 3 mmol/L EDTA, 2 mmol/L sodium vanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L DTT] for 15 minutes on ice. Cell debris was removed by centrifugation and protein concentration was determined with Bio-Rad protein assay dye (Bio-Rad, Hercules, CA). JNK was immunoprecipitated from 500-μg protein extract by using 2-μg anti-JNK1 antibody (C-17, Santa Cruz Biotechnology) at 4°C for 90 minutes with agitation, and then the immune complex was pulled down by addition of 50-μL Protein A beads at 4°C for 90 minutes with agitation. Following immunoprecipitation, beads were washed once with kinase lysis buffer and twice with assay buffer [20 mmol/L HEPES (pH 7.5), 20 mmol/L β-glycerophosphate, 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 50 μmol/L sodium vanadate, and 1 μg/mL leupeptin]. Beads were drained and resuspended in 40 μL of assay buffer containing 2 μg of glutathione *S*-transferase-Jun and 20 μmol/L ATP and 3 μCi [γ-^{32}P]ATP. The kinase reaction was carried out at 30°C for 30 minutes and stopped by adding 4× sample buffer, followed by boiling for 5 minutes. Samples were separated by 12% SDS-PAGE and kinase activity was detected by autoradiography.

Bisulfite modification and methylation-specific PCR. LMP1-expressing plasmid DNA (pT7E) and E-cadherin promoter reporter −1,008/+49 were cotransfected into MCF7 cells (5×10^6 per 35-mm dish) by Lipofectamine 2000 reagent (Invitrogen). Transfected cells were harvested 24 hours after transfection and the genomic DNA was isolated for bisulfite modification as described in ref. 20. For methylation-specific PCR (E-cadherin promoter) analysis, 100 ng of modified DNA in a final volume of 50 μL were incubated with 1× PCR buffer (1.64 mmol/L NH_4SO_4, 6.4 mmol/L $MgCl_2$, 100 mmol/L 2-mercaptoethanol), 200 nmol/L deoxynucleotide triphosphate, and 10 pmol primers for E-cadherin. The PCR products were isolated and cloned into pUC-T vector (Yeastern, Taipei, Taiwan) and the DNA sequences were determined by autosequenceing.

Immunohistochemistry and *in situ* hybridization. Immunohistochemical staining was done on 5-μm-thick consecutive sections of formalin-fixed, paraffin-embedded tissue specimens. Samples were deparaffinized and dehydrated for the immunohistochemistry and *in situ* hybridization studies. For a detailed protocol, refer to Supplementary Materials and Methods. Staining score for immunohistochemistry was confirmed by three pathologists (Y.L., C.S., and C-L.C.). Statistical analysis was done with SigmaPlot 2004 software (SYSTAT Software, Inc., San Jose, CA).

Results

CTAR2 YYD domain is essential for LMP1-mediated activation of *dnmt1* promoter activity. To investigate the LMP1-mediated signaling that is involved in the activation of *dnmt1* gene, luciferase reporter *dnmt*-P1-Luc and LMP1-expressing constructs were cotransfected into NPCTW02 cells. A dose-dependent activation of dnmt-P1 promoter was observed when increasing amounts of LMP1 were added (Supplementary Fig. S1). It has been established that CTAR1 and CTAR2, located at the cytoplasmic domain of LMP1, are two critical regions responsible for several LMP1-mediated signaling pathways. To map the corresponding domain of LMP1 that is involved in DNMT1 promoter activation, a series of LMP1 and LMP1 mutant constructs, as depicted in Fig. 1*A*, were cotransfected with reporter p*dnmt*P1-Luc. Promoter activities were analyzed by measuring the relative luciferase units and normalized by β-galactosidase activity. Figure 1*B* shows that LMP1 activates p*dnmt*P1 promoter activity ~2-fold when compared with vector control. Deletion of the LMP1 three terminal

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amino acids YYD (ΔYYD-378 or ΔYYD) and CTAR2 (ΔCTAR2-335 or ΔCTAR2), but not mutant CTAR1 (mCTAR1), abolishes LMP1-mediated activation of the P1 promoter. These results confirm the involvement of LMP1 in *dnmt1* gene activation and further suggest that the YYD domain plays an important role in this activation process.

It has been shown that the YYD domain of LMP1 mediates JNK (16), p38/MAPK (15), and NF-κB (21) signaling pathways. To further evaluate which signaling pathway(s) is involved in LMP1-mediated activation of *dnmt1*, we used various specific inhibitors and dominant negative mutants for JNK, p38/MAPK, and NF-κB signaling pathways to block the LMP1-mediated *dnmt1* activation. As shown in Fig. 1*C*, transient cotransfection of an LMP1-expressing vector with the P1 reporter construct into NPC cells resulted in activation of P1 by LMP1. However, this activation was inhibited by JNK inhibitor, SP600125 (5 μmol/L), but not by MEK1 inhibitor, PD98059 (10 μmol/L), NF-κB inhibitor, BAY 11-7082 (5 μmol/L), and the vehicle control (DMSO). Similarly, LMP1-mediated *dnmt1* activation was blocked when cells were cotransfected with a dominant-negative mutant construct of JNK1, pcDNA-Flag-JNK1 (APF), but not by a dominant-negative mutant construct of p38 MAPK, pCMV-Flag-p38 (APF), and a constitutive active form of I-κB, pCMV-Flag-IκB-S32A,S36A. Together, these results indicate that JNK signaling pathway, but not p38/MAPK and NF-κB, is responsible for LMP1-mediated *dnmt1* activation. Similarly, the inhibition of *dnmt1* gene at transcriptional level was also observed in SP600125-treated NPCTW02-LMP stable cell clone. Real-time RT-PCR revealed that induction of *dnmt1* promoter activity by LMP1 (1.6-fold) was totally abolished by the addition of JNK inhibitor (Supplementary Fig. S2). The inhibitors and dominant-negative mutants used in these experiments had no inhibitory effect on the LMP1 protein expression as shown by Western blot analyses with anti-LMP1 antibody (S12). These results collectively suggest that the LMP1-YYD domain may mediate JNK signaling, which plays a critical role in the activation of *dnmt1* gene expression.

LMP1-mediated DNMT expression is inhibited by siRNAs targeting JNK pathway in nasopharyngeal epithelial cells. To further confirm that LMP1 induces DNMT1 expression in nasopharyngeal epithelial cells, we compared the protein and RNA expression of DNMT1 in NP69 (immortalized normal nasopharyngeal epithelial cells) and NP69-LMP1 (an NP69 cell line that stably expresses LMP1). As examined by Western blot analysis with DNMT1-specific antibody, we found that DNMT1 protein level was significantly increased in NP69-LMP1 cells (~4-fold) when compared with that of NP69 cells (Fig. 2*A*). On the contrary, the E-cadherin protein expression was suppressed ~4-fold in NP69-LMP1 (Fig. 2*A*). In addition, when NP69 cells were transiently transfected with LMP1-expressing vector (pCMV-LMP1), the *dnmt1* mRNA level, measured by quantitative real time RT-PCR, increased ~3-fold when compared with vector control (pCMV-Flag2; Fig. 2*B*). These data are consistent with the *dnmt1* mRNA level detected in NP69-LMP1 (~2-fold increase) when compared with NP69 (data not shown). These results suggest that both protein and mRNA levels of DNMT1 can be induced by LMP1 expression in nasopharyngeal epithelial cells.

The induction of DNMT1 in NP69-LMP1 cells can be intervened by the addition of siRNAs targeting JNK signaling mediators, including JNK and c-Jun, and siRNAs targeting LMP1 signaling mediators, including TRADD, an LMP1-YYD domain interacting protein, and LMP1 itself (Fig. 2*C*). The level of endogenous *dnmt1* mRNA, measured by quantitative real time RT-PCR, was reduced to 20% (si-JNK), 40% (si-Jun), 60% (si-TRADD), and 50% (si-LMP1), respectively, when compared with the control nontarget siRNA. Western blot analyses were done to verify that all the siRNAs have the ability to knock down at least 75% of their specific targets. Apparently, different siRNAs have slightly different performance in reducing the endogenous *dnmt1* transcript. Nevertheless, these

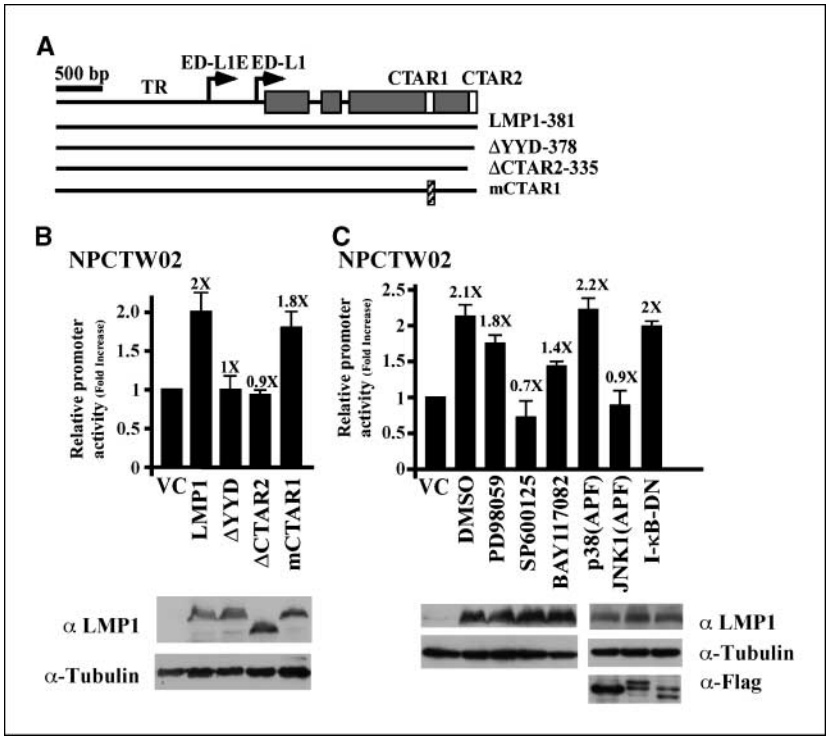

Figure 1. Identification of the signaling pathway involved in LMP1-mediated *dnmt1* promoter activation. *A,* schematic representation of LMP1 and its mutants. CTAR1, CTAR2, and the last three amino acids, YYD, are specified. Plasmids expressing full-length, deletion of YYD (*ΔYYD*), deletion of CTAR2 (*ΔCTAR2*), and CTAR1 mutation (*mCTAR1*) of LMP1 are illustrated. *B,* various LMP1 constructs and vector control (*VC*) were cotransfected with p*dnmt*P1-Luc reporter into NPCTW02 cells to dissect the domain on LMP1 that is important for *dnmt1* promoter activation. Relative luciferase activity was determined as fold increase when compared with vector control and normalized with β-galactosidase activity. Western blotting analyses were done to confirm the expression of LMP1 (S12) and equal protein loading (anti-tubulin). *C,* NPCTW02 cells were cotransfected with p*dnmt*P1-Luc reporter and full-length LMP1 in the presence and absence (DMSO) of signal transduction inhibitors, PD98059 (10 μmol/L), SP600125 (5 μmol/L), and BAY 11-7082 (5 μmol/L), and dominant negative mutants, pcDNA-Flag-JNK1 (APF), pCMV-Flag-p38 (APF), and pCMV-Flag-IκB-S32A,S36A. *Columns,* mean of at least five independent, duplicate experiments; *bars,* SD. Western blot analyses were done to confirm the expression of LMP1 (S12) and dominant negative mutants (anti-Flag), and equal protein loading (anti-tubulin).

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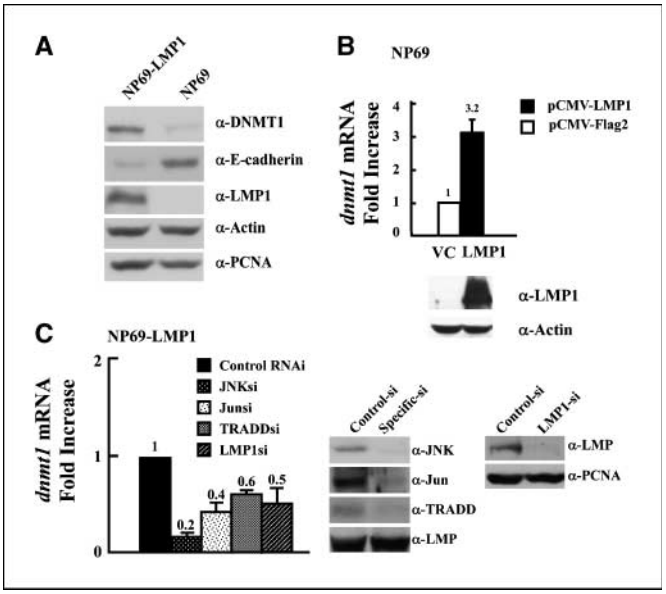

Figure 2. LMP1-mediated DNMT1 activation is inhibited by JNK signaling pathway–related dsRNAs. *A,* Western blot analyses in NP69 and NP69-LMP1 cells. In NP69-LMP1 cells, LMP1 and DNMT1 are overexpressed whereas the expression of E-cadherin is suppressed. *B,* NP69 cells were transiently transfected with either pCMV-LMP1 (■) or vector (pCMV-Flag2; □). The endogenous *dnmt1* mRNA levels were determined by real-time RT-PCR. Normalization was done by using PCNA. *C,* NP69-LMP1 cells were transfected with different dsRNAs, including nontarget control RNAi, JNK-si, Jun-si, TRADD-si, and LMP1-si; the endogenous *dnmt1* mRNA levels were determined by real-time RT-PCR and normalized by PCNA. *Columns,* mean of at least three independent, duplicate experiments; *bars,* SDs. Western blot analyses were done to confirm >75% reduction of the original protein expression and specificity for each dsRNA target.

data further indicate that LMP1-mediated DNMT1 activation involves JNK signaling.

JNK/AP-1 signaling is involved in LMP1-mediated *dnmt1* gene activation. To further determine if the JNK-AP-1 pathway directly affects *dnmt*1 promoter activity, potential transcription responsive element within the P1-mini promoter (−254 to +308) region was analyzed. An AP-1 site (TGACCCA), a target for the Fos and Jun family transcription factor, located within the −195 position of the *dnmt1*-P1-mini promoter, was predicted with the TFSEARCH program.[8] To elucidate if this site is the responsive element for *dnmt1* promoter activation by LMP1, NPCTW02 cells were cotransfected with LMP1-expressing plasmid (pT7E), three *dnmt1*-P1-mini deletion reporter constructs with AP-1 site deletion (−188dnmt1p1mini, −75dnmt1p1mini, and −15dnmt1p1mini), and one AP-1 site-specific mutant (mAP-1-P1mini), of which the AP-1 sequence had been changed to TG*GT*CCA. As shown in Fig. 3*A*, wild-type P1-mini was activated ∼1.6-fold by LMP1. In contrast, deletion of AP-1 site in −188-P1mini, −75-P1mini, and −15-P1mini prevented LMP1 from activating the *dnmt1* promoter. More specifically, mAP-1-P1mini was no longer activated by LMP1 (fold increase for luciferase activity was statistical significant when compared with P1mini; $P < 0.05$). Results indicated that the LMP1 responsive element is located within the AP-1 site (−201 to −195) on *dnmt1*-P1-mini promoter.

[8] http://www.cbrc.jp/research/db/TFSEARCH.html.

Activation of transcription factor c-Jun involves the phosphorylation of c-Jun by JNK. To test whether the JNK activity is stimulated by LMP1, LMP1 293 Tet-on inducible cells were treated with doxycycline, followed by *in vitro* JNK kinase assay. As shown in Fig. 3*B*, in the presence of doxycycline, LMP1 induced JNK kinase activity; however, such induction was blocked by the treatment with JNK inhibitor SP600125. Similarly, using LMP1 293 Tet-on inducible cells, the expression of phosphorylated c-Jun was elevated by 10-fold in the presence of LMP1; on the other hand, such expression elevation was reduced to one fifth of the original activation level by the addition of JNK inhibitor SP600125 (Supplementary Fig. S3). Thus, LMP1 mediates both the activation of both JNK kinase and phosphorylation of c-Jun.

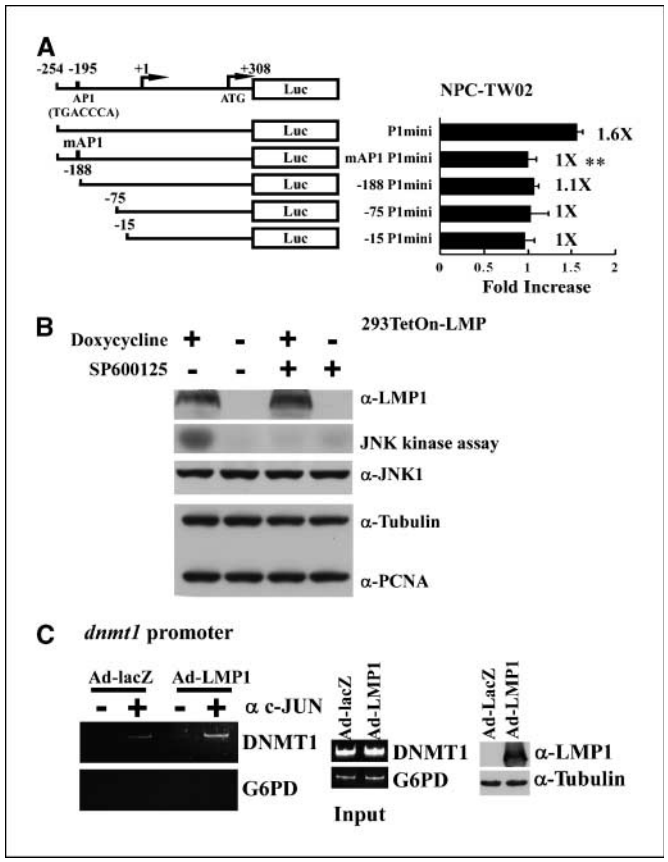

Figure 3. LMP1 induces DNMT1 expression via the JNK/AP-1 signaling pathway. *A,* identification of the responsible element on *dnmt1*-P1-mini promoter. NPCTW02 cells were cotransfected with various lengths of pdnmt-P1-mini-Luc promoter (−188, −75, and −15) or with AP-1 site mutant (−188, mAP-1) and full-length LMP1. Promoter activities were presented as fold increase, which was normalized by vector control. *Columns,* mean of at least three independent, duplicate experiments; *bars,* SD. *B,* JNK kinase assay. Cell extracts from 293 Tet-on LMP1 cells treated with JNK inhibitor SP600125 (5 μmol/L) or left untreated in the presence or absence of doxycycline were used for JNK kinase assay, separated in 10% SDS-PAGE and subjected to autoradiography. Western blot analyses using anti-JNK, anti-tubulin, and PCNA were done as control. *C,* LMP1-induced c-Jun binding on *dnmt1* proximal promoter. NPCTW02 cells were infected with recombinant adenovirus expressing either LMP1 or its control LacZ. Cells were fixed with formaldehyde 12 hours postinfection as described. Chromatin immunoprecipitation assay was done with anti-c-Jun antibody to immunoprecipitate the protein-DNA complex, followed by PCR reaction using a specific dnmt1 primer set to amplify a 300-bp DNA, or glucose-6-phosphate dehydrogenase (G6PD) primer set as negative control. PCR was done to indicate equal amount of the input DNA-protein in Ad-LMP1- and Ad-LacZ-infected cells. Western blot analysis was done to confirm the expression of LMP1 in Ad-LMP1-infected cells.

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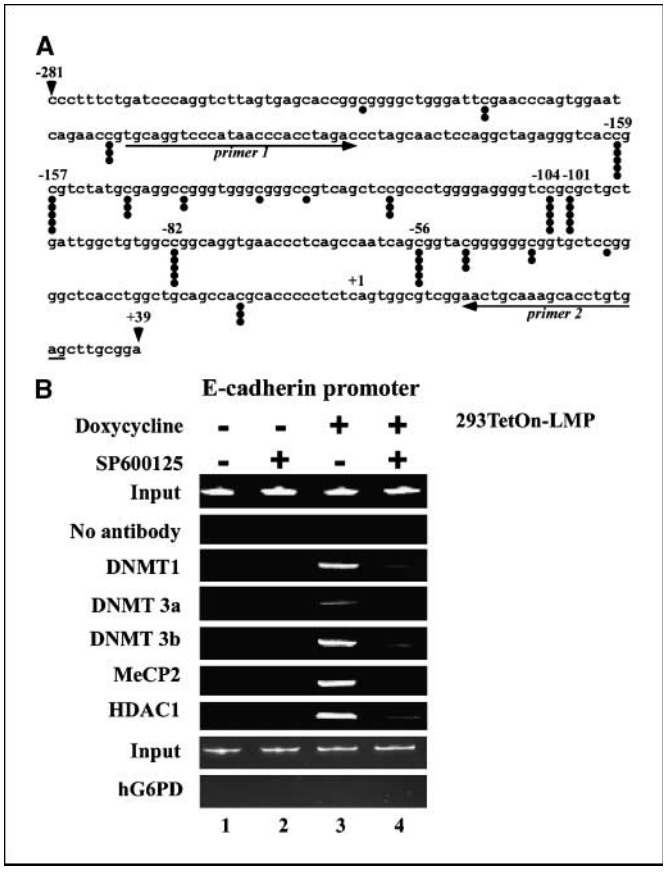

Figure 4. LMP1 mediates hypermethylation of E-cadherin promoter. *A,* bisulfite DNA sequencing analysis on E-cadherin proximal promoter from −281 to +39. MCF7 cells were transiently cotransfected with LMP1-expressing DNA (pT7E) or vector with E-cadherin promoter reporter pEad-(−1,008/+49). Genomic DNA was isolated 24 hours posttransfection for bisulfite modification. Modified DNA was then amplified with methylation-specific primer sets. PCR products were purified and cloned into pUC-T vector. Eight independent clones were sequenced. *Black dot,* methylation that occurs in one clone at specific CpG dinucleotide on E-cadherin promoter. *B,* LMP1 mediates the recruitment of a transcription repression complex on E-cadherin promoter. Chromatin immunoprecipitation assay was conducted in 293 Tet-on LMP1 cells treated with SP600125 or left untreated in the presence or absence of doxycycline with anti-DNMT1, anti-DNMT3a, anti-DNMT3b, anti-MeCP2, and anti-HDAC1 antibodies or without antibody. *Top,* dnmt1 PCR product of input cross-linked DNA-protein. A negative control primer set of G6PD was included in chromatin immunoprecipitation assay.

To show that the activated c-Jun physically binds to the *dnmt1* promoter in the presence of LMP1, chromatin immunoprecipitation assay using anti-c-Jun antibody was conducted. From cells infected with the LMP1-expressing recombinant adenovirus, a differential higher level of *dnmt1* promoter DNA was detected in the chromatin immunoprecipitation assay as compared with that of the control virus (Fig. 3*C*), indicating that LMP1 induces *dnmt1* promoter activation through phosphorylation of c-Jun transcription factor. And this activated phospho-c-Jun, in turn, binds to the *dnmt1* promoter AP-1 site and transcriptionally activates the promoter.

JNK inhibitor blocks LMP1-mediated formation of transcription repression complex on E-cadherin proximal promoter. We have previously shown that LMP1 can induce hypermethylation of endogenous E-cadherin proximal promoter (9). To confirm the methylation status of exogenous E-cadherin promoter in the presence of LMP1 *in vitro*, E-cadherin promoter reporter and LMP1-expressing plasmid (pT7E) or control plasmid (pUC18) were cotransfected into MCF-7 cells and the exogenous E-cadherin promoter methylation status was assayed by bisulfite sequencing. Genomic DNA was extracted from cells 24 hours posttransfection and was treated with sodium bisulfite, converting deoxycytosine, but not the 5-methylcytosine residues, into uracil through deamination. Methylation-specific PCR was done to distinguish the unmethylated DNA from methylated DNA by two sets of primers (20). Interestingly, methylated PCR product can only be detected in the LMP1-expressed MCF-7 cells (data not shown). Methylated PCR products were isolated and cloned into pUC-T vector and the DNA sequence was determined by autosequencing. Eight independent clones were examined; the methylated C residues (ranging from −281 to +39 of E-cadherin promoter) in each clone were represented by a solid spot as depicted in Fig. 4*A*. There were 18 CpG methylation sites detected and the methylation percentage for each site varied from 12.5% (1 of 8) to 62.5% (5 of 8). These data strongly support that LMP1 can induce hypermethylation on specific CpG sites on E-cadherin promoter.

It has been well documented that DNA methylation, in particular at the promoter region, recruits protein factors, including DNMTs, and transcription repression complex such as methyl-CpG binding protein 2 (MeCP2), which binds tightly to methylated residues (22), and histone deacetylase (HDAC), an essential component in methylation-dependent transcriptional repression (23). To determine whether induction of DNA methylation and transcriptional repression complex formation can occur on E-cadherin promoter in an LMP1-dependent manner, LMP1 293 Tet-on cells were treated with doxycycline or left untreated and subjected to chromatin immunoprecipitation assay with antibodies specific for DNMT1, DNMT3a, DNMT3b, MeCP2, and HDAC1. Our results showed that only in doxycycline-treated cells (Fig. 4*B, lane 3*), and not in the untreated cells (Fig. 4*B, lane 1*), was E-cadherin promoter DNA fragment encompassing −169 to +31 detected by PCR in the chromatin immunoprecipitation assay with anti-MeCP2, anti-DNMT1, anti-DNMT3a, and anti-DNMT3b antibodies, indicating that the methylation protein complexes were formed within the E-cadherin promoter in the presence of LMP1. Additionally, the chromatin immunoprecipitation analysis also revealed that HDAC1 is part of the LMP1-induced DNA methylation on E-cadherin promoter; this is consistent with the previous observation that DNMT3a and DNMT3b interact with HDAC1 (24). Our data support that there is a cross talk between DNA methylation, histone deacetylation, and transcription repression. Thus, the data provide direct evidence that LMP1 induces DNA methylation and formation of transcriptional repression complexes containing DNMT1, DNMT3a, DNMT3b, MeCP2, and HDAC1 on E-cadherin promoter, which leads to transcriptional repression and silencing of E-cadherin. However, the formation of such LMP1-induced repression complex was inhibited when the JNK inhibitor SP600125 was added (Fig. 4*B, lane 4*), suggesting that the drug inhibits the LMP1-mediated JNK activation of DNMT1, accordingly blocking methylation of E-cadherin promoter, and consequently prevents the binding of MeCP2, DNMT1, DNMT3a, DNMT3b, and HDAC1 to the promoter region. Thus, the results presented here confirm that LMP1 induces DNA methylation and transcription repression on E-cadherin promoter via JNK signaling.

Correlation between LMP1, phospho-c-Jun, and DNMT1 expression in NPC biopsy tissues. To further investigate the *in vivo* correlation between LMP1, phospho-c-Jun, and DNMT1 expression, we examined clinical NPC biopsy samples from 32 patients after staining with LMP1, phospho-c-Jun, and DNMT1 antibodies individually. As shown in Fig. 5, LMP1, an integral

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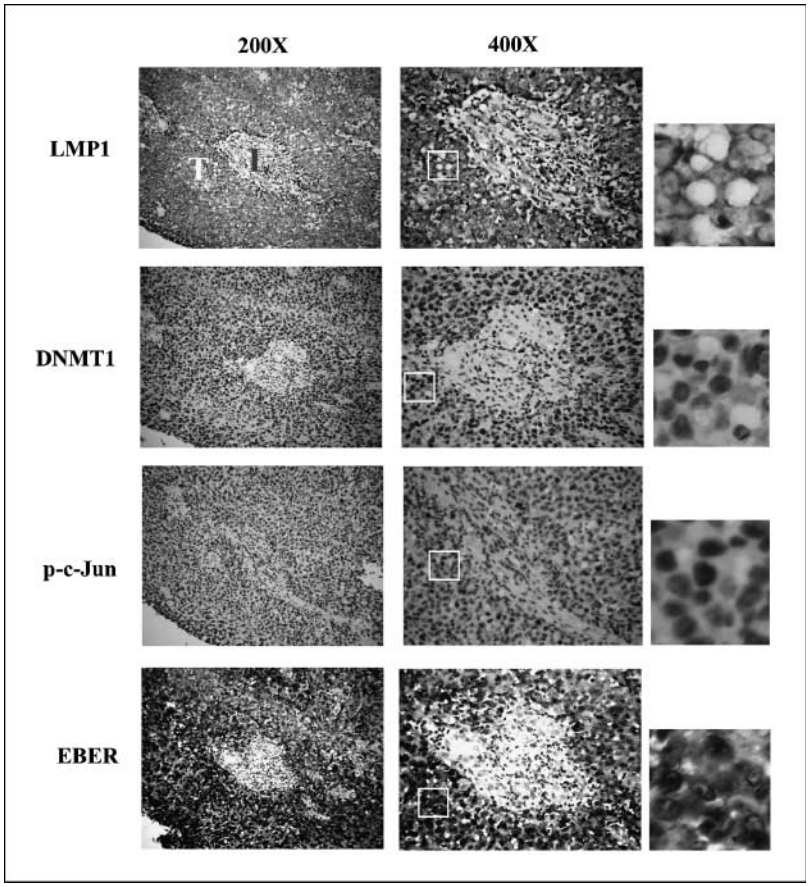

Figure 5. Immunohistochemical staining (LMP1, DNMT1, and phospho-c-Jun) and *in situ* hybridization (EBER) of formalin-fixed, paraffin-embedded consecutive NPC sections at different magnifications (×200 and ×400). The expression of LMP1, DNMT1, phospho-c-Jun proteins, and EBER RNAs are detected in similar region in NPC tumor cells. Strong expressions of DNMT1, phospho-c-Jun, and EBER are detected in the nuclei, whereas LMP1 is detected at the cell membrane. *T,* tumor cells; *L,* infiltrated lymphocytes. *Right,* enlarged figures.

membrane protein that can be detected at the surface of the cell membrane, is expressed in 18 of 32 (56.25%) NPC tissues. Both phospho-c-Jun and DNMT1 are nuclear proteins and are highly expressed in 27 of 32 (84.38%) and 31 of 32 tumor samples (97%) examined, respectively. All 32 NPC tumor samples (100%) are EBV positive as confirmed by *in situ* hybridization using EBV noncoding RNAs [EBV-encoded RNA (EBER)] as probe. From the staining pattern of these consecutive tissue sections, we can conclude that the region with tumor cells (signified by the enlarged nucleus) that are EBV and LMP1 positive have significant phospho-c-Jun and DNMT1 expression (Fig. 5; Supplementary Fig. S4*A* for colored figure). Interestingly, the adjacent nontumor tissue, including the normal epithelial cells, plasma cells, and infiltrated lymphocytes, which are EBV and LMP1 negative, has little or no phospho-c-Jun and DNMT1 expression as shown in Supplementary Fig. S4*B*. For the qualitative assessment of immunohistochemical staining of

Table 1. Clinicopathologic features and expression of LMP1, DNMT1, phospho-c-Jun, and EBER in 32 NPC biopsies

	% Positive cells (score)				Positive cases	Positive rate (%)
	0	1+	2+	3+		
Tumor						
LMP1	14	2	9	7	18	56.25
DNMT1	1	2	14	15	31	97
Phospho-c-Jun	5	4	14	9	27	84.38
EBER	0	1	15	16	32	100
Adjacent tissue						
LMP1	29	3	0	0		
DNMT1	28	4	0	0		
Phospho-c-Jun	30	2	0	0		
EBER	32	0	0	0		

NOTE: Total number of NPC patients, $n = 32$. Scoring system: 0, 0% to <10% positive staining; 1+, 11% to 30% positive staining; 2+, 31% to 60% positive staining; 3+, >60% positive staining.

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tumor and nontumor cells, a 0 to 3+ scoring system was used to represent the percentage of the positive cells, as shown in Table 1. A moderately positive correlation was found between LMP1 and DNMT1 expression (Pearson correlation $R = 0.58$; Supplementary Fig. S5) and between LMP1 and phospho-c-Jun ($R = 0.63$). However, significantly positive correlations between (*i*) DNMT1 and EBER expression ($R = 0.91$; Supplementary Fig. S5), (*ii*) phospho-c-Jun and EBER expression ($R = 0.75$), and (*iii*) phospho-c-Jun and DNMT1 expression ($R = 0.75$) are observed. These clinical data support our findings that LMP1 expression correlates with high phospho-c-Jun and DNMT1 expression.

Discussion

LMP1, an EBV oncoprotein, is required for EBV-mediated transformation. The higher migration ability of LMP1-expressed cells correlates with the repression of E-cadherin (9, 25), an adhesion molecule that is often lost in many cancers including NPC (26); nevertheless, the molecular mechanism was not clear. Previously, we have shown that LMP1 is responsible for the down-regulation of E-cadherin expression by inducing DNMTs, resulting in hypermethylation of E-cadherin promoter (9). To continue our last study, here we provide detailed evidence on how LMP1 confers DNMT induction at molecular level.

LMP1 mainly activates the major promoter, P1, of the *dnmt1* gene. Deletion of CTAR2, or specifically the last three amino acids, YYD, resulted in failure to activate the P1 promoter. To date, there are at least three well-defined signaling pathways, NF-κB, p38/MAPK, and JNK, which are elicited by LMP1 COOH-terminal domain (13, 27). According to the studies on inhibitors and dominant negative mutants and si-targets, we have identified the JNK pathway, but not the NF-κB or p38/MAPK signaling pathway, which contributes to LMP1-mediated P1 *dnmt1* gene activation. JNK is one of the three subfamilies of MAPK and is characterized as a stress-activated protein kinase in response to growth stimuli, cellular transformation, and tumor cell metastasis (28–30). The activated kinase subsequently phosphorylates its direct target, c-Jun, a component of AP-1 complex, forming homodimer or heterodimeric complexes with c-Fos. Finally, the phosphorylated c-Jun binds to and transactivates the promoter containing the AP-1 site. LMP1 induction of JNK kinase activity may be regarded as a growth advantage to EBV latent infected cells due to the versatile nature of this important signaling pathway. However, LMP1-induced *dnmt1* activation and JNK kinase activity can be specifically blocked by the inhibitor SP600125 (5 μmol/L). The compound preferentially blocks c-Jun phosphorylation and has no effect on other MAPKs, such as ERKs, p38/MAPK, and IκB kinases (ref. 31; data not shown). To confirm the involvement of JNK signaling in regulating LMP1-mediated *dnmt1* promoter activity, we searched for the potential AP-1 site on *dnmt1* promoter. As expected, we located an AP-1 responsive element within the −201 to −195 site of the *dnmt*-P1-mini promoter. The importance of this particular AP-1 site was confirmed by the *in vitro dnmt1* promoter reporter assay (Fig. 4*A*), in which the mutated AP-1 site on *dnmt1* promoter abolishes the LMP1-mediated *dnmt1* activation. Furthermore, chromatin immunoprecipitation assay showed that in the presence of LMP1, more c-Jun physically binds to the AP-1 site on *dnmt1* promoter (Fig. 4*C*). Accordingly, we show for the first time that DNMT1 is a direct target of LMP1-mediated JNK signaling.

Our previous report showed that LMP1 additionally activates DNMT3a and DNMT3b (9). In this study, DNMT3a and DNMT3b are detected in the LMP1-induced repression complex; yet, the formation of this complex is inhibited by SP600125 (Fig. 4*B*). An ~2- to 3-fold decrease in DNMT3a and DNMT3b mRNA levels was detected in the presence of SP600125 (data not shown). Sequence analyses of DNMT3a and DNMT3b promoters reveal the presence of AP-1 sites that are potentially regulated by LMP1. Thus, LMP1 may also activate these two promoters via JNK activation.

We identified earlier an LMP1-mediated DNMT1 target gene, E-cadherin (9), an adhesion molecule that mediates cell-cell contact and is important for tissue morphogenesis, cell polarity, and tumor invasiveness (32). On DNMT1 activation, both transcriptional and translational levels of E-cadherin are significantly reduced. DNMT1 catalyzes the methylation of CpG dinucleotides on DNA, which leads to suppression of gene expression. Loss of membranous E-cadherin expression results in enhanced cell migration activity (9, 33), which significantly correlates with tumor invasion, advanced disease stage, and tumor metastasis (34). Thus, E-cadherin is considered as a tumor suppressor gene (35–39). In this current study, chromatin immunoprecipitation assay reveals that LMP1 mediates induction of a repression complex containing DNMT1, DNMT3a, DNMT3b, MeCP2, and HDAC1 on E-cadherin proximal promoter. This finding is consistent with a previous report showing that DNMT3a and DNMT3b interact with HDAC1 (24). At present, the sequential order for recruiting these transcription repression factors remains unclear. Again, the formation of this repression complex is inhibited by SP600125, suggesting that JNK signaling is the key

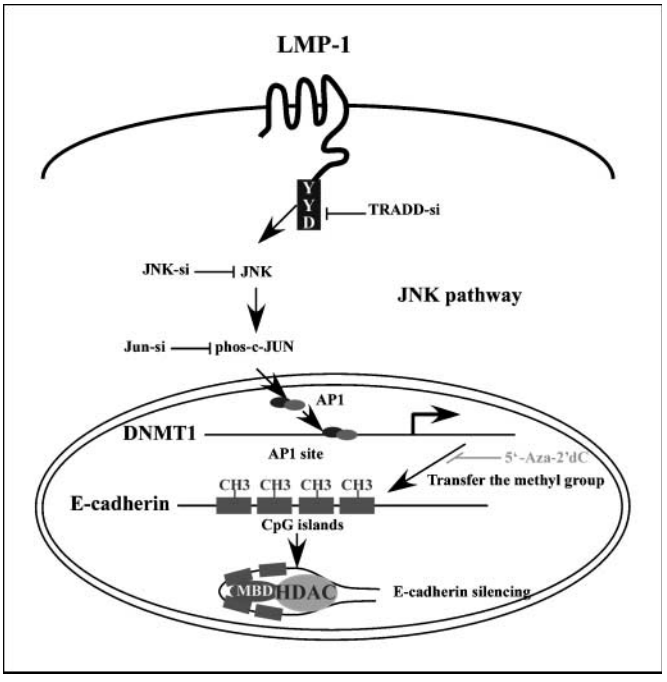

Figure 6. A model for LMP1-mediated DNMT1 activation via JNK/AP-1 signaling. Activated DNMT1 hypermethylates E-cadherin promoter and recruits a transcriptional repression complex leading to gene silencing. The YYD domain of LMP1 activates the JNK signaling pathway and activated JNK, in turn, phosphorylates transcription factor c-Jun. Phosphorylated c-Jun of AP-1 complex binds and transactivates the *dnmt1* promoter. Elevated DNMT1 expression leads to hypermethylation of E-cadherin gene and formation of a transcriptional repression complex including DNMTs, methyl-binding proteins (*MBD*), and HDAC. This LMP1-mediated DNMT1 activation can be blocked by JNK inhibitor SP600125, dominant negative mutant (*DN-JNK*), and dsRNAs (JNK-si, c-Jun-si, TRADD-si, and LMP1-si).

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for triggering the LMP1-DNMT1–mediated gene silencing cascade. One may speculate that DNMTs bind to the E-cadherin first; however, it is difficult to predict which factor, HDAC or MeCP2, would be the next one to be recruited.

We investigated the correlation between the expression of LMP1, DNMT1, phospho-c-Jun, and EBER in 32 NPC biopsies. Clinical data in this study also support that LMP1 expression or EBV infection correlates with JNK signaling and DNMT1 overexpression. Indeed, elevated DNMT expression may be functionally important for NPC tumor formation because activation of DNA methyltransferases has been reported in other human malignant diseases (40–43). The positive rate for LMP1 expression detected by immunostaining was moderate ($\sim$56%) and variable when compared with that of DNMT1 (>90%) and phospho-c-Jun (84.38%) in NPC biopsies. Among the LMP1-positive biopsies, the staining pattern for LMP1 is heterogeneous, which is similar to previous reports (44–46), suggesting that only a subset of NPC cells express LMP1. Using a more sensitive RT-PCR–based detection, several reports revealed much higher frequencies (>80%) of LMP1 expression (47, 48). In addition, >70% of NPC patients' sera are positive for anti-LMP1 (49). Taken together, the positive rate for LMP1 in NPC is likely underestimated due to the sensitivity and specificity of the detection method. Apart from these limitations, we do observe in the consecutive NPC sections that most of the LMP1-positive cells are also DNMT1 and phospho-c-Jun positive, and these three proteins coexpress in a similar tissue region but at different cellular compartments, as shown in Fig. 5. Although the correlation between LMP1 and DNMT1 and between LMP1 and phospho-c-Jun in our study is considered moderate (R = 0.58 and R = 0.63, respectively), this may not reflect the real situation when the underestimated LMP1 expression is considered. It is interesting to note that the noncoding EBV EBER RNA expression correlates well to DNMT1 (R = 0.91) and phospho-c-Jun (R = 0.75) expression. Because EBERs do not code for proteins, it is unlikely that EBER expression has direct effect on DNMT1 and phospho-c-Jun activation, although this possibility cannot be ruled out. This, on the other hand, suggests that besides LMP1, EBV infection per se or other EBV latent genes may have an additive effect on DNMT1 and phospho-c-Jun up-regulation.

A model for this LMP1-JNK-DNMT1 signaling is then proposed based on our findings (Fig. 6). Taken together, our data clearly show that there is an important link between LMP1-mediated JNK signaling, epigenetic gene regulation, and gene silencing. Conceivably, E-cadherin is not the only gene of which the transcription is being down-regulated by LMP1-DNMT1. It would be of interest for us to identify other genes that are regulated by similar mechanism. Indeed, an earlier study shows that promoters of two other tumor suppressor genes, *p16* and *RASSF1A*, are hypermethylated in NPC (50). The issue of whether DNA methylation of these two promoters is mediated by LMP1 is still under investigation. Our data suggest that HDAC1 and DNMTs may be potential dual targets for treating EBV LMP1-positive tumors. Thus, elucidation of the mechanism by which LMP1 regulates DNA methyltransferases is critical for the development of therapeutic agents.

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