Twist and Epithelial-Mesenchymal Transition Are Induced by the EBV Oncoprotein Latent Membrane Protein 1 and Are Associated with Metastatic Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC), an EBV-associated malignancy, is highly metastatic compared with other head and neck tumors, perhaps because of its viral link. Here, we show that the principal EBV oncoprotein, latent membrane protein 1 (LMP1), induces epithelial-mesenchymal transition (EMT) via Twist, a master transcriptional regulator in embryogenesis and newly implicated in metastasis, which, in turn, are likely to contribute to the highly metastatic character of NPC. LMP1 could induce EMT and its associated cell motility and invasiveness in a cell culture model, whereas expression of Twist small interfering RNA reversed LMP1-induced EMT. In diverse EBV-infected cell lines, expression of Twist correlates with expression of LMP1. Dominant-negative LMP1 could suppress Twist expression in EBV-positive cells, whereas LMP1 could induce Twist in EBV-negative nasopharyngeal cells. LMP1 signals through the nuclear factor-κB pathway, and an IκB superrepressor inhibited induction of Twist by LMP1. Finally, in human NPC tissues, expression of Twist and LMP1 is directly correlated and expression of Twist is associated with metastasis clinically. These results suggest that induction of Twist by a human viral oncoprotein LMP1 directly contributes to the metastatic nature of NPC. [Cancer Res 2007;67(5):1970–8]

Introduction

Nasopharyngeal carcinoma (NPC) is notorious among head and neck cancers for its highly metastatic character (1). Carcinoma often spreads clinically before forming a mass in the primary lesion at early stages of the disease (1). Also, no human carcinoma is as consistently associated with EBV as NPC, and EBV, the first human tumor virus, is detected in most NPCs and is intimately associated with its oncogenesis (1–3). The close association of EBV with several invasive malignancies, especially NPC, B-cell lymphoproliferative diseases as well as some types of Hodgkin's disease, and invasive breast cancers (2), has raised the question of whether a tumor virus could contribute to the invasive character of tumors.

Latent membrane protein 1 (LMP1) is the principal EBV oncoprotein. LMP1 is required for immortalization of B lymphocytes (4) and has attracted great interest because it is the only EBV latency gene capable of transforming rodent fibroblasts (5). The oncogenic character of LMP1 is underscored by its ability to induce lymphomas in transgenic mice (6). LMP1 is a membrane protein that acts as a constitutively active receptor-like molecule in the absence of ligand. On the level of signal transduction, these effects of LMP1 on cell growth and proliferation can be attributed in part to its tumor necrosis factor α receptor (TNFR) II (7) and CD40-mimicking functions (8). LMP1 has a significant role in initiating EBV-associated lymphoproliferative disease and EBV-related malignancies (9). LMP1 is detected in at least 70% of NPCs at the protein level and in virtually all at the transcriptional level (10).

Although most studies of LMP1 have focused on its primary oncogenic role in EBV-related malignancies, more recently LMP1 has been implicated also in their metastatic properties (11–13). EBV latency types 2 and 3–related malignancies exemplified by NPC and B-cell lymphoproliferative disease, respectively, express LMP1 and show invasive and metastatic phenotypes; on the other hand, type 1 EBV malignancies, such as Burkitt lymphoma and a subset of stomach carcinoma, which do not express LMP1, are characterized initially by localized growth (14). NPCs with high LMP1 expression are reported to show an increased tendency toward metastasis compared with those with low LMP1 expression (12, 13, 15–18). Further, we have produced evidence in recent years that LMP1 can up-regulate multiple invasion, angiogenesis, and metastasis factors, and clarified the molecular mechanisms of their induction (12, 13, 16, 17, 19–27).

Epithelial-mesenchymal transition (EMT) is a process whereby epithelial cells lose polarity and cell-to-cell adhesion and undergo dramatic remodeling of the cytoskeleton (28). Concurrent with loss of epithelial cell adhesion and cytoskeletal components, cells undergoing EMT acquire expression of mesenchymal components and a migratory phenotype. EMT was first recognized in embryogenesis in the early 1980s. Today, evidence is growing that carcinoma cells activate the dormant EMT program in promoting cell migration, invasion, and metastasis (28–30); however, its pathogenesis in human carcinomas is obscure.

Distinctive among head and neck cancers is the undifferentiated histopathology of NPC (1, 31), and EBV is associated with this unique histopathology. Based on degree of differentiation, NPC is classified into three subtypes: WHO type I is keratinizing squamous cell carcinoma, the common type in other head and neck cancers, type II is nonkeratinizing carcinoma, and type III is undifferentiated carcinoma (32). Most NPCs in endemic regions are classified as types II and III (1, 31). The association of EBV with type I NPC is less consistent; however, monoclonal EBV episomes are detected in almost all types II and III NPC tissues (3). Notably, types II and III

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tumor cells, which are always associated with EBV, exhibit phenotypes similar to cells that have undergone EMT and are composed of single or single-file rows of tumor cells that have lost typical epithelial tissue structure. Histologically, they show diffusely infiltrative growth. Indeed, the incidence of metastasis in type III NPC is greater than in other types (33). Based on these observations, we hypothesized that EBV might also be involved in EMT.

Recently, we found that the transcriptional factor Twist—a highly conserved basic helix-loop-helix protein that is essential for early embryogenesis—promotes EMT and plays an essential role in metastasis in a breast tumor model (34). Twist has also been suggested to have oncogenic properties. Overexpression of Twist in rhabdomyosarcomas inhibited Myc-induced apoptosis and interfered with p53 tumor suppression (35). Up-regulation of Twist is associated with malignant transformation in T-cell lymphoma (36). Forced expression of Twist triggers resistance of human cancer cells to drugs that inhibit microtubule formation, such as Taxol and vincristine (37). Further, following our report (34), expression of Twist has been implicated in promotion of metastasis and invasive pathologic subtypes in several types of carcinomas (37–39). However, the mechanism of Twist activation and its upstream signaling pathway during tumorigenesis remain enigmatic.

Based on this background, we explored whether EMT could be induced by the EBV oncogene LMP1 and its association with Twist in this context. Further, we took advantage of an established tumor virus with its many known intracellular signaling pathways to explore the mechanistic link between EBV LMP1 and Twist, and how induction of Twist by LMP1 might contribute to the highly metastatic character of NPC. We first show that LMP1 can activate the EMT program via Twist. We show next that in EBV-infected cell culture models, up-regulation of Twist depends on LMP1, and LMP1 can directly induce Twist via nuclear factor-κB (NF-κB) in nasopharyngeal epithelial cells. Finally, expression of Twist correlates with LMP1 protein expression in human NPC tissues as well as with metastasis.

Materials and Methods

NPC tissues. Ten frozen NPC tissues from Institut Gustave Roussy (Paris, France) were used for analysis of LMP1 and Twist proteins by Western blotting. Thirty-seven NPC paraffin-embedded specimens from Fukui Prefectural Hospital (Fukui, Japan), provided by Dr. Yasuharu Kaizaki (Fukui Prefectural Hospital), were used for immunohistochemical analysis.

Cell cultures. KH-1 and KH-2 cells are EBV-positive type II cell lines derived from fusion of KR-4 (an EBV-positive type III lymphoblastoid cell line) and HeLa cells (human cervical carcinoma cell line; gifts of Dr. Maria Masucci, Karolinska Institute, Stockholm, Sweden; ref. 40). MDA-MB-231 and EBV-infected MDA-MB-231 clones (C4A3, C1D12, C2G6, and C3B4) were described previously (41). The parental MDA-MB-231 cells and C4A3 clone are LMP1 negative. C1D12, C2G6, and C3B4 are LMP1-positive clones, and LMP1 expression is strongest in the C3B4 clone. Ad-AH cells, kindly provided by Dr. Erik K. Flemington (Tulane University, New Orleans, LA), are an EBV-negative human nasopharyngeal cell line (42). Madin-Darby canine kidney cells (MDCK) and HeLa cells were from the American Type Culture Collection (Manassas, VA). Cells were maintained as previously described (25).

Plasmids and small interfering RNA. PcDNA3-based LMP1 plasmid (14) and LMP1 mutants (LMP-PQAA, LMP-IID and LMP-DM; ref. 43) have been described. LMP-PQAA has point mutations in COOH-terminal activation region 1 (CTAR1). LMP-IID has point mutations in CTAR2. LMP-DM has point mutations in both CTAR1 and CTAR2 and acts as dominant-negative form of LMP1. A FLAG-tagged superrepressor mutant form of IκBα [srIκBα (S32A/S36A)], which prevents translocation of NF-κB into the nucleus, was provided by Dr. Albert Baldwin (University of North Carolina, Chapel Hill, NC; ref. 22). Twist cDNA is subcloned into the pBabe-Puro vector (34). The Twist small interfering RNA (siRNA)–expressing U6 lentivirus system has been described (34, 44).

Transient and stable transfection. Cells were transfected with the use of Fugene6 transfection reagent (Roche Diagnostics) following the manufacturer's instructions. Stable cell lines expressing LMP1 were established by cultivating Ad-AH cells and MDCK cells in the presence of 500 μg/mL G418.

Lentiviral production and infection of target cells. The production of lentiviruses and pBabe-amphotropic viruses and infection of cells have been described (34, 44). siRNA-infected cells were selected with 2 μg/mL puromycin.

Western blot analysis. Proteins were extracted from NPC tissues or cultured cells, and 100 μg of cell lysates were loaded and analyzed by Western blotting as described before (24). The following antibodies were used as primary antibodies: mouse LMP1 monoclonal antibody from DAKO (Glostrup, Denmark); rabbit Twist polyclonal antibody, epithelial marker antibodies (mouse E-cadherin monoclonal antibody, rabbit α-catenin polyclonal antibody, mouse β-catenin monoclonal antibody, and rabbit γ-catenin polyclonal antibody), and two mesenchymal antibodies (goat vimentin polyclonal antibody and rabbit N-cadherin polyclonal antibody) were from Santa Cruz Biotechnology (Santa Cruz, CA); the other two mesenchymal antibodies (mouse fibronectin monoclonal antibody and mouse α-smooth muscle actin monoclonal antibody), mouse Flag M2 monoclonal antibody, and mouse γ-tubulin monoclonal antibody were from Sigma (St. Louis, MO).

Northern blot analysis. RNA preparation and Northern analysis were carried out as described before (24, 25).

Luciferase reporter assay. Assays were done as described (22). Luciferase reporter vector containing Twist promoter is from Dr. Louise R. Howe (Cornell University, New York, NY; ref. 45). Transfection efficiency was monitored by *Renilla* luciferase activity with the Dual Luciferase assay kit (Promega, Madison, WI).

Invasion assay and cell wound–migration assay. The invasion assay was done with the use of Biocoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA) as described previously (21). We established these indices to evaluate *in vitro* invasiveness: percentage invasion and invasion index. Indices were calculated as follows: % invasion = number of cells invading through Matrigel-coated membrane / number of cells migrating through uncoated control insert membrane. Invasion index was calculated as % invasion of tested cells / % invasion of control cells (MDCK + vector + control siRNA cells). Cell wound–migration assay was done as previously described (17). Migration was assessed by measuring distances between wound edges.

Immunofluorescence. Procedures were done as previously described (46). Primary antibodies for Twist, E-cadherin, and vimentin are the same as used for Western blotting.

Immunohistochemical analysis. Immunohistochemistry was done as previously described (16, 17, 47). Primary antibodies for LMP1 and Twist are the same as for Western blotting. Previously identified LMP1-positive NPC and breast cancer cells served as positive controls for LMP1 (16, 17) and Twist (48), respectively. NPC specimens were evaluated independently by two authors (T.H. and S.K.) without knowledge of the clinical data and reviewed by a pathologist (R.S.). Two examiners each selected two representative fields of >200 tumor cells and counted both the stained cells and the total number of tumor cells. The average percentage of stained cells was used to calculate the LMP1 and Twist expression scores. Stainings were repeated at least twice in sequential sections to assess reproducibility.

Statistical analysis. Pearson's correlation coefficient was used to analyze correlations between the expression of LMP1 and Twist in NPC. Expression of Twist in relation to clinical data was analyzed with the Mann-Whitney *U* test. The differences in wound migration and invasion indices between MDCK cell clones were analyzed by the paired *t* test.

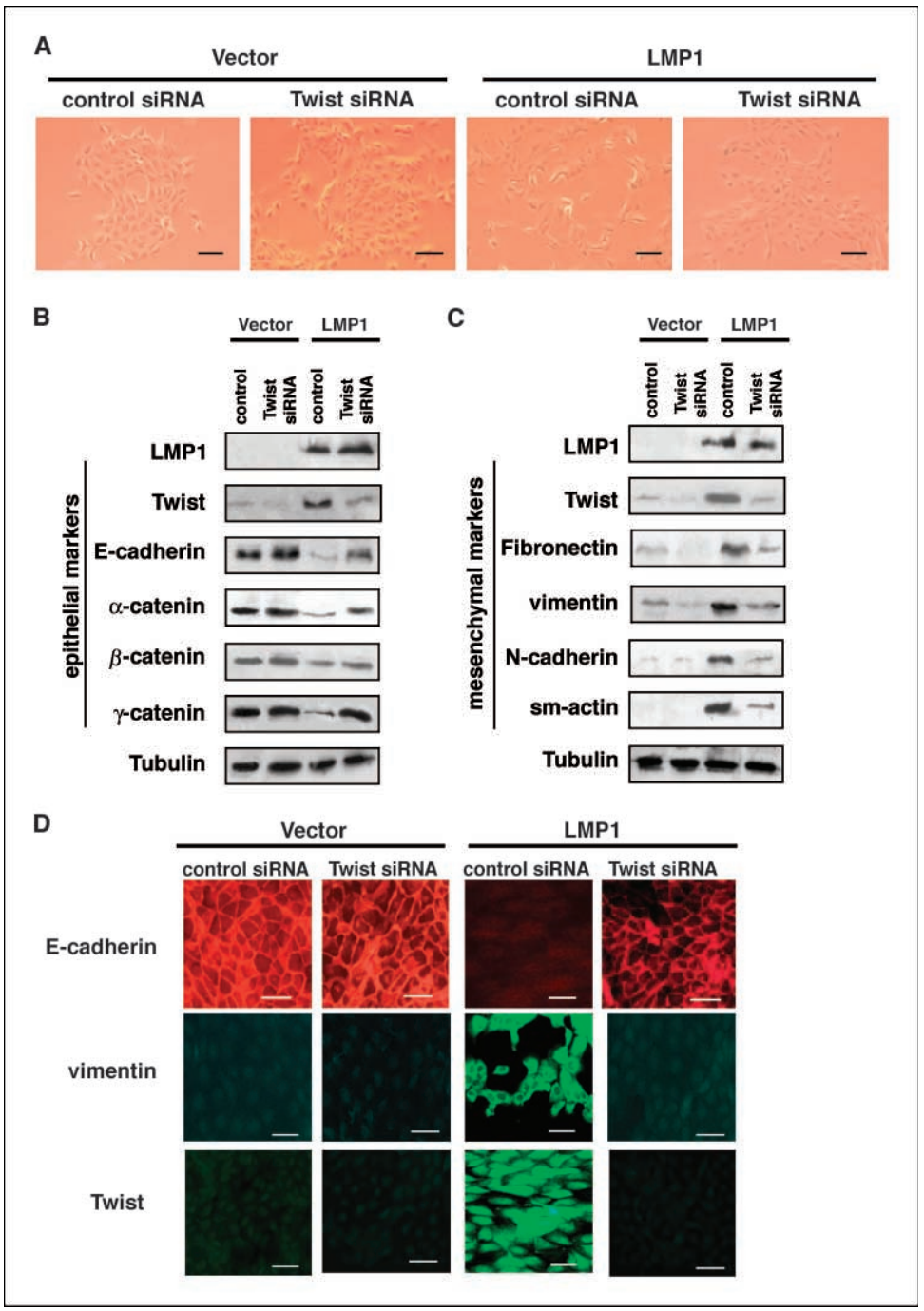

Figure 1. LMP1 induces EMT via Twist. *A,* morphologic changes in MDCK epithelial cells induced by transformation with LMP1 could be reversed by Twist siRNA. MDCK cell clones, 2×10^5 cells per dish, were plated onto 35-mm plastic dishes and observed after 24 h. Twist siRNA and control siRNA were transduced by the lentiviral system to generate stable clones. Representative clones. *Bar*, 50 μm. *B,* expression of epithelial marker proteins E-cadherin, α-catenin, β-catenin, and γ-catenin, together with LMP1 and Twist in MDCK cell clones shown by Western blotting. *C,* expression of mesenchymal marker proteins, fibronectin, vimentin, N-cadherin, and smooth muscle actin (*sm-actin*) was examined by Western blotting together with LMP1 and Twist in the MDCK cell clones. *D,* immunofluorescence staining for a representative epithelial marker, E-cadherin, and a mesenchymal marker, vimentin, in MDCK cell clones together with staining for Twist. *Bar*, 20 μm.

Results

LMP1 induces EMT. EMT has been newly implicated in dissemination of carcinoma cells (28). We explored whether EBV LMP1 could affect EMT and, if so, whether and how Twist is involved, using MDCK epithelial cells as in our previous study (34). MDCK cells provide a model system for EMT. Parental and MDCK cells transfected with control plasmid exhibited characteristic cobblestone morphology. In contrast, LMP1 transfectants lost cell-to-cell adhesion and acquired long spindle-shape morphology. These changes represent hallmarks of EMT. Results with a representative clone are shown in Fig. 1*A*.

To determine whether molecular changes associated with EMT could be detected, we examined both epithelial and mesenchymal markers. As shown in Fig. 1*B*, the expression level of epithelial markers, E-cadherin, α-catenin, β-catenin, and γ-catenin, decreased upon transfection of LMP1. In contrast, the expression of mesenchymal markers, fibronectin, vimentin, N-cadherin, and smooth muscle actin, which correlates positively with EMT, was induced in response to LMP1 expression (Fig. 1*C*). Immunofluorescence studies also showed that E-cadherin disappeared from cell membranes, and vimentin was induced in the cytoplasm of MDCK cells by LMP transfection (Fig. 1*D*). Hence, both the morphologic and molecular changes indicated that LMP1 is capable of inducing EMT. Further, we examined whether EMT induced by LMP1 accompanies up-regulation of Twist. The results show that Twist protein was clearly up-regulated by

LMP1 as detected by Western blotting and immunofluorescence (Fig. 1*B–D*).

Twist is required for the induction of EMT, cell migration, and invasiveness by LMP1. We recently identified *Twist* as a novel EMT-related gene (30, 34). Therefore, we examined whether the ability of the viral oncoprotein to induce EMT is attributable to Twist. We probed the significance of Twist expression by transducing Twist siRNA through a lentiviral system into LMP1-transformed MDCK cells and generated stable clones, and then tested whether down-regulation of Twist could lead to any changes in EMT. As shown in Fig. 1*A*, inactivation of Twist in LMP1-transformed MDCK cells resulted in changes from scattered and fibroblast-like shapes to tightly packed cobblestone morphology, characteristics of mesenchymal-to-epithelial transition, the reverse of EMT (28). Furthermore, expression of epithelial markers significantly increased, whereas the levels of mesenchymal markers decreased (Fig. 1*B* and *C*). Immunofluorescence studies also showed features of mesenchymal-to-epithelial transition (Fig. 1*D*). Hence, both morphologic and molecular changes showed that suppression of Twist expression in LMP1-transformed MDCK cells resulted in the reversion of EMT. These results indicate that Twist is required for LMP1-induced EMT.

To test whether this EMT is mirrored in up-regulation of metastatic properties in a cell culture model, we examined alterations of cellular motility and invasiveness. First, we carried out a wound-induced migration assay to assess the contribution of LMP1 and Twist to cellular motility. Control MDCK cells showed tight cell-to-cell adhesion and did not migrate away from the monolayers. In contrast, LMP1-transformed cells migrated into the wounded area away from the monolayer edges. Further, suppression of expression of Twist with siRNA resulted in evident reduced cell migration (Fig. 2*A* and *B*). Next, we studied cell invasiveness with the use of a Matrigel invasion chamber system. As shown in Fig. 2*C* and *D*, LMP1-expressing MDCK cells were more invasive in this assay than pcDNA3-transfected cells, suggesting that LMP1 up-regulates invasiveness of MDCK cells. Further, introduction of Twist siRNA clearly inhibited LMP1-induced invasiveness. Thus, LMP1-induced EMT was reflected in the up-regulation of cellular motility and invasiveness via Twist.

The level of Twist protein is increased in latently EBV-infected cells. We investigated further the link between Twist and LMP1. First, we examined the endogenous levels of Twist protein in a set of EBV-infected cell lines. KH-1 and KH-2 are type II latently infected adherent cell lines derived by fusion of an EBV-infected lymphoblastoid suspension cell line, KR4, with adherent HeLa cells (40). The expression level of Twist protein is significantly higher in KR4, KH-1, and KH-2 cells than in the EBV-negative parental HeLa cells (Fig. 3*A*). We also tested a set of EBV-infected breast cancer

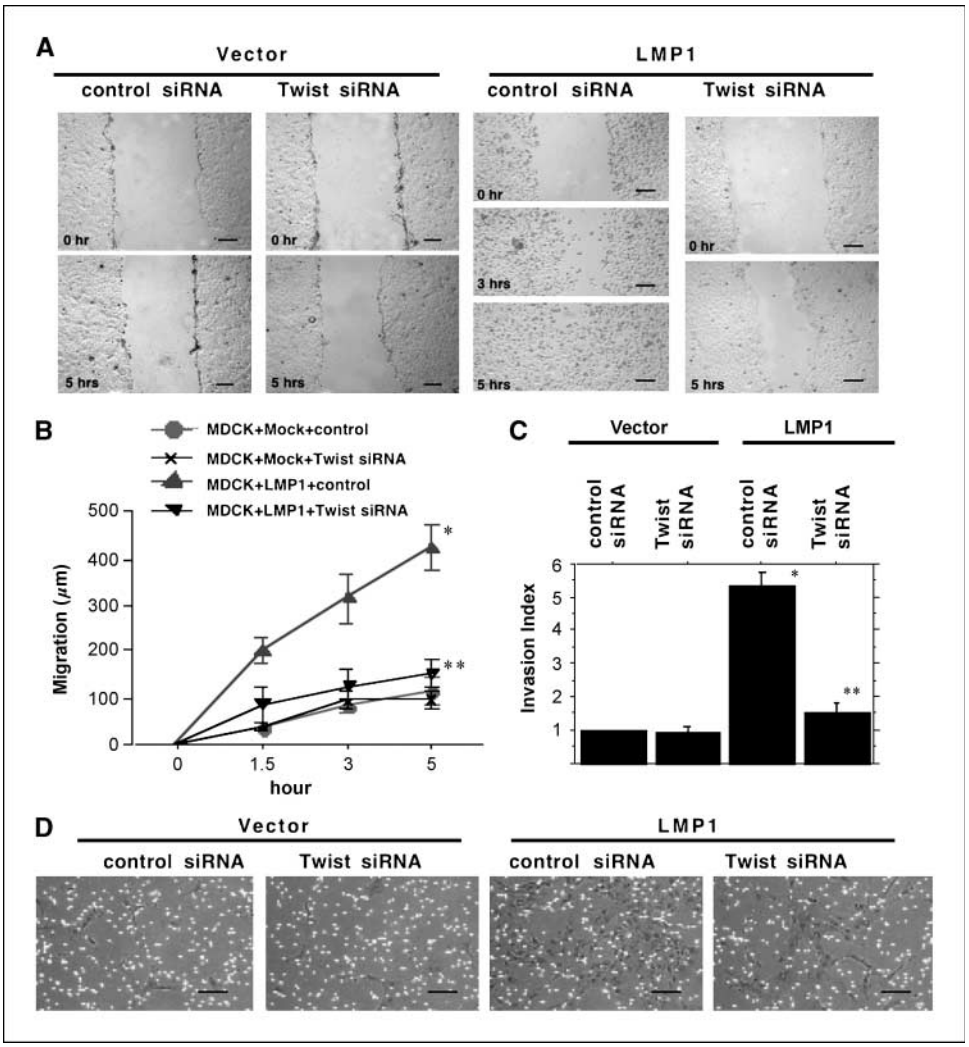

Figure 2. LMP1 up-regulates cellular motility and invasiveness via Twist. *A,* scrape-wound migration assay shows that enhanced motility in LMP1 transformants is down-regulated by silencing Twist. Twist siRNA and control siRNA were transduced by the lentiviral system. Confluent monolayers of MDCK cell clones were scraped with a plastic pipette, and wound-induced migration of cells was observed for 5 h after scraping. Typical wounds for each cell clone. *Bar*, 200 μm. *B,* migration of LMP1-transformed cells is significantly down-regulated by Twist siRNA. Migration was measured at five locations in triplicate after 1.5, 3, and 5 h, and average migration distances are plotted. *Points,* average distances; *bars,* SD. Differences were tested by paired *t* test. *, $P < 0.05$ at all time points compared with control cells (MDCK + vector + control siRNA cells). **, $P < 0.05$ at all time points compared with MDCK + LMP1 + control siRNA cells. *C,* invasion indices, calculated from counts of cells invading through Matrigel-coated membrane, show that enhanced invasiveness in LMP1 transformants is significantly down-regulated by silencing Twist. Cells were from same experiment as in Fig. 3*A* and *B*. Significance was tested by paired *t* test. *, $P = 0.0029$, compared with control cells (MDCK + vector + control siRNA cells). **, $P = 0.0007$, compared with MDCK + LMP1 + control siRNA cells. *D,* invasiveness of LMP1-transformed cells is reduced by Twist siRNA. After 72 h in Matrigel invasion assays, cells adherent on lower surface of filters were fixed and stained. Five representative fields were photographed by light microscopy (magnification, ×100) and counted in triplicate for invasion indices. Representative photos. *Bar*, 50 μm.

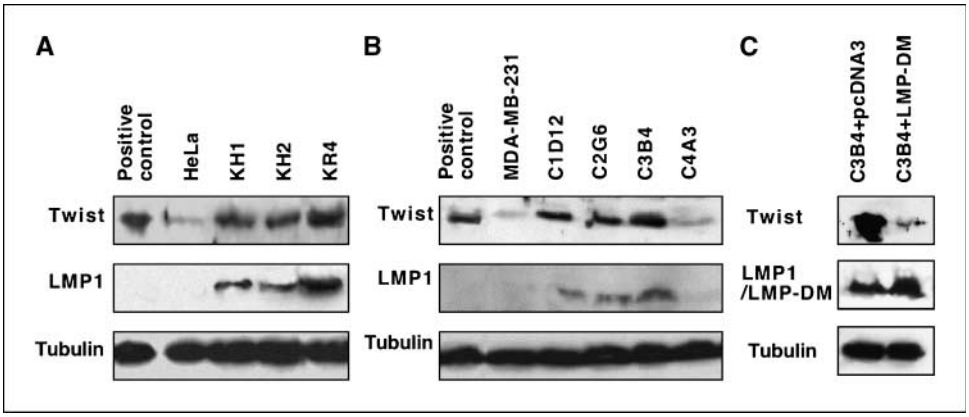

Figure 3. The expression of Twist is associated with expression of LMP1 in latently EBV-infected cells. *A,* levels of Twist are increased in types 2 and 3 latently EBV-infected cells and correspond to level of LMP1 as detected by Western blotting. KH-1 and KH-2 type 2 cell lines were derived by fusion of type 3 KR-4 and HeLa cells. HeLa cells transfected with Twist cDNA served as positive control. *B,* Twist protein levels correlate with LMP1 expression in EBV-infected breast cancer cell lines. MDA-MB-231 is an EBV-negative breast cancer cell line. C4A3, C1D12, C2G6, and C3B4 are EBV-infected clones derived from the MDA-MB-231 parental cell line. C1D12, C2G6, and C3B4 express LMP1. *C,* transfection of LMP-DM, a dominant-negative form of LMP1, into C3B4 cells down-regulates the expression of Twist.

cell lines that express different levels of LMP1. MDA-MB-231 is an EBV-negative breast cancer cell line, and C4A3, C1D12, C2G6, and C3B4 are EBV-infected clones derived from it (41). As shown in Fig. 3*B*, the Twist level is greater in the LMP1-positive clones C1D12, C2G6, and C3B4, but not in LMP1-negative C4A3 cells or in the parental line MDA-MB-231. These data suggest a tight correlation between levels of Twist and LMP1 in two distinct cell types infected by EBV.

LMP1 induces expression of Twist protein and mRNA in nasopharyngeal epithelial cells. To examine whether LMP1 is responsible for induction of Twist, we transfected LMP-DM, a dominant-negative form of LMP1, into the breast cancer cells (i.e., C3B4) that express the highest levels of both endogenous LMP1 and Twist. Expression of LMP-DM significantly reduced the level of Twist protein (Fig. 3*C*), suggesting that LMP1 is required for the induction of Twist in EBV-infected cells.

To explore whether LMP1 can directly induce Twist, we transfected LMP1 expression vector into Ad-AH cells, an EBV-negative nasopharyngeal epithelial cell line. In transient transfection assays, the level of Twist was up-regulated by LMP1 (Fig. 4*A*). Moreover, Twist induction by LMP1 seemed to be dose dependent. Stable expression of LMP1 in Ad-AH cells also resulted in an increased level of Twist compared with control cells (Fig. 4*B*). Furthermore, we found that LMP1 also induced higher levels of Twist mRNA in the same stably transfected Ad-AH cells by Northern blot analysis (Fig. 4*C*). The results indicate that LMP1 is capable of inducing expression of Twist protein and mRNA in nasopharyngeal epithelial cells.

The LMP1 signaling domains CTAR1 and CTAR2 both induce Twist through NF-κB in nasopharyngeal epithelial cells. Two cytoplasmic regions of LMP1, CTAR1 and CTAR2, are well-characterized signaling domains of LMP1 that are essential for directly mediating expression of many genes implicated in transformation and metastasis (49). We investigated which CTAR was involved in induction of Twist. In Ad-AH cells, Twist was not induced by the LMP-DM construct, which has point mutations inactivating both CTAR1 and CTAR2. In contrast, Twist was induced by both the LMP-IID and LMP-PQAA constructs, but to a lesser extent than by the wild-type LMP1 protein (Fig. 5*A*); the first construct has point mutations inactivating the CTAR2 YYD motif, whereas the second construct has mutations inactivating the CTAR1 PXQXT motif (43). Therefore, we concluded that LMP1 could activate Twist through both CTAR1 and CTAR2 regions.

LMP1 CTARs can activate several frequently used signaling molecules via the activation of NF-κB in common, including in Ad-AH cells (21, 49). When cotransfected with LMP1, srIκB (S32A/

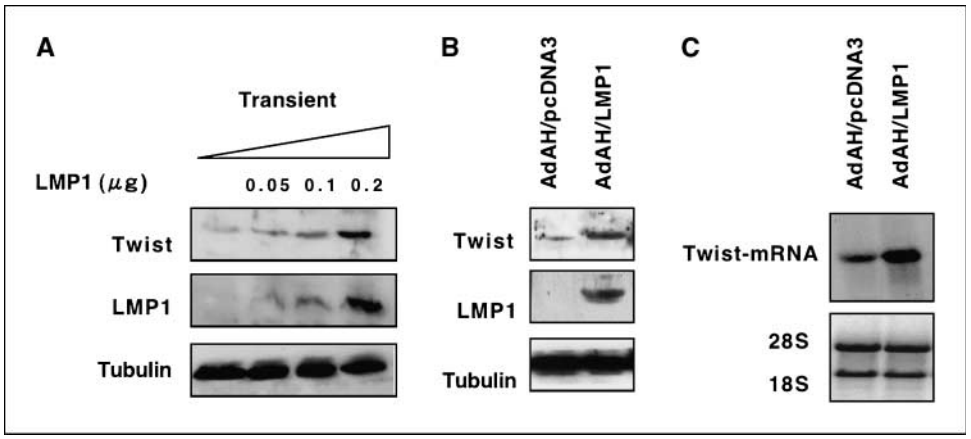

Figure 4. LMP1 induces expression of Twist protein and mRNA in nasopharyngeal epithelial cells. *A,* induction of Twist by LMP1 is dose dependent in Ad-AH cells, an EBV-negative nasopharyngeal epithelial cell line, detected by Western blotting. Tubulin is loading control. *B,* expression of Twist is increased in Ad-AH cells stably expressing LMP1. *C,* LMP1 up-regulates Twist mRNA levels. Extracts from the same cells described in Fig. 5*B* were analyzed by Northern blotting.

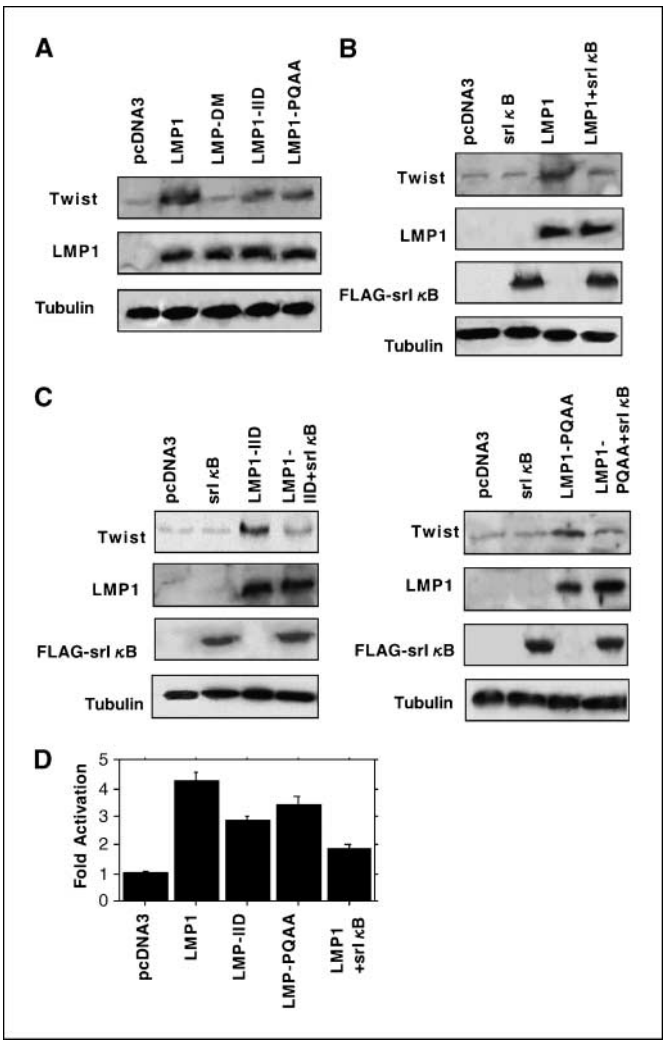

Figure 5. Both LMP1 COOH-terminal signaling domains induce Twist via NF-κB. *A,* both CTAR1 and CTAR2 of LMP1 partially induce Twist in Ad-AH nasopharyngeal epithelial cells. LMP-IID has point mutations in the CTAR2 YYD motif; LMP-PQAA has point mutations in the CTAR1 PXQXT motif; and LMP-DM has point mutations in both CTAR1 and CTAR2. *B,* NF-κB is involved in induction of Twist by LMP1. srIκB (S32A/S36A) suppressed induction of Twist by LMP1. *C,* LMP1 CTAR1 and CTAR2 partially induce Twist through NF-κB. Twist levels were analyzed after coexpression of srIκB (S32A/S36A) with LMP-IID or LMP-PQAA in Ad-AH cells. *D,* LMP1 induces Twist promoter activity. CTAR2 mutant LMP-IID and CTAR1 mutant LMP-PQAA both partially induced Twist promoter activity, and the activity was repressed by the coexpression of srIκB (S32A/S36A). Relative luciferase activity from two independent experiments prepared in triplicate.

S36A) repressed endogenous and LMP1-activated NF-κB transcriptional activity (data not shown; ref. 43). As shown in Fig. 5*B* and *C*, srIκB suppressed induction of Twist protein by LMP1 as well as by either LMP1-IID or LMP1-PQAA. Furthermore, LMP1 induced Twist promoter activity by both LMP-IID and LMP-PQAA in luciferase reporter assays (Fig. 5*D*). Coexpression of srIκB (S32A/S36A) repressed the induced Twist activity (Fig. 5*D*). These results show that both LMP1 CTAR1 and CTAR2 induce Twist through NF-κB in nasopharyngeal epithelial cells.

Expression of Twist is associated with LMP1-positive NPC. The studies in cell culture models suggested that induction of Twist by LMP1 contributes to tumor cell invasion and metastasis. Therefore, finally, we examined whether there is any correlation between expression of Twist and LMP1 in NPC. Ten tissue samples of NPC selected at random from Hospital Saint-Louis, Paris, France, were analyzed by Western blotting for endogenous levels of Twist and LMP1. As shown in Fig. 6*A*, six of seven LMP1-positive NPCs clearly expressed Twist. On the other hand, in only one of three LMP1-negative NPCs was Twist weakly detected. Further, we analyzed Twist and LMP1 expression by immunohistochemistry in 37 human NPC tissues from Fukui Prefectural Hospital. In normal epithelium of nasopharynx, Twist protein was not detected. In contrast, Twist protein was clearly overexpressed in NPC tumor nests. Twist protein was detected mainly in the nuclei (Fig. 6*B*). LMP1 protein was detected in membranes and cytoplasm of the tumor cells, consistent with previous reports (Fig. 6*B*; refs. 16, 17). The expression of LMP1 and Twist was usually high or low in parallel in the same case. Case I shows a representative result in which both LMP1 and Twist are highly expressed. By way of contrast, case II is representative of results when LMP1 and Twist were not expressed. The distribution of all 37 cases according to LMP1 and Twist levels is graphed in Fig. 6*C*. Expression of Twist showed a linear dependence on the expression of LMP1 ($r = 0.779$; $P < 0.0001$; Fig. 6*C*). These results combined with those in the cell culture systems suggest that LMP1 is a major inducing signal for Twist in NPC.

Twist levels in NPC tissues correlate directly with metastasis. Clinically, regional lymph node metastasis is the most frequent finding in NPC, reflecting the highly metastatic character of the malignancy (1). We therefore analyzed the association of expression scores of Twist with metastasis. In 26 cases with lymph node metastasis, the mean Twist expression score was 49.9 ± 27.9 (mean ± SD). In contrast, in 11 cases without metastasis, the score was 11.1 ± 16.0. Thus, the expression of Twist in metastasis-positive cases is significantly higher than in metastasis-negative cases ($P = 0.0001$; Fig. 6*D*).

Discussion

Although levels of expression of the transcriptional factor Twist correlate directly and strikingly with degree of metastasis in a mouse model of breast cancer (34) and in several human carcinomas (37, 38, 48), the induction signal for Twist in such tumors is largely unknown. Moreover, whether, and if so how, a human tumor viral oncogene could induce expression of this newly implicated metastasis factor has not yet been examined. The major findings reported here are first and foremost that Twist is up-regulated and associated with the principal EBV oncogene LMP1 in one of the most invasive EBV-associated malignancies, NPC. Indeed, NPC stands out among head and neck tumors in invasive and metastatic propensity (1). Moreover, the viral oncoprotein LMP1 can clearly induce the pleotropic alterations that comprise EMT, via Twist, and Twist siRNA can reverse such changes both at molecular and morphologic levels. The significance of these results is strikingly underscored by the correlation between expression of Twist and metastasis in NPC itself.

EMT has recently emerged as a key step in cancer metastasis and progression (28), and its significance was expanded by the addition of evidence of the key role of Twist in EMT induction and metastasis (30, 34). However, how EMT is induced during cancer progression is not well understood. Besides transforming growth factor β and receptor tyrosine kinase/Ras signaling, autocrine factors and Wnt-, Notch-, and NF-κB–dependent pathways are reported to contribute to EMT (29). Transcriptional

regulators such as Snail and Twist are newly implicated in the mechanism of EMT mainly through the repression of E-cadherin (28). We previously studied EMT in a breast cancer model. Here, we focus on the association of the EBV oncogene LMP1 with EMT, because NPC has distinctive EBV-related undifferentiated histopathology and highly metastatic character. Indeed, EBV LMP1 clearly caused morphologic and molecular changes of EMT in cultured epithelial cells and this EMT was reversed by suppressing Twist. The importance of Twist in metastasis is underscored by the higher levels of the protein detected in NPC tissues from patients with metastasis. This report is the first to show the induction of EMT by a viral oncogene and clarifies the vital role of Twist in this context. These results not only are important in explaining the metastatic character of EBV-related malignancies such as NPC, but also provide new insight for the pathogenesis of EMT in human carcinomas. Monoclonal EBV episomes have been detected in all hyperplastic or preneoplastic nasopharyngeal cells, and LMP1 protein was expressed in 100% of preinvasive NPC lesions (10). These findings might suggest that EMT is also induced early in the onset of this malignancy and may culminate in the distinctive histopathology and early dissemination that characterize NPC. Although EMT as a major metastasis-promoting mechanism in human carcinomas is a new concept and not fully established (50), investigation of evidence for EMT in NPC, especially with its association with EBV, might give further clues to the mechanism of EMT in human carcinomas.

The endogenous level of Twist protein corresponded with that of LMP1 in two distinct types of EBV-positive cell lines. Also, expression of dominant-negative LMP1 could reduce Twist levels in EBV-positive epithelial cells. Further, LMP1 induced expression of Twist protein in nasopharyngeal epithelial cells. The association between expression of Twist and LMP1 in NPC tissues further supports the existence of such an induction mechanism. LMP1 increased the mRNA level and the promoter activity of Twist, and Twist protein seemed not to be stabilized by LMP1 in studies with the potent proteasomal inhibitor MG132 (last data not shown). Thus, we conclude that LMP1 increases the level of Twist protein by induction of its transcription. These effects on Twist are not only important in themselves, but they also provide a reliable means of inducing Twist. LMP1 has an activated TNFR-mimicking function and thus constitutively activates NF-κB (7, 50). The proximal signaling domain of LMP1, CTAR1, interacts with TNFR-associated factors and activates NF-κB (51). The distal domain, CTAR2, interacts with TNFR-associated death domain and also

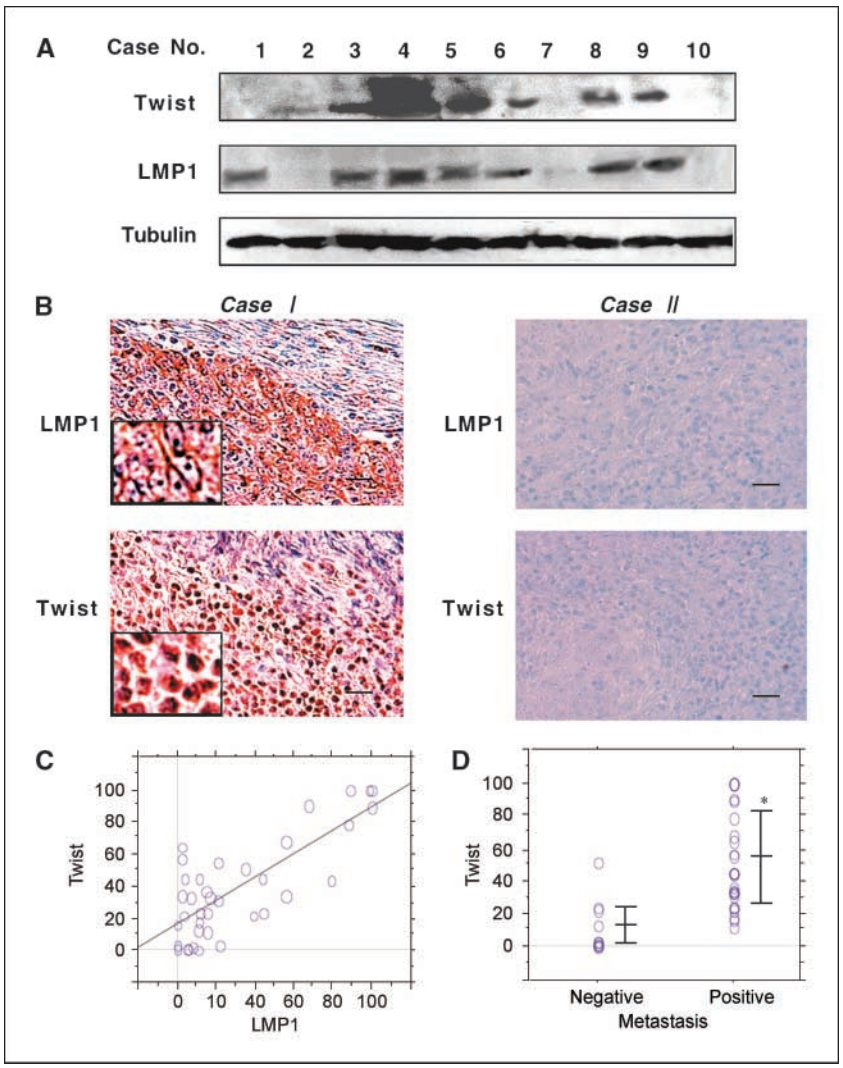

Figure 6. Levels of LMP1 correlate directly with Twist, and expression of Twist correlates directly with metastasis in NPC. *A,* levels of LMP1 and Twist proteins in NPC tissues. Ten randomly selected NPC tumor specimens were analyzed for LMP1 and Twist by Western blotting. Tubulin is used as loading control. *B,* expression of LMP1 and Twist in NPC tissues, detected by immunohistochemistry. LMP1 protein is visible at cell membrane and in cytoplasm of nests of tumor cells. Twist protein is localized mainly in nuclei of tumor cells. Case I, representative result showing high LMP1 and Twist expression. Case II, representative result with no expression of LMP1 and Twist. *Bar*, 50 μm. *C,* expression scores for LMP1 and Twist in all 37 NPC specimens. Average percentages of tumor cells staining for LMP1 and Twist are plotted (see Materials and Methods). Expression scores for LMP1 and Twist correlated significantly: Pearson correlation coefficient, $r = 0.779$; $P < 0.0001$. *D,* expression scores for Twist correlate with cervical lymph node metastasis. Twist score in metastasis-positive category was 49.9 ± 27.9; score in negative category was 11.1 ± 16.0. *, $P = 0.0001$, significance by Mann-Whitney *U* test. *Columns,* mean; *bars,* SD.

mediates NF-κB signaling (52). CTAR2 also induces the activity of activator protein-1 (AP-1) transcriptional factor via a signaling pathway that involves c-jun NH_2-terminal kinase 1 (53). Further, LMP1 activates the p38–mitogen-activated protein kinase pathway both through the CTAR1 and CTAR2 domains (54). Taking advantage of this established tumor virus and the many known intracellular signaling pathways of its principal oncoprotein, we have clarified the molecular mechanisms of induction of a constellation of metastasis-related factors by LMP1, including matrix metalloproteinase-9 (MMP-9) via NF-κB and AP-1 (16, 23, 26), c-Met proto-oncogene via ets-1 (17), MMP-1 via ets-1(19), and several angiogenic factors such as interleukin-8 via NF-κB (27), vascular endothelial growth factor via cyclooxygenase-2 (22), and hypoxia-inducible factor 1α by stabilizing Siah1 E3 ubiquitin ligase (20). Finally, how dormant Twist can be reactivated in cancer progression has been a key question to be answered and has remained quite obscure. In *Drosophila* embryos, Twist is induced by an interleukin-1–like TOLL receptor through NF-κB activation during mesoderm formation (55). BMP, Wnt, and fibroblast growth factor pathways are known to modulate vertebrate neural crest development where expression of Twist is essential (56). Our current study clearly provides one answer to this question by demonstrating the induction of Twist by the EBV oncogene LMP1 via NF-κB, and provides immediate insights into how Twist can be activated. The findings also single out a potential new target, Twist, for molecularly directed therapy.

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