

Alterations of Biologic Properties and Gene Expression in Nasopharyngeal Epithelial Cells by the Epstein-Barr Virus–Encoded Latent Membrane Protein 1

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SUMMARY: Undifferentiated nasopharyngeal carcinoma (NPC) is closely associated with EBV infection, and the EBV-encoded latent membrane protein 1 (LMP1) is frequently detected in NPC. However, little is known about the pathologic roles of LMP1 in this disease. Recently, we reported the morphologic transformation and increased expression of the LAMC2 and ITG α 6 genes in LMP1-expressing NPC cell lines. In this study, we further examine the effects of LMP1 in an immortalized nasopharyngeal epithelial cell line called NP69. This cell line was established from primary nonmalignant nasopharyngeal epithelial cells and may represent a model of premalignant nasopharyngeal epithelial cells. LMP1 induced many phenotypic changes in NP69 cells. These include morphologic transformation, increased cell proliferation, anchorage-independent growth, resistance to serum free-induced cell death, and enhanced cell migration and invasion. In addition, expression array analysis identified 28 genes that demonstrated a more than 2-fold difference in expression of NP69 cells expressing LMP1 when compared with a vector control. Two of the up-regulated genes (*VEGF* and *vimentin*) identified have been previously reported as LMP1 targets. The majority of the identified genes are associated with cell growth, differentiation, cell shape, and invasion. The present findings support the proposed roles of LMP1 in promoting cell transformation, migration, and invasion in premalignant nasopharyngeal epithelial cells. The present study also indicates the activation of the Ras/MAPK pathway in LMP1-expressing cells, which may be involved in mediating some of the transforming effects of LMP1 observed in nasopharyngeal epithelial cells. (*Lab Invest* 2003, 83:697–709).

The poorly differentiated or undifferentiated nasopharyngeal carcinoma (NPC) is common in Southern China. This cancer is characterized by a high incidence of lymph node metastasis (Sham et al, 1990) and is closely associated with EBV infection (Rickinson and Kieff, 1996). A causal role of EBV in NPC has been suggested, and the EBV-encoded latent membrane protein 1 (LMP1) is believed to be important in mediating the transformation properties of EBV in NPC development. LMP1 is detected in about 65% of EBV-positive NPC (Fahraeus et al, 1988). A clinical study of NPC has shown that LMP1-positive tumors are more progressive than

LMP1-negative tumors in growth and prone to invade lymph nodes (Hu et al, 1995). In in vitro studies, LMP1 is essential for EBV-induced immortalization of B lymphocytes. LMP1 is also able to transform fibroblasts (Rickinson and Kieff, 1996) and induce various phenotypic changes in epithelial cells (Dawson et al, 1990; Rickinson and Kieff, 1996). LMP1 may also be involved in metastasis of tumor cells by induction of matrix metalloproteinase-9 (MMP-9) (Yoshizaki et al, 1998), Ets1 (Kim et al, 2000), and COX-2 (Murono et al, 2001) as well as reduction of E-cadherin expression (Fahraeus et al, 1992). However, few studies have been conducted to investigate the effects of LMP1 in nasopharyngeal epithelial cells, which are the natural host cells of EBV infection. In our previous study of LMP1 in NPC cells, we observed that LMP1 could induce morphologic transformation and increase expression of ITG α 6 and LAMC2, which may facilitate tumor metastasis (Lo et al, 2001a). To further determine the pathologic effect of LMP1 in NPC, a detailed study of the effects of LMP1 on the biologic properties and gene expression profile in an SV40T-immortalized nasopharyngeal epithelial cell line,

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NP69, established in our laboratory (Tsao et al, 2002) were investigated.

Results

LMP-Induced Morphologic Change in NP69 Cells

Expression of LMP1 in NP69 cells induced a morphologic change from a typical epithelial cobblestone to an elongated and fibroblastoid shape. The NP69-LMP1 cells also grew in a scattered pattern with a marked reduction in cell-cell contact (Fig. 1).

LMP1 Increased Cell Proliferation and Reduced Serum Dependence

In medium supplemented with growth factors, the NP69-LMP1 cells have a shorter doubling time (66 hours) compared with control NP69-pLNSX cells (87 hours doubling time) (Fig. 2A). Cell cycle analysis also indicated that the proportion of S phase cells in NP69-LMP1 (34%) was higher than that in the NP69-pLNSX control (24%) (Fig. 2, C and D). When growth factors were withdrawn from the medium, NP69-LMP1 cells were more resistant to cell death and their proliferation could still be detected at Day 7, with an average doubling time of 123 hours (Fig. 2B). In contrast, the control NP69-pLNSX cells underwent

growth arrest by Day 7, with a marked decrease in cell numbers (Fig. 2B). Also, after removal of growth factors for 36 hours, a marked reduction in the percentage of S phase cells in control NP69-pLNSX (16%) (Fig. 2E) compared with NP69-LMP1 cells (48%) was detected (Fig. 2F). Both NP69-LMP1 and NP69-pLNSX cells eventually underwent cell death after prolonged growth in medium without growth factors.

LMP1 Enhanced Anchorage-Independent Growth

In soft agar assay (Fig. 3), the NP69-LMP1 cells were able to undergo anchorage-independent growth by forming 42 colonies, of an average 5×10^4 cells (Table 1). In contrast, the control NP69-pLNSX cells failed to form any colonies in three independent experiments (Table 1).

LMP1-Induced Cell Migration and Invasion

In a scrape-wound migration assay, the NP69-LMP1 cells migrated to the wound surface on the plastic dish after 10 hours, forming an uneven growth edge (Fig. 4, C and D). By contrast, the control NP69-pLNSX cells demonstrated slower migration ability and the wound edge remained smooth (Fig. 4, A and B). In a collagen gel invasion assay, after growing the cells for 8 days, the control NP69-pLNSX cells formed spheroid shapes inside the collagen gel matrix (Fig. 4E), which is typical of noninvasive cells. By contrast, the NP69-LMP1 cells formed branching tubules through the collagen gel (Fig. 4F), which is commonly observed in cells with high invasive properties. In a Boyden chamber invasion assay (Fig. 5A), the number of NP69-LMP1 cells passing through the matrigel coated membrane was about 5-fold more than that of control cells (Fig. 5B) after 24 hours of incubation.

Determination of Gene Expression Profiles of LMP1-Expressing NP69 Cells by cDNA Array

The gene expression profile of NP69-LMP1 cells was examined by Atlas Human Cancer 1.2 arrays (Clontech) and was compared with that of the control NP69-pLNSX cells at similar passage numbers. A total of 28 genes showed significant change of expression level in NP69-LMP1 cells (>2-fold difference). Seventeen genes were overexpressed, whereas 11 were suppressed (Tables 2 and 3).

Confirmation of Differentially Expressed Genes Identified in LMP1-Expressing Cells

By quantitative real-time RT-PCR, the up-regulation of *VEGF*, *ET2*, *RAD*, *RhoGDI-2*, *MT3-MMP*, *FN1*, *Axl* and its ligand, *Gas6*, as well as the down-regulation of *K14*, *K19*, *Notch1*, and *JAG2* was confirmed in NP69-LMP1 cells (Fig. 6). Overexpression and down-regulated expression of these genes were also observed in at least two of the four NPC cell lines (CNE1, CNE2, SUNE1, and HK1) expressing LMP1 (Fig. 6).

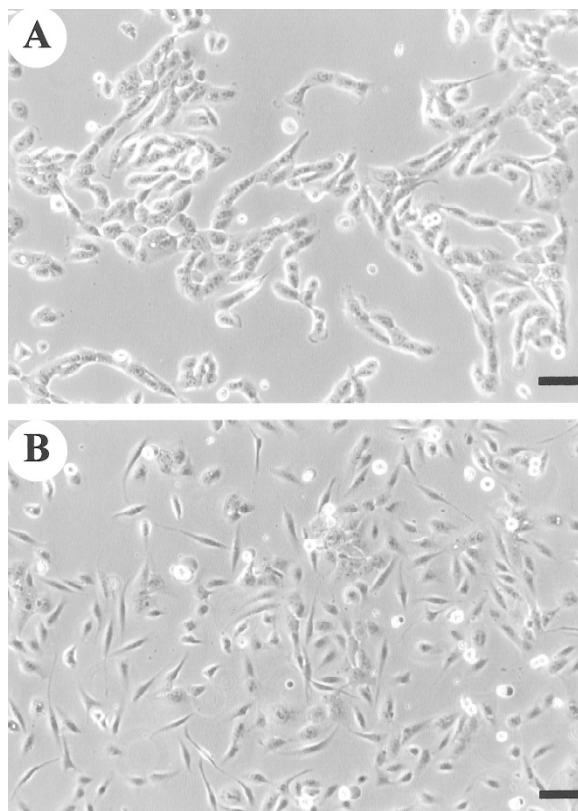


Figure 1.

Morphologies of NP69-immortalized nasopharyngeal epithelial cells. A, Vector control NP69-pLNSX cells showed epithelial cobblestone morphology. B, Latent membrane protein 1 (LMP1)-expressing NP69 cells (NP69-LMP1) exhibited elongated and fibroblast-like shape. All panels are of the same magnification; scale bar, 10 μ m.

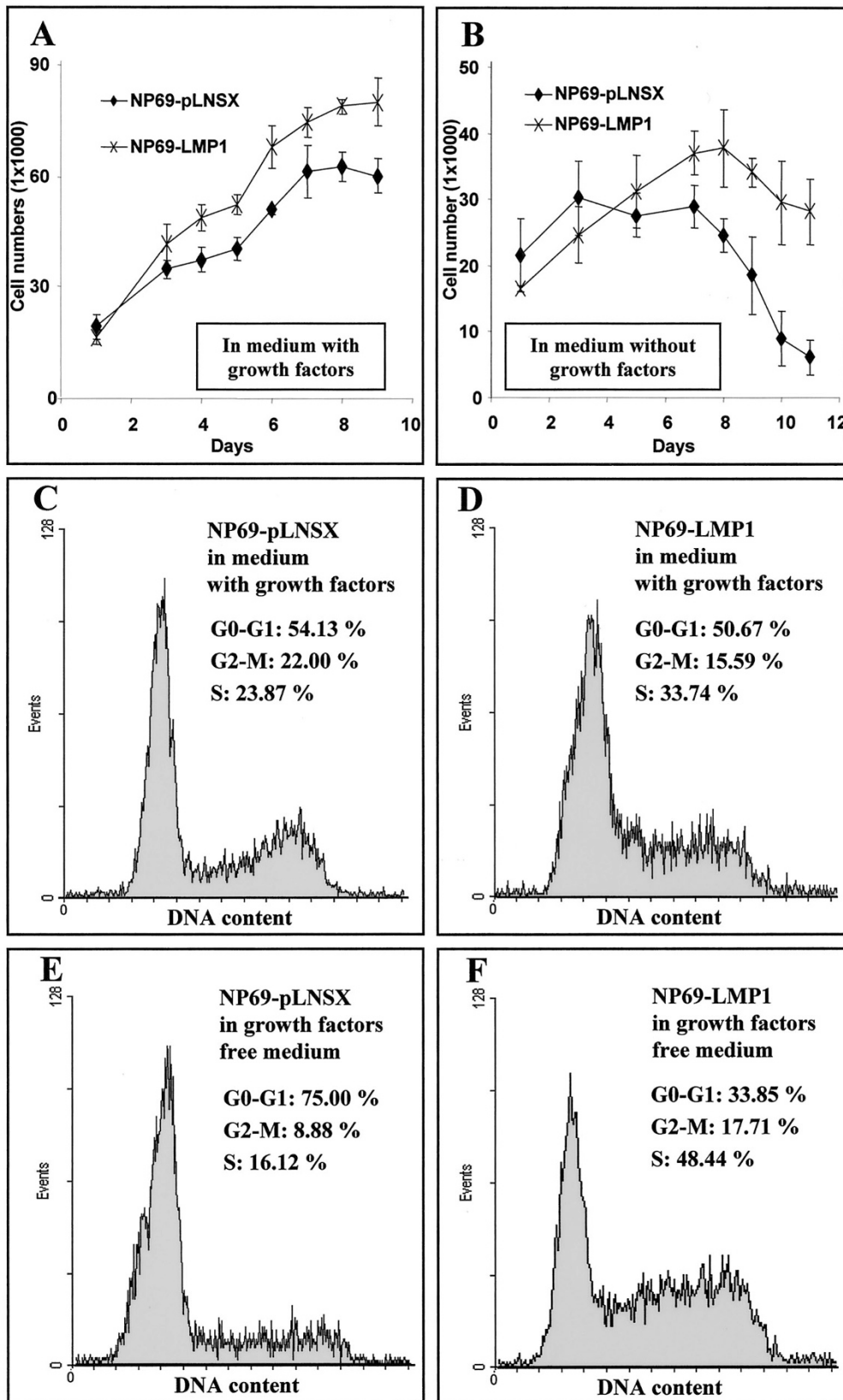


Figure 2.

Effect of LMP1 on the growth curve of NP69-immortalized nasopharyngeal epithelial cells. A, Control NP69-pLNSX cells and LMP1-NP69 cells were cultured in medium supplemented with growth factors. B, Control NP69-pLNSX cells and LMP1-NP69 cells were cultured in medium without growth factors. Effect of LMP1 on the cell cycle of NP69 cells. C and D, Control NP69-pLNSX cells and LMP1-NP69 cells were cultured in medium with growth factors for 36 hours. E and F, Control NP69-pLNSX cells and LMP1-NP69 cells were cultured in growth factor-free medium for 36 hours.

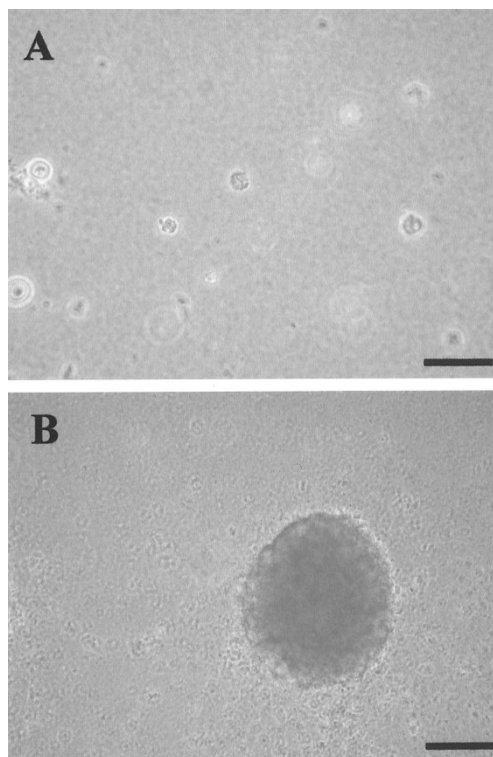


Figure 3. Anchorage-independent growth. A, Vector control NP69-pLNSX cells failed to form soft agar clones after 3 weeks of incubation. B, LMP1-expressing NP69 (NP69-LMP1) cells formed clones in soft agar. All panels are of the same magnification; scale bar, 10 mm.

Table 1. Anchorage-Independent Growth of the Immortalized Nasopharyngeal Epithelial Cell Line, NP69, Expressing LMP1

Cell line	Well	Cloning efficiency in soft agar ^a
NP69-pLNSX	All 6 wells examined ^b	0
NP69-LMP1	1-1	43
	1-2	55
	2-1	30
	2-2	36
	3-1	52
	3-2	34
	Mean	41.67
	SD	10.13

^a Three independent experiments were performed, and each cell line was plated in duplicate. In each well, 50,000 cells were plated. The numbers represent the number of clones in each well.

^b No clone was found in three independent experiments in which duplicate wells were counted.

Western Blot Analysis of LMP1-Expressing Cells

By Western blotting (Fig. 7), stable expression of the LMP1 protein in all LMP1-infected cell lines was confirmed. In addition, protein expression of vimentin and VEGF was increased in LMP1-expressing NP69 and in at least two of four NPC cell lines expressing LMP1 when compared with their control counterparts. Protein expression of E-cadherin was decreased in

LMP1-expressing NP69, CNE1 and CNE2 cell lines. Furthermore, using antibodies specific to phosphorylated kinases of the Raf/MAPK signal pathway, Western blot analysis indicated the activation of Raf, MEK1/2, and Erk1/2 kinases in LMP1-expressing NP69 and two NPC cell lines (CNE1 and SUNE1) expressing LMP1 (Fig. 8).

Discussion

This is the first article that uses an immortalized nasopharyngeal epithelial cell line (NP69) system to study the pathologic role of LMP1 in NPC development. The immortalized NP69 cells were established from primary culture of nonmalignant nasopharyngeal epithelial cells that had not been infected and modified by EBV. They are nontumorigenic in nude mice and do not exhibit anchorage-independent growth in soft agar (Tsao et al, 2002). Thus, they provide a good model for examining the biologic properties of EBV genes including LMP1. In this study, we demonstrated that LMP1 could induce many phenotypic changes in NP69 cells. By cDNA array analysis, the gene expression profile of NP69 cells expressing LMP1 was determined (Table 2 and 3). To reduce the chances of false-positive results, only those genes that demonstrated at least a 2-fold difference in expression between NP69 cells expressing LMP1 and their empty vector control cells were considered. Consistent with previous studies of other workers, two of the up-regulated genes (*VEGF* and *vimentin*) identified here (Table 2) have been demonstrated to be induced by LMP1 (Birkenbach et al, 1989; Murono et al, 2001). The identification of two known LMP1 target genes provided some validation of our experimental approach. Interestingly, the majority of the differentially expressed genes identified correlated with the biologic alterations observed in NP69-LMP1 cells. Our present findings support a major role of LMP1 in enhancing survival and malignant properties in premalignant nasopharyngeal epithelial cells. The significance of these differentially expressed genes, their relationships with the biologic changes in NP69-LMP1 cells, and the possible involvement of these genes in the pathogenesis of NPC are discussed below.

Cell Growth and Survival

In this study, LMP1 expression in NP69 cells induced cell proliferation and enhanced resistance to serum free-induced apoptosis (Fig. 2). These observations are in line with previous reports showing that LMP1 reduced serum dependence in human keratinocytes (Hu et al, 1993) and Jurkat T cells (Kawanishi, 1997), although the mechanisms involved are not clear. Here, both *Axl* and *Gas6* were demonstrated to be up-regulated by LMP1 (Fig. 6). *Axl* is a receptor tyrosine kinase that can induce cellular proliferation and transformation after binding with its ligand, *Gas6* (Mark et al, 1996). The increased expression of *Axl* and *Gas6* may contribute to the higher proliferation rate and enhanced survival ability upon withdrawal of growth

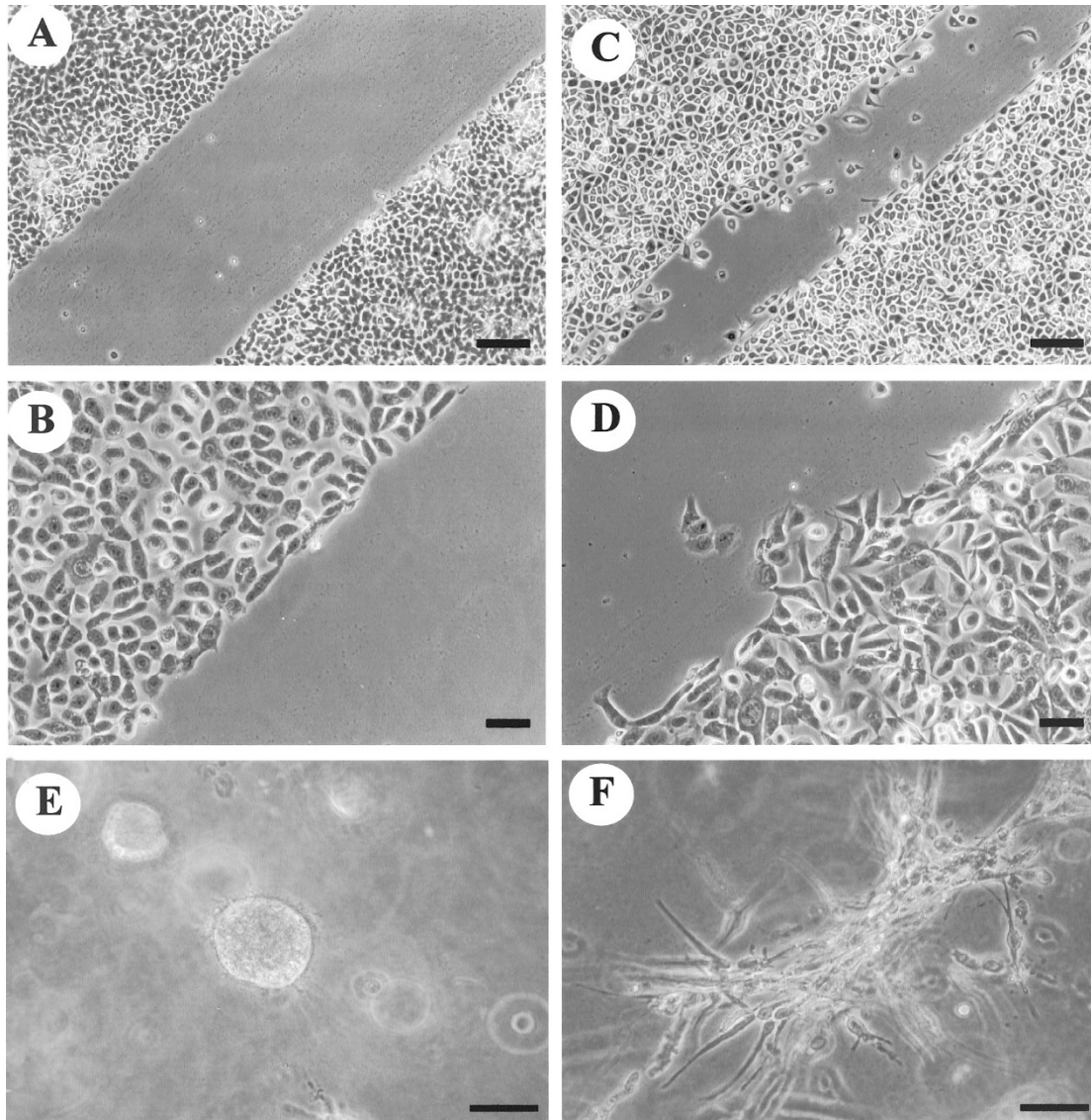


Figure 4.

Motility analysis in scrape-wound migration assay. Confluent monolayers of control NP69-pLNSX cells and NP69-LMP1 cells were scraped by a plastic pipette. After 8 hours of incubation in serum-free medium (A and B), the NP69-pLNSX cells formed smooth wound edges. C and D, NP69-LMP1 cells migrated to form uneven growth edges. Scale bar: A and C, 20 mm; B and D, 10 mm. Invasion analysis in three-dimensional collagen gel. NP69-pLNSX and NP69-LMP1 cells were cultured in a three-dimensional collagen gel matrix for 8 days. E, Control NP69-pLNSX cells formed spheroid shapes. F, NP69-LMP1 cells formed branching tubules through the collagen gel. Scale bar, 10 mm in magnification.

factors observed in NP69-LMP1 cells. Further study is warranted to confirm the involvement of this pathway in cell growth and survival of LMP1-expressing nasopharyngeal epithelial cells.

Epithelial Mesenchymal Transformation

The effect of LMP1 in morphologic alterations has been noted previously in human epithelial cells (Fahreus et al, 1990). Our present data also indicated a spindle shape and scattered growth pattern in LMP1-expressing NP69 cells (Fig. 1). The mechanism involved in this morphologic change induced by LMP1 is poorly understood. Interestingly, we observed altered gene expression of some cytoskeletal proteins and their associated proteins in NP69-LMP1 cells. These

include down-regulation of *keratin 14 (K14)*, *keratin 19 (K19)*, *desmoplakin I and II* and *desmocollin 3A/3B* as well as up-regulation of *vimentin* (Tables 2 and 3). Cytokeratins are the common intermediate filaments expressed in epithelial cells, including nasopharyngeal epithelium. Their association with cell adhesion structures such as desmoplakin and desmocollin are involved in maintaining cell shape and adhesion in epithelial cells. Down-regulation of these cytoskeleton-associated proteins may contribute to the altered morphology observed in LMP1-expressing NP69 cells. In contrast, vimentin, which is predominantly a mesenchymal skeletal protein commonly expressed in fibroblasts, was up-regulated in NP69-LMP1 cells (Fig. 7). In this study, the up-regulation of

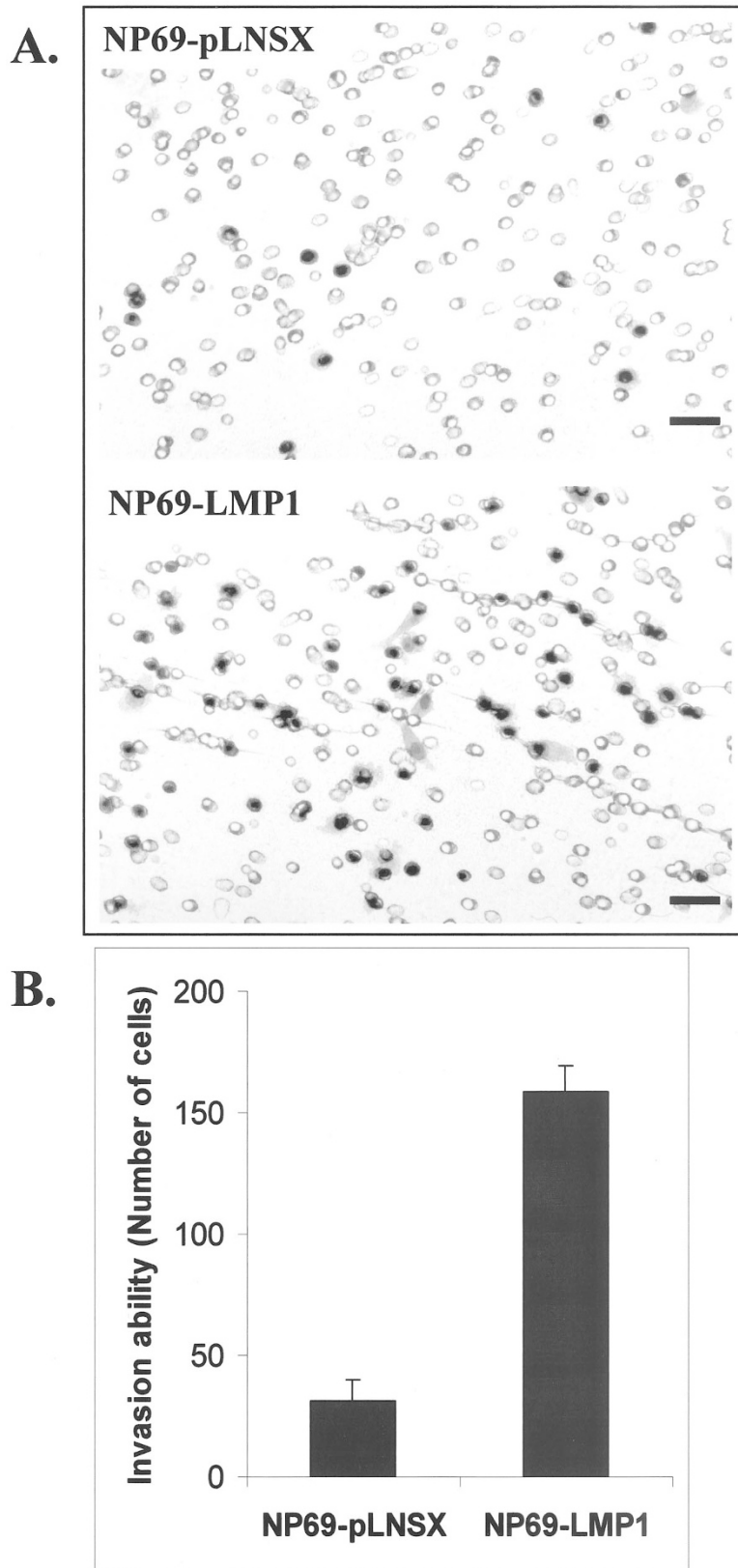


Figure 5.

Invasion analysis in the matrigel gel Boyden chamber assay. A, Lower surfaces of the membrane to which NP69-pLNSX (upper panel) and NP69-LMP1 (lower panel) cells traversed. Scale bar, 30 μ m. B, Invasion ability was measured by counting the number of cells that traversed the Matrigel coated membrane in 24 hours.

Table 2. Genes Up-Regulated in LMP1-Expressing NP69 Cells Detected by cDNA Array

Gene descriptions	Ratio (L/C) ^a	Putative functions
rho GDP dissociation inhibitor 2 (RhoGD12)	9.91	Commonly expressed in hematopoietic cells. Member of Rho GDI family, maybe for GTPase activity modulator.
Fibronectin 1 (FN1)	9.74	Extracellular matrix protein, for promoting cell adhesion and morphology.
Tyrosine-protein kinase receptor (AXL)	5.21	Oncogene, for regulating cell proliferation and transformation.
Membrane-type MMP-3 (MT-MMP-3 or MMP-16)	5.16	Metalloproteinase, for degrading extracellular matrix.
Vitronectin precursor (VTN)	4.91	Intermediate filament protein, for cell attachment and spreading.
Endothelin 2 (ET2)	4.75	Cytokine, function as vasoconstricting or anti-inflammatory agent.
Vascular endothelial growth factor (VEGF)	4.56	Cytokine, angiogenic factor for inducing cell proliferation and migration.
Neutrophil gelatinase-associated lipocalin (NGAL)	4.36	Associated with neutrophil gelatinase, functions as a modulator of inflammation.
Vimentin (VIM)	4.12	Intermediate filament protein, for cytoskeleton framework of the cells.
GTP-binding protein ras associated with diabetes (RAD)	4.10	Ras-related GTPase, maybe for regulating cytoskeleton.
SL cytokine precursor; FLT3 ligand (FLT3LG)	3.97	Ligand of FLT3 receptor, for stimulating proliferation of hematopoietic progenitor cells.
Transforming growth factor- β 2 (TGF- β 2)	3.08	Natural and potent growth inhibitors in normal cells but stimulate the invasiveness of tumor cells.
Thrombospondin 2 (THBS2)	2.65	Matrix adhesion receptors, for cell adhesion and migration.
Leukocyte interferon-inducible peptide	2.60	Extracellular matrix protein, for inflammation.
Transforming growth factor- β (TGF- β)	2.58	Natural and potent growth inhibitors in normal cells but stimulate the invasiveness of tumor cells.
NDR protein kinase	2.29	Nuclear kinase, for regulating growth and morphology.
High mobility group protein (HMG-I)	2.12	Chromosomal protein, for regulating transcription, but also secreted for inflammation and tumor metastasis.

^a Expression ratio: expression intensity in LMP1-NP69 cells/expression intensity in pLNSX-NP69 cells.

vimentin and the down-regulation of K14 and K19 in LMP1-expressing nasopharyngeal epithelial cells suggest that LMP1 facilitates the switch of intermediate filament expression from cytokeratins to vimentin, an epithelial-mesenchymal transformation (EMT) process commonly observed in tumor invasion and metastasis (Portella et al, 1998). The underlying mechanism of this mesenchymal transformation induced by LMP1 is unknown. Some studies have indicated that transforming growth factor- β (TGF- β) is involved in the EMT process in normal mammary epithelial cells, which is accompanied by reorganization of cytoskeleton and decreased expression of E-cadherin and cytokeratins but increased expression of vimentin (Miettinen et al, 1994). TGF β and TGF β 2 were also up-regulated by LMP1 in this study (Table 2). Together with findings by Western blotting that E-cadherin was reduced in LMP1-expressing cells (Fig. 7), the EMT change mediated by LMP1 expression may facilitate invasion in premalignant nasopharyngeal epithelial cells.

Cell Migration and Invasion

The invasive property of NP69-LMP1 cells was indicated by their high rate of invasion and migration (Figs. 4 and 5). In particular, the invasion process involves degradation of the extracellular matrix in which proteinase must be produced. Increased expression of MMP-9 proteinase by LMP1 has been reported previously (Yoshizaki et al, 1998) and has been indicated in LMP1-positive NPC tissues (Horikawa et al, 2000). Additionally, the increased expression of MT3-MMP in LMP1-expressing nasopharyngeal epithelial cells was observed in the present study (Fig. 6). The MT3-MMP is a membrane-bound matrix metalloproteinase and is capable of degrading extracellular matrix (Takino et al, 1995). It also possesses the potential to mediate the growth and invasion behavior of cancer cells in vivo (Kitagawa et al, 1999). Kang et al (2000) have reported that overexpression of MT3-MMP enhances the invasive growth of MDCK cells in colla-

Table 3. Genes Down-Regulated in LMP1-Expressing NP69 Cells Detected by cDNA Array

Gene descriptions	Ratio (C/L) ^a	Putative functions
Cytokeratin 14 (K14)	12.49	Intermediate filament protein, for cytoskeleton framework of the cells.
Jagged2 (JAG2)	10.70	Ligand of NOTCH, for regulating cell fate determination and differentiation.
Cytokeratin 19 (K19)	8.88	Intermediate filament protein, for cytoskeleton framework of the cells.
Notch protein homolog 1 (NOTCH1)	6.50	Transmembrane receptor, regulating cell fate determination and differentiation.
Tissue inhibitor of metalloproteinase 3 (TIMP3)	5.38	MMP inhibitor, binding to the active sites of MMPs for MMPs inhibition.
Hepatoma-derived growth factor (HDGF)	5.20	Growth factors, cytokines and chemokines.
Desmocollin 3A/3B (DSC3) + desmocollin 4 (DSC4)	4.99	Desmosomal molecule, facilitating the attachment of juxtaposed cells.
Desmoplakin I and II (DPI and DPII)	4.57	Desmosomal plaque molecule, for linking keratin to the cytoskeleton.
BIGH3	4.02	Microfilament protein.
CD9 antigen or leukocyte antigen MIC3	3.80	Extracellular protein, involved in cell adhesion, motility and signaling.
Growth arrest and DNA-damage-inducible protein (GADD45)	3.58	Apoptosis-associated protein, for stimulating DNA excision repair and inhibiting S phase entry.

^a Expression ratio: expression intensity in pLNSX-NP69 cells/expression intensity in LMP1-NP69 cells.

gen gels. MT3-MMP may be another target of LMP1 in enhancing the invasive property of NPC cells.

Cell Differentiation

We report here for the first time, the down-regulation of Jagged 2 and Notch1 in LMP1-expressing nasopharyngeal epithelial cells (Fig. 6). Jagged family proteins are ligands of Notch receptors. Jagged/Notch signaling is important in regulating the balance between cell differentiation and stem cell proliferation (Artavanis-Tsakonas et al, 1999). Previous study has indicated that inhibiting mouse Notch1 expression prevents hormone-induced differentiation of 3T3-L1 cells (Ruiz-Hidalgo et al, 1999). Another study in transgenic mice has also shown that deletion of the Notch1 gene resulted in deregulated expression of multiple differentiation markers and marked epithelial hyperplasia, which are early steps of the carcinogenic process (Rangarajan et al, 2001). EBV infection is closely associated with the undifferentiated and poorly differentiated NPC. Inhibition of terminal differentiation of epithelial cells and reduction of keratin expression had been reported in human epithelial cell lines expressing LMP1 (Dawson et al, 1990; Fahraeus et al, 1990). Previous study in LMP1 transgenic mice has also shown that LMP1 could induce hyperplasia in epidermis (Wilson et al, 1990). It would be interesting to determine whether Jagged/Notch is a target pathway disrupted by LMP1 to deregulate the normal differentiation process, which may promote tumor progression.

In line with the inhibition of differentiation by LMP1, reduced expression of keratins (K14 and K19) was observed in LMP1-expressing cells (Fig. 6). Keratins

are commonly used as differentiation markers and specific types of keratins are expressed in epithelial cells at different stages of differentiation. Alteration of keratin expression is common in neoplasms because of a deranged differentiation program (van der Velden et al, 1993). A previous report has shown the absence of K5/K14 antibody staining in preinvasive and most invasive breast carcinomas (Jarasch et al, 1988). Loss of K19 had also been reported in dedifferentiated MDCK-C7 cells (Pollack et al, 1997). In addition, weak expression of K19 was found in poorly differentiated carcinoma of the stomach (Stammberger and Baczako, 1999). In this study, reduction of *K14* and *K19* as well as *JAG2* and *Notch1* in LMP1-expressing cells implies a close association of LMP1 with the undifferentiated phenotype of nasopharyngeal epithelial cells. It remains to be determined whether LMP1 directly induced the undifferentiated phenotype of nasopharyngeal epithelial cells or the association is a result of clonal expansion of undifferentiated clones expressing LMP1.

Activation of Ras/MAPK Signal Activation

The identification of *VEGF*, *fibronectin*, and *Gas6* as LMP1 up-regulated genes (Fig. 6) was of particular interest. Expression of these three genes has been indicated in Ras/MAPK signal activation (Danen and Yamada, 2001; Goruppi et al, 1999; Yu and Sato, 1999), suggesting that LMP1 is able to activate the Ras/MAPK signal pathway. In an earlier study, LMP1 was proposed to be functionally similar to activated Ras in cellular transformation (Dawson et al, 1990). Activation of the Ras/MAPK pathway by LMP1 has also been reported to be essential for cellular trans-

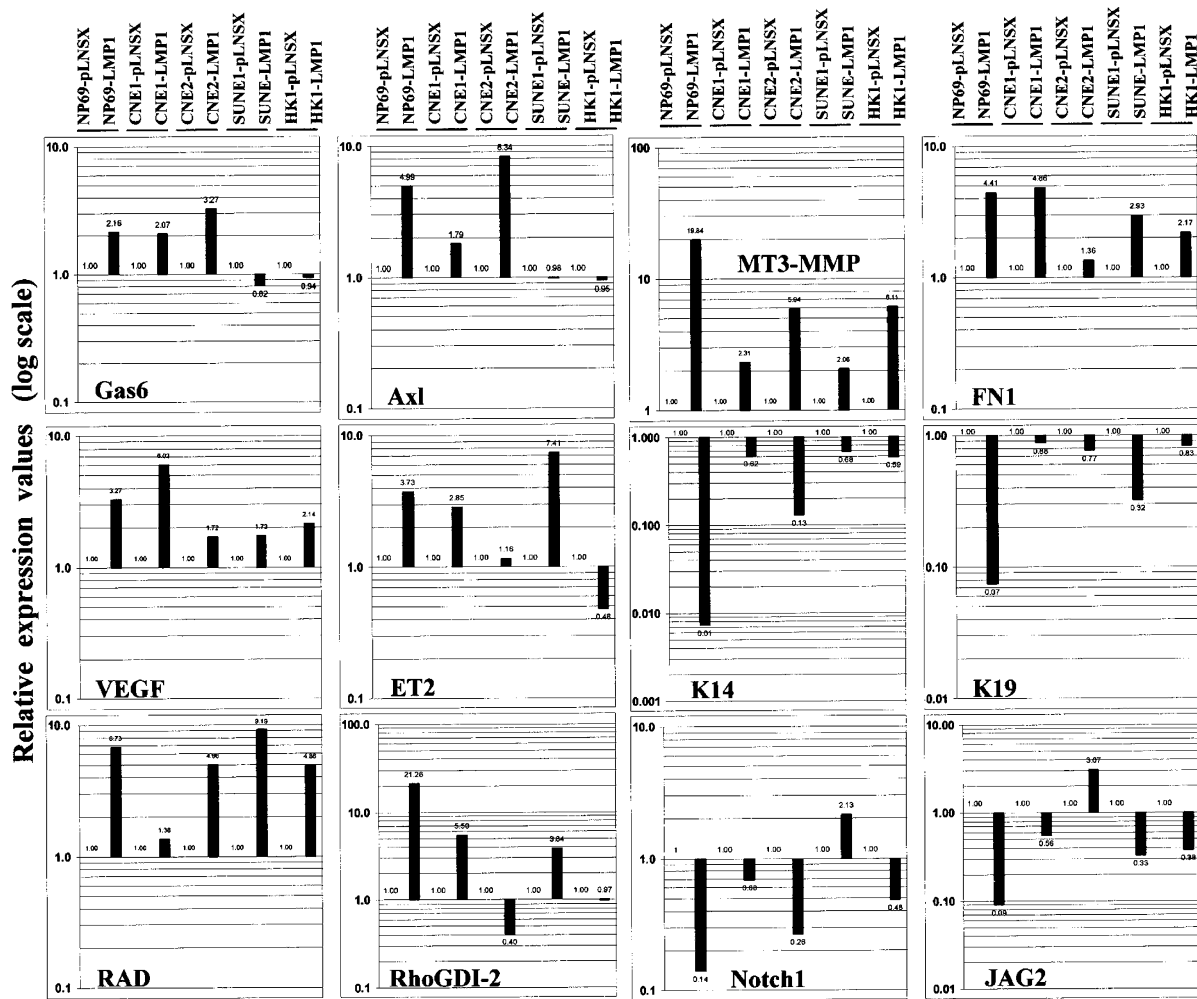


Figure 6.

Quantitative real time RT-PCR analysis. PCR products were detected by measuring the increase in fluorescence caused by the binding of SYBR Green to double-strand DNA PCR products. Detection was performed at the extension step of each amplification cycle. Each PCR reaction was optimized to ensure that a single band was amplified and that no bands corresponding to primer-dimers were present.

formation of rodent fibroblasts (Roberts and Cooper, 1998). In the present study, the Ras/MAPK signal kinases—Raf, MEK and Erk—were activated in LMP1-expressing nasopharyngeal epithelial cells (Fig. 8). The activation of the Ras/MAPK signal pathway by LMP1 may mediate some of the transformation properties of NP69-LMP1 cells, notably the anchorage-independent growth and enhanced proliferation.

Interestingly, the cDNA array hybridization used in our system was able to detect differential expression of some but not all of the other genes previously reported to be up-regulated by LMP1. For example, up-regulation of VEGF and vimentin expression by LMP1, which have been previously reported (Birkenbach et al, 1989; Murono et al, 2001), were detected in NP69-LMP1 cells by the cDNA array but not epithelial growth factor receptor, MMP-9, and IL-8 (Eliopoulos et al, 1999; Fahraeus et al, 1992; Miller et al, 1998). One explanation is that SV40T in the immortalized nasopharyngeal epithelial cells may also affect the expression levels of

these genes and mask the effect of LMP1 on their expression. However, using RT-PCR, we were able to confirm the up-regulated expression of these genes in NP69-LMP1 (data not shown). Similarly, down-regulation of E-cadherin, which has also been reported to be a downstream event of LMP1 (Yoshizaki et al, 1998), was also confirmed in NP69-LMP1 cells by Western blotting (Fig. 7) but not by cDNA array. This suggests that the cDNA array hybridization may have limitations in detecting low copy differences in the cellular mRNA level.

In summary, using an immortalized nasopharyngeal epithelial cell line model, we have demonstrated the relationship between biologic alterations and differentially expressed genes induced by LMP1. The present findings support the major role of LMP1 in enhancing cell survival and promoting tumor invasion in nasopharyngeal epithelial cells. This is also supported by clinical observation of the high expression of LMP1 in premalignant lesions of NPC (Pathmanathan et al, 1995), which may facilitate their conversion into invasive NPC cells.

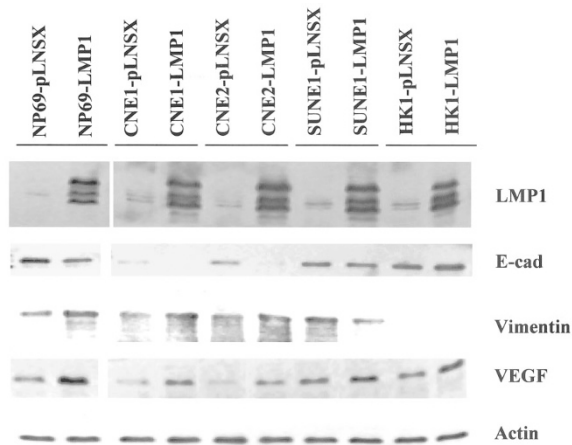


Figure 7.

Western blot analysis of LMP1, E-cadherin, vimentin, and VEGF in nasopharyngeal epithelial cell lines with and without LMP1 expression. Fifty micrograms of g protein extracts from each sample was separated in 10% SDS-PAGE and transferred onto a nitrocellulose membrane for blotting with specific antibodies. Actin protein was detected as loading control. *E-cad* = E-cadherin.

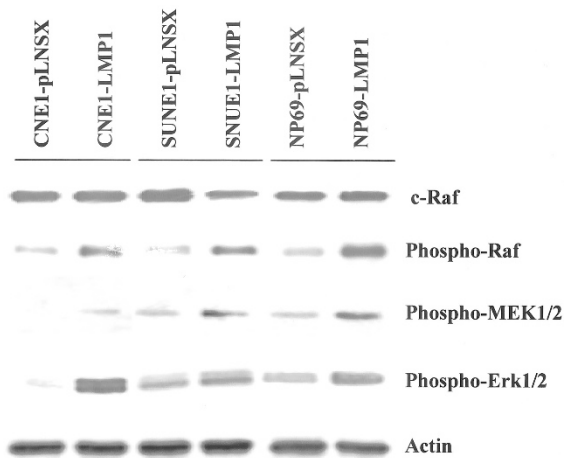


Figure 8.

Western blot analysis of the phosphokinases involved in the Ras/MAPK signal pathway in nasopharyngeal epithelial cell lines with and without LMP1 expression. Twenty- to 40- μ g protein extracts were separated in SDS-PAGE and transferred onto nitrocellulose membrane for blotting with antibodies specific to the phospho-form of Raf, MEK1/2, and Erk1/2 kinases. Actin and c-Raf proteins were detected as internal controls.

Materials and Methods

Cell Lines

The SV40T-immortalized nasopharyngeal epithelial cell line (NP69) was grown in low calcium (0.1 mM) MCDB151 medium supplemented with 1% dialyzed fetal bovine serum and other growth supplements. Details of the growth medium used in this cell line have been described previously (Tsao et al, 2002). The NPC cell lines, CNE1, CNE2, SUNE1 (Teng et al, 1996) (kindly provided by Dr. Z. Zheng, Zhong Shan Medical Science University), and HK1 (Huang et al, 1980), were routinely maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Expression of LMP1

LMP1 expression in NP69 cells was achieved by infecting the cells with pLNSX-LMP1 retrovirus. Control cells were infected with the empty vector pLNSX at the same passage number. The retroviral vector expressing LMP1 (pLNSX-LMP1) was kindly provided by Dr. L. Cao, University of Hong Kong (Yang et al, 2000). The retroviral infection of LMP1 in nasopharyngeal epithelial cells has been previously described (Lo et al, 2001a). The successfully infected cells were selected by 400 μ g/ml of G418, and the resistant clones were pooled and designated as NP69-LMP1. Expression of LMP1 in the resistant clones was confirmed by Western blotting (Fig. 7). Control cells infected with the pLNSX vector were designated as NP69-pLNSX.

Cell Cycle Analysis

Cell cycle analysis was performed on an Epic Elite flow cytometer (Beckman-Coulter) and analyzed using the ModFit LT2.0 software (Coulter, Hialeah, Florida). Cells (5×10^5) were fixed in ice-cold 70% ethanol for at least 1 hour. Before analysis, fixed cells were washed with PBS, treated with RNase (1 μ g/ml), and stained with propidium iodide (50 μ g/ml) for 30 minutes at 37° C.

Growth Curve Analysis

For growth curve analysis, 2×10^5 cells per well were inoculated onto a 24-well plate. The cells were trypsinized every 1 or 2 days, and the number of viable cells was counted with the trypan blue exclusion method.

Soft Agar Cloning Assay

For soft agar assay, the cells were seeded into semi-solid agar medium (base layer, 0.6%; upper layer, 0.3%) at a density of 5×10^3 cells per well in 6-well plates. After 3 weeks of incubation at 37° C with 5% CO₂, the number of colonies formed was determined.

Scrape-Wound Migration Assay

For scrape-wound migration assay, confluent cultures grown on 6-well plates were scraped with a yellow pipette tip to make a wound. The scraped culture was maintained in serum-free medium to slow the growth rate. The morphology of individual cells in the migrating front was monitored at 8 to 12 hours under a phase-contrast microscope.

Boyden Chamber Invasion Assay

The Boyden chamber invasion assay was performed using a transwell chamber (6.4 mm in diameter with 8.0- μ m pore size; Millipore) coated with Matrigel (Becton Dickinson) according to a previously published method (Albini et al, 1987). Cells (5×10^4) in 200 μ l of serum-free medium with 0.1% BSA were inoculated into a chamber that was put into a well of a 24-well

plate. After 24 hours of incubation, the cells migrating on the lower surface were fixed in methanol and stained with hematoxylin and eosin.

Collagen Gel Invasion Assay

For collagen gel assay, a collagen mixture was prepared by mixing seven volumes of ice-cold type I collagen solution (Collaborative Research) with two volumes of $5\times$ concentrated culture medium and one volume of 0.05 N NaOH containing 2.2% NaHCO_3 and 200 mM HEPES. Cells were suspended in this collagen mixture at 5×10^4 cells/ml. After gel setting, the cells were maintained in medium with growth factors. The pattern of the colony was examined and photographed at Day 8.

Western Blotting

The detailed procedures of Western blotting have been described previously (Lo et al, 2001b). Briefly, the cells were lysed in cell lysis buffer (Cell Signaling). The cell lysates (25–100 μg of protein) were separated in 10% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane (BioRad, Richmond, California) for blotting. The primary antibodies used were LMP1 (1:1000 dilution; Dako, Carpinteria, California), E-cadherin (1:2000), c-Raf (1:1000; Transduction Laboratories (Lexington, Kentucky), vimentin (1:100; Sigma, St. Louis, Missouri), VEGF (1:100; Santa Cruz Biochemicals, Santa Cruz, California), phospho-Raf, phospho-MEK1/2, and phospho-Erk1/2 (Cell Signaling). All phospho-antibodies were diluted 1:1000.

cDNA Array Hybridization

Atlas Human Cancer 1.2 Arrays (Clontech, Palo Alto, California) containing 1176 genes were used in this study to identify the gene expression profiles of the LMP1-expressing NP69 cells. Total RNA was isolated using TRIzol reagent (Invitrogen, San Diego, California). RNA was then treated with DNase I (Invitrogen) to remove DNA contamination. Preparation and hybridization of radioactively labeled cDNA from total RNA were performed according to the protocol provided by Clontech. The hybridization signal was detected with a PhosphorImager, and the signal intensity at each cDNA spot was quantified using AtlasImage 1.01 software (Clontech). Signal intensities were calculated by subtracting the background signal and then normalized to the spots of positive controls on the array.

Quantitative Real-Time RT-PCR

First-strand cDNA was synthesized from DNase-treated total RNA with oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen), followed by PCR amplification using the corresponding gene-specific primers. Primer sequences are as follows: (a) 5'-CGGAATCTGGTCATCAAGGT-3' (sense) and 5'-AGGGTTTATAGGCTGCACGA-3' (antisense) for *Gas6*; (b) 5'-GGGATCCACTGTGAGAACA (sense)

and 5'-CACACCAGTGCACAAGGTTTC-3' (antisense) for *Notch1*; and (c) 5'-CCGCAGACGTGTAAATGTTCT-3' (sense) and 5'-CGGCTTGTCACATCTGCAAGTA-3' (antisense) for *VEGF*. For other gene-specific primers used in this study, the primer sequence information was acquired from Clontech. Expression of *GAPDH* (5'-CTCAGACACCATGGGGAA-3' (sense) and 5'-ATGATCTTGAGGCTGTTG-3' (antisense)) was used as an internal control. Real-time PCR was performed in a 25- μl reaction, which included 1 μl of cDNA template, 50 mM KCl, 1.5 to 3 mM MgCl_2 , 10 mM Tris-HCl, pH 9.0, 0.05 mM of each dNTP, 0.2 mM of each forward and reverse oligo primer, 2.5 U of *Taq* DNA polymerase (Amersham, Arlington Heights, Illinois), and $0.1\times$ SYBR Green I (Molecular Probes, Eugene, Oregon), using an iCycler iQ Multi-Color Real Time PCR Detection System (BioRad). Each PCR reaction was optimized to ensure that a single PCR product was amplified and no product corresponding to primer-dimer pairs was present. PCR reactions of each template were performed in duplicate in one 96-well plate for each pair of gene-specific primers. The PCR cycling conditions were as follows: 95° C for 3 minutes followed by 50 cycles of three steps at 95° C for 30 seconds, gene-specific annealing temperature for 30 seconds, and then 72° C for 30–40 seconds.

The relative fold change method was used to determine the relative quantitative gene expression for each gene studied compared with the *GAPDH* control (Winer et al, 1999). The relative fold change or relative gene expression value = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t\text{Target[LMP1]}} - C_{t\text{GAPDH[LMP1]}}) - (C_{t\text{Target[pLNSX]}} - C_{t\text{GAPDH[pLNSX]}})$. $C_{t\text{Target[LMP1]}}$ = threshold cycle of the target gene examined in the LMP1-expressing cells; $C_{t\text{GAPDH[LMP1]}}$ = threshold cycle of the *GAPDH* gene in the LMP1-expressing cells; $C_{t\text{Target[pLNSX]}}$ = threshold cycle of the target gene examined in the control vector pLNSX cells; and $C_{t\text{GAPDH[pLNSX]}}$ = threshold cycle of the *GAPDH* gene in the control vector pLNSX cells.

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