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Epstein-Barr Virus Latent Membrane Protein 1 Induces Cancer Stem/Progenitor-Like Cells in Nasopharyngeal Epithelial Cell Lines[▽]

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Recent studies suggest the existence of cancer stem cells (CSC) and cancer progenitor cells (CPC), although strict definitions of neither CSC nor CPC have been developed. We have produced evidence that the principal oncoprotein of Epstein-Barr virus (EBV), latent membrane protein 1 (LMP1), which is associated with human malignancies, especially nasopharyngeal carcinoma (NPC), promotes tumor cell invasion and metastasis, as well as the epithelial-mesenchymal transition (EMT). However, whether LMP1 is involved in the development of CSC/CPC is still unclear. This study investigates whether the expression of EBV-LMP1 is related to the development of CSC/CPC. Analysis of cancer stem cell markers reveals that LMP1 induces the CD44^{high} CD24^{low} CSC/CPC-like phenotype as well as self-renewal abilities in LMP1-expressing epithelial cell lines. In addition, we show here that LMP1 induction in epithelial cells causes high tumorigenicity and rapid cellular proliferation. Furthermore, we found that LMP1 expression increased the expression of several CPC markers as well as producing increased levels of EMT markers. Our findings indicate that LMP1 can induce a CPC-like rather than a CSC-like phenotype in epithelial cells and suggest that LMP1-induced phenotypic changes contribute to the development of NPC.

Recent studies have proposed that solid tumors are organized as a hierarchy composed of a spectrum of phenotypically distinct cells at different stages of maturation (25, 26). The concept that cancer stem cells (CSC) include primitive rare CSC and cancer progenitor cells (CPC) has been developed (25). At the apex of the hierarchy are primitive rare CSC, which possess extended self-renewal capabilities that allow them to perpetuate themselves and develop into CPC. CPC have only limited self-renewal abilities and can, in turn, differentiate into various types of cancer cells. *In vivo*, the primitive rare CSC rarely divide, whereas CPC proliferate rapidly.

CD44^{high} CD24^{low} cells from breast cancer tissues were found to be more tumorigenic in immunodeficient mice than CD44^{low} CD24^{high} cells, and the results of these observations were used to support the CSC hypothesis for solid tumors (1). Immortalized human mammary epithelial cells undergoing an epithelial-mesenchymal transition (EMT) are CSC-like, as evidenced by their CD44^{high} CD24^{low} phenotype and their capacity for self-renewal and for forming mammospheres (20). Thus, the conjunction of EMT and the CD44^{high} CD24^{low} phenotype appears to be important for the understanding of CSC-like characteristics, although CSC have been characterized in solid tumors by using a variety of stem cell markers (9, 10, 33).

Epstein-Barr virus (EBV) is a human herpesvirus that is associated with several malignancies, such as Burkitt lymphoma (BL), Hodgkin lymphoma (HL), gastric cancer, and, most importantly, nasopharyngeal carcinoma (NPC) (24). In all these tumors, EBV infection is predominantly latent. In latent infection, expressed EBV genes are restricted to six EBV nuclear antigens (EBNA1, -2, -3A, -3B, -3C, and -LP), three latent membrane proteins (LMP1, -2A, -and 2B), and two small nonpolyadenylated RNAs (EBER1 and -2). On the basis of the patterns of expression of these genes, latency is classified into three types. In type I latency, exemplified by BL, only EBNA1 and EBERs are expressed. In type II latency, exemplified by HL and NPC, EBNA1, EBERs, and the three LMPs are expressed. In type III latency, the full set of EBNAs and LMPs as well as EBERs are expressed, as in EBV B-cell lymphoproliferative diseases (24).

LMP1, a member of the tumor necrosis factor receptor superfamily, is considered to be a potent oncogenic protein because of its transforming effects on rodent fibroblasts and because it is essential for the immortalization of B lymphocytes and epithelial cells (8, 30). We have shown that LMP1 induces the expression of an array of cellular invasion and metastasis factors (15, 29, 34). In particular, LMP1 can induce EMT via

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Twist or Snail (11, 12), which coincides with the acquisition of CSC properties (20). Several LMP1 variants, which originate from different types of EBV isolates and diseases, have been reported (14, 19, 22). Among these variants, CAO-LMP1 was originally isolated from a patient with NPC and has been reported to be more tumorigenic than other variants (8, 21, 22).

The aim of this study is to investigate whether the expression of an EBV-associated oncoprotein, LMP1, contributes to the development of CSC/CPC, by analyses with CAO-LMP1 which is associated with NPC. We also examine whether LMP1 affects tumorigenicity, cell proliferation, and the expression of CSC/CPC markers, including an EMT phenotype and stem cell-like genes, in epithelial cells. Finally, we propose here the new concept that LMP1 contributes to the generation of CPC-like but not CSC-like cells.

MATERIALS AND METHODS

Cell cultures. KR4, KH1, KH2, HeLa, C666-1, and AdAH cells have been described previously (4, 7, 29). C666-1, MDA-MB-231, and KR4 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), penicillin, and streptomycin. The other cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS, penicillin, and streptomycin.

Tumor sphere assay. For the tumor sphere assay, cells were seeded at a density of 1×10^3/ml in uncoated plastic dishes with a serum-free expansion medium (Stemcell Technologies, Vancouver, Canada) with epidermal growth factor and basic fibroblast growth factor (10 ng/ml each; PeproTech, Rocky Hill, NJ). Cells were cultured for 14 days, until sphere formation was observed. The number of sphere formations was analyzed with a *t* test. A *P* value under 0.05 was considered to be significant.

Plasmids and retroviral infection. The LMP1 expression vectors pSG5-CAO-LMP1 and pMRX-CAO-LMP1-IRES-GFP have been described previously (14, 23). From these plasmids, cDNA of the CAO-LMP1 fragment digested by EcoRI and NotI was introduced into the same site of the pFB-Neo vector (Stratagene, Santa Clara, CA), and the resultant plasmid was named pFB-LMP1 (CAO-LMP1). Retroviruses were produced as described previously (23). AdAH cells were infected with these viruses in the presence of Polybrene. Stable cell lines were established by cultivating AdAH cells in the presence of G418 (Invitrogen, Carlsbad, CA). After culturing for 7 days with G418, we selected single clones using cloning paper (Sigma-Aldrich, St. Louis, MO).

siRNA transfection. LMP1 and negative-control small interfering RNAs (siRNA) were chemically synthesized by Hokkaido System Sciences (Sapporo, Hokkaido, Japan). The sequences of LMP1 siRNA and negative-control siRNA have been described elsewhere (3). The EBV-positive NPC cell line C666-1 was seeded into 60-mm-diameter dishes at 6×10^5 cells per dish on the day before transfection. Lipofectamine 2000 (Invitrogen) was used for transfection, with a final siRNA concentration at 100 nM for 72 h, for subsequent experiments.

RT-PCR analysis. Total RNA was extracted using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany) and was reverse transcribed with SuperScript III (Invitrogen). The resulting cDNAs were used for amplification of LMP1 and β-actin cDNA by the use of *Taq* DNA polymerase (Takara Bio, Ootu, Japan) as described previously (22). For SYBR green real-time reverse transcriptase PCR (RT-PCR), reactions were performed in triplicate using QuantiFast SYBR green PCR (Qiagen) on a LightCycler system (Roche, Mannheim, Germany). All primers were made using the QuantiTect primer assay (Qiagen). All quantitations were normalized to an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative quantitative value for each target gene compared with the calibrator for that target is expressed as $2^{-(C_T - C_C)}$ (C_T and C_C are the mean threshold cycle differences for the target and control, respectively, after normalization to GAPDH). The relative expression levels of samples are presented by a semilog plot.

Flow cytometry and cell sorting. Cells were labeled with the following anti-human antibodies: phycoerythrin (PE)-conjugated anti-CD24, anti-CD29, and anti-CD49b, fluorescein isothiocyanate (FITC)-conjugated anti-CD49f, allophycocyanin (APC)-conjugated anti-CD44 and anti-CD90, PE-conjugated anti-CD117, APC-conjugated anti-CXCR4 (BD Pharmingen, San Diego, CA), FITC-conjugated anti-EpCAM (Abcam, Cambridge, MA), and PE-conjugated anti-ABCG2, anti-CD166 (R & D Systems, Minneapolis, MN), and anti-CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Labeled cells were detected using a JSAN cell sorter (Bay Bioscience, Kobe, Japan). Appropriate isotypes of nonrelated antibodies were used as controls. For cell sorting, a cocktail of PE-conjugated anti-CD24 and APC-conjugated anti-CD44 was used. MDA-MB-231 or MCF-7 cells were used for positive staining of CD44 or CD24, respectively (28). Labeled cells were analyzed and sorted using JSAN. For the positive population, only the 5% of most brightly stained cells were selected. For side population (SP) analysis, cells (1×10^6/ml) were incubated in prewarmed Hanks' balanced salt solution with 2% fetal bovine serum, 10 mM HEPES buffer, and 1% penicillin-streptomycin, containing freshly added Hoechst 33342 (final concentration, 5 μg/ml), for 90 min or 120 min at 37°C with intermittent mixing. Cells were incubated with the Hoechst dye with or without verapamil (300 μM). At the end of incubation, cells were spun down and resuspended in ice-cold buffer. Propidium iodide (PI) (1 mg/ml) was added before flow cytometric analysis.

Immunoblotting. Immunoblotting was carried out as described previously (29). Antibodies against the following proteins were used: LMP1 (Dako, Glostrup, Denmark), KLF4 (H-180), Oct4 (C-10), E-cadherin (G-10), cytokeratin 14 (CK14), and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), nestin (10C2; Novus Biologicals, Littleton, CO), c-myc (9E10; BioLegend, San Diego, CA), fibronectin (IST-4; Sigma-Aldrich), Nanog (1E6C4; Cell Signaling, Danvers, MA), vimentin (V9), CD24 (SN3), and CK18 (Thermo Scientific, Cheshire, United Kingdom), ABCG2 (BCRP1) and CD44 (EPR1013Y) (Millipore, Temecula, CA), CK19 (Abcam), and N-cadherin (BD Bioscience Pharmingen).

Cell proliferation assay. A total of 1.5×10^3 cells were seeded into a 96-well plate, and cell proliferation was performed using a CellTiter 96 Aqueous One solution cell proliferation kit (Promega, Madison, WI) as described previously (35). Four samples for each group were used for analysis. Similarly, the cell proliferation rate was determined with a CellTrace CFSE (carboxyfluorescein succinimidyl ester) cell proliferation kit (Invitrogen). Cells (5×10^6/ml) were harvested, washed twice in phosphate-buffered saline (PBS), and then stained with 20 μM CFSE for 15 min at 37°C. Residual CFSE was removed by washing twice with PBS, and cells were seeded in 6-well plates and were grown in DMEM with 1% fetal bovine serum. CFSE fluorescence intensity was measured by fluorescence-activated cell sorter (FACS) analysis every 48 h for 4 days.

Soft-agar colony formation assay. A soft-agar assay was performed by seeding cells in a layer of 0.35% agar–DMEM–FBS over a layer of 0.5% agar–DMEM–FBS. Cultures were maintained at 37°C. On day 21 after seeding, cells were fixed with pure ethanol containing 0.05% crystal violet, and the colony-forming efficiency was quantified by light microscopy.

Injection of cells into immunodeficient mice. AdAH-pFB-Neo or AdAH-pFB-LMP1 cells in a volume of 0.1 ml of PBS were inoculated subcutaneously into female BALB/c-nu/nu or NOD/SCID mice aged 6 weeks (Charles River Japan, Yokohama, Japan). The research plan was approved by the Ethics Committee of the Laboratory for Animal Experiments, School of Medicine, Kanazawa University. All the mice were sacrificed at 12 weeks after injection.

Fluorescence immunostaining. Fluorescent immunostaining was performed as described previously (35). Antibodies against LMP1, Twist, Snail, E-cadherin, vimentin, fibronectin, N-cadherin, CK14 (Santa Cruz Biotechnology), CK18 (Thermo Scientific), and CK19 (Abcam) were used for staining.

RESULTS

LMP1-expressing cells exhibit a CD44^{high} CD24^{low} phenotype that enhances self-renewal properties. There are several reports that only cells with the CD44^{high} CD24^{low} phenotype exhibit CSC/CPC properties (1, 20). We first investigated hybrid somatic cell lines generated from EBV-negative epithelial cells (HeLa) and EBV-positive type III lymphoblastoid cells (KR4) (7). The fusion cell lines KH1 and KH2 exhibit type II EBV latency, as do NPC cells. As shown in Fig. 1A, these cell lines strongly express LMP1. Flow cytometric analysis showed that both KH1 and KH2 cells contained subpopulations of cells with a CD44^{high} CD24^{low} expression pattern, whereas HeLa control cells had a CD44^{low} CD24^{high} profile (Fig. 1B). On the other hand, KR4 cells had a CD44 CD24 profile quite different from that of KH1 and KH2 cells, although levels of LMP1 expression were similar. Only a small portion of KR4 cells expressed both CD44 and CD24. KR4 cells express type III EBV latency genes, in contrast to type II latency in KH1 and

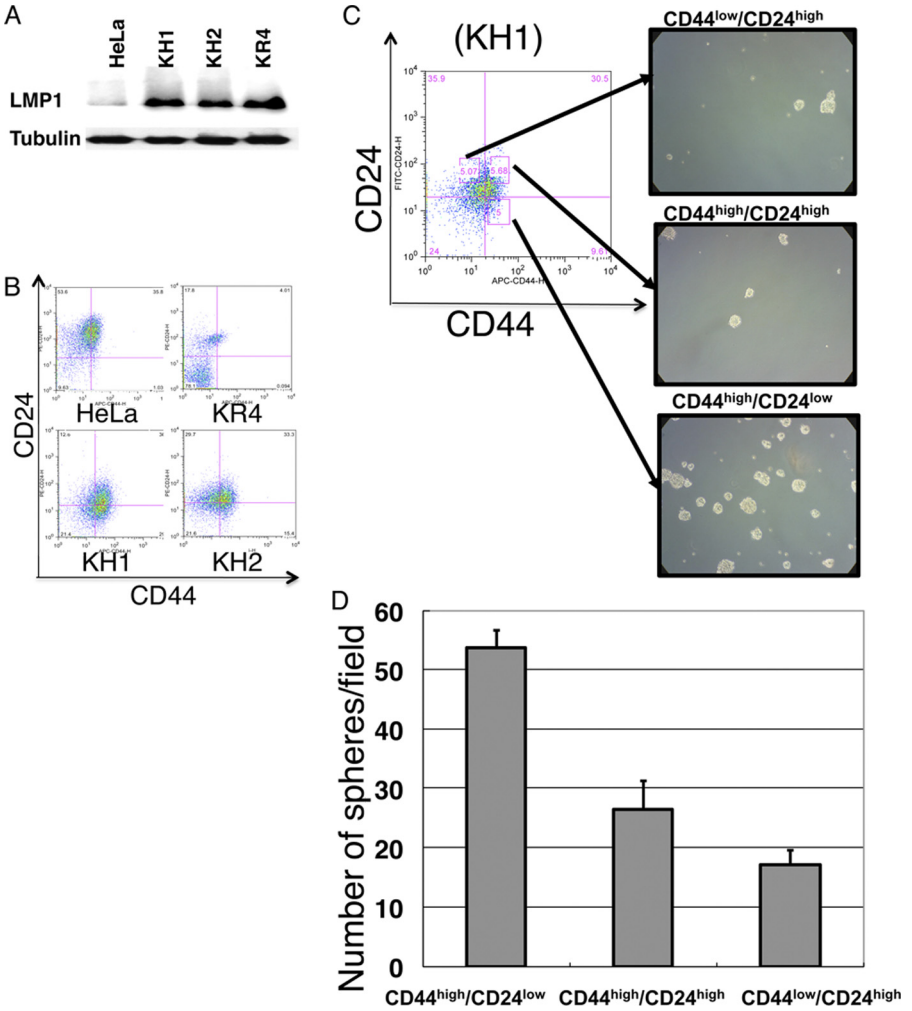

FIG. 1. LMP1-expressing cells exhibit a CD44^{high} CD24^{low} profile associated with stem cells. (A) KR4 is an EBV-positive type III lymphoblastoid cell line. KH1 and KH2 are type II cell lines derived from the fusion of KR4 and HeLa cells. Levels of LMP1 expression by these cell lines were revealed by Western blotting. (B) FACS analysis of the cell surface markers CD44 and CD24 in these cells. (C) (Left) CD44^{high} CD24^{low} cells and CD44^{low} CD24^{high} cells were separated by FACS. (Right) Phase-contrast images of tumor spheres seeded by CD44^{low} CD24^{high} (top) and CD44^{high} CD24^{low} (bottom) cells. (D) Quantification of tumor spheres formed by cells from the FACS-separated CD44^{high} CD24^{low}, CD44^{high} CD24^{high}, and CD44^{low} CD24^{high} populations of KH1 cells. The data are mean numbers of tumor spheres per 2,000 seeded cells ± standard deviations ($P < 0.001$).

KH2 cells. The difference in EBV gene products between type II latency and type III latency may affect CD44 and CD24 expression.

Next, we examined whether cells with the CD44^{high} CD24^{low} phenotype exhibited enhanced self-renewal properties in our cell lines by generating tumor spheres. Because the KH1 and KH2 cell lines have similar CD44/CD24 expression patterns, KH1 cells were used as the representative cell line for the experiment. The size and number of the tumor spheres reflect the ability of self-renewal *in vitro*. After sorting for CD44^{high} CD24^{low}, CD44^{high} CD24^{high}, and CD44^{low} CD24^{high} groups, KH1 cells were cultured in suspension with serum-free medium to generate tumor spheres (Fig. 1C). As shown in Fig. 1D, CD44^{high} CD24^{low} cells formed almost 3 times more tumor spheres than CD44^{high} CD24^{high} or CD44^{low} CD24^{high} cells ($P < 0.05$).

These data prompted us to investigate the role of LMP1 in the phenotypic change of epithelial cells from CD44^{low} CD24^{high} to CD44^{high} CD24^{low} and in the acquisition of the self-renewal property.

LMP1 induces morphological change and a CD44^{high} CD24^{low} profile as well as the self-renewal property in nasopharyngeal epithelial cells. To further assess whether LMP1 expression actually enhances CSC/CPC properties, human epithelial cell lines stably expressing LMP1 were tested. In this study, we focused on an LMP1 variant, CAO-LMP1, as a representative form of LMP1, since CAO-LMP1 has been detected most frequently in NPC worldwide and has been reported to be more tumorigenic than other variants (8, 23).

To establish stable epithelial cell lines that express LMP1, AdAH cells (an EBV-negative nasopharyngeal epithelial cell line) were infected either with a retrovirus vector carrying LMP1 or with an empty retrovirus vector, followed by selection in neomycin. Finally, a representative clone with the strongest

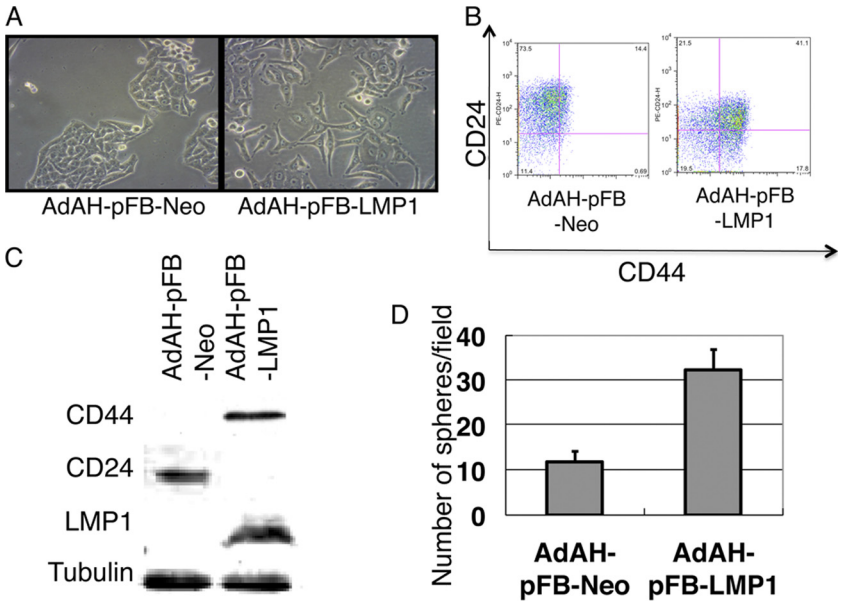

FIG. 2. LMP1-expressing nasopharyngeal epithelial cells express the $CD44^{high}$ $CD24^{low}$ phenotype. (A) Morphological changes in newly established AdAH nasopharyngeal epithelial cell lines expressing retroviral vector pFB-Neo or pFB-LMP1 in standard growth medium. Phase-contrast images of representative clones are shown. Magnification ×100. (B) FACS analysis of the cell surface markers CD44 and CD24 in the cells described in the legend to panel A. (C) AdAH cells expressing pFB-Neo or pFB-LMP1 were analyzed by Western blotting for CD44, CD24, LMP1, and tubulin. (D) Quantification of tumor spheres formed by AdAH cells with or without LMP1 expression. The data are mean numbers of tumor spheres per 2,000 seeded cells ± standard deviations ($P < 0.001$).

LMP1 expression was selected. Although the morphology of the empty-vector-infected cells did not change, LMP1-expressing AdAH cells changed morphologically into fibroblast-like, spindle-shaped cells (Fig. 2A). The LMP1-expressing clone tends to grow in a scattered pattern, in contrast to the cobblestone-like pattern of the LMP1-negative clone and parental AdAH cells.

To further clarify whether LMP1 signaling affects the CD44/CD24 expression profiles of these cells, flow cytometric and Western blot analyses of AdAH-pFB-Neo and AdAH-pFB-LMP1 cells were performed on the basis of CD44 and CD24 expression. The distributions of the cell surface markers CD44 and CD24 in the newly established cell lines with or without LMP1 are shown in Fig. 2B and C. AdAH-pFB-LMP1 expressed high CD44 but low CD24 levels compared with those of the AdAH-pFB-Neo control.

Finally, we tested whether LMP1 expression in nasopharyngeal epithelial cells enhanced self-renewal properties. We examined tumor sphere formation with AdAH-pFB-Neo and AdAH-pFB-LMP1 cells. AdAH-pFB-Neo and AdAH-pFB-LMP1 cells were cultured in suspension with serum-free medium to generate tumor spheres. As shown in Fig. 2D, AdAH-pFB-LMP1 cells formed more than twice as many tumor spheres as the control ($P < 0.05$). These data show that LMP1 expression in epithelial cells induces this self-renewal property.

LMP1 promotes rapid cell growth and high tumorigenicity. To examine the cellular effects of LMP1, the growth kinetics of AdAH cells expressing LMP1 were examined over 7 days (Fig. 3A). The growth of AdAH-pFB-LMP1 cells was more rapid than that of AdAH-pFB-Neo cells (Fig. 3A). This result is compatible with previous reports that LMP1 causes rapid cellular growth (8, 23).

To assess the proliferation rate at the single-cell level, AdAH-pFB-Neo and AdAH-pFB-LMP1 cells were stained with CFSE; this stain is evenly distributed in the cytoplasm and is stoichiometrically distributed to the daughter cells during cell division. The intensity of CFSE was clearly lower in AdAH-pFB-LMP1 cells than in AdAH-pFB-Neo cells (Fig. 3B). This result supports findings that LMP1 induces rapid cellular proliferation.

Anchorage-independent cell growth is frequently used as an assay for tumorigenicity in CSC/CPC studies (16). Tumorigenic cells can form more colonies in semisolid soft-agar medium than less tumorigenic cells. Using this assay, we investigated whether LMP1 affects colony formation in such a medium. AdAH-pFB-Neo or AdAH-pFB-LMP1 cells were plated onto a semisolid agar medium. Cells were analyzed by light microscopy 21 days posttransduction. LMP1-expressing AdAH cells generated more and larger colonies in soft agar than LMP1-negative cells (Fig. 3C).

Finally, the effect of LMP1 on tumorigenicity was assessed by inoculating AdAH cells into BALB/c-nu/nu mice. LMP1-expressing cells (5×10^6 cells), but not mock-expressing AdAH cells, formed palpable tumors in inoculated sites (5/8 mice [62.5%] inoculated with LMP1-expressing cells had tumors versus 0/8 mice [0%] in the control group [Table 1]). However, when the number of injected cells was reduced to 5×10^5, tumors did not develop with either LMP1-positive or LMP1-negative cells.

To further characterize the tumorigenicity of the LMP1-expressing cells, NOD/SCID mice, which have a more severe

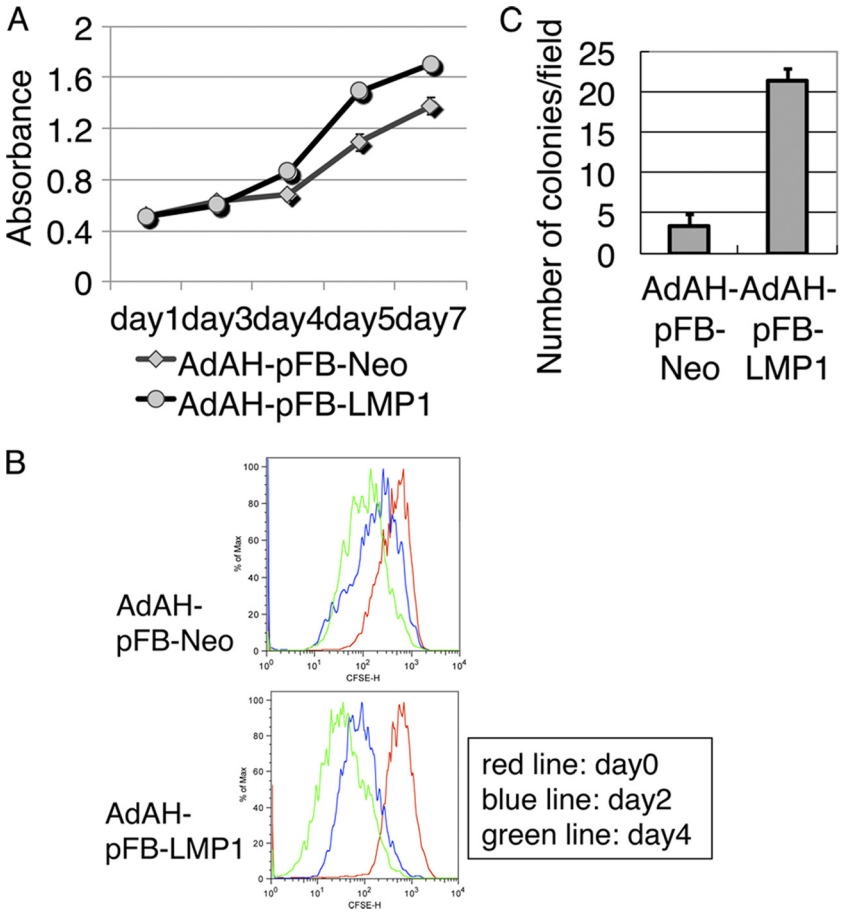

FIG. 3. LMP1 induces rapid cell growth and high anchorage-independent growth. (A) Growth curves of AdAH cells expressing pFB-Neo or pFB-LMP1. The proliferation activities of LMP1 clones were significantly higher than those of the controls ($P < 0.05$). (B) CFSE intensity as measured by FACS at day 0 (red lines), day 2 (blue lines), and day 4 (green lines) in AdAH-pFB-Neo and AdAH-pFB-LMP1 cells. (C) Quantification of the anchorage-independent growth of AdAH cells expressing pFB-Neo or pFB-LMP1. The data are mean numbers of colonies per field ± standard deviations ($P < 0.001$).

immunodeficiency than BALB/c-nu/nu mice, were inoculated with AdAH-pFB-LMP1 or AdAH-pFB-Neo cells. With large numbers of cells (1×10^7), palpable tumors developed in almost all cases (Table 2). However, when lower numbers of cells (1×10^6 to 1×10^4) were inoculated, only the LMP1-expressing AdAH clone formed tumors (Table 2). These results indicate that LMP1 enhances the tumorigenicity of human epithelial cells in culture.

We conclude that LMP1 has substantial ability to induce CSC/CPC properties in human epithelial cells.

LMP1 silencing reduces CD44^{high} CD24^{low} properties, accompanied by lowering of the cellular proliferation rate in naturally derived EBV-positive NPC cell lines. To further verify that LMP1 could affect CD44^{high} CD24^{low} properties with rapid cellular proliferation in physiological NPC cell lines, we made LMP1-silencing systems by chemical synthesis of siRNA in C666-1 cells. C666-1 is a naturally occurring carcinoma cell line derived from an NPC biopsy specimen, and EBV genes, including LMP1, are expressed (4). First, we detected LMP1 transcriptional expression to identify the LMP1 siRNA that produced the desired reduction of LMP1 expression. As shown in Fig. 4A, LMP1 siRNA could reduce LMP1 expression in C666-1 cells, whereas control siRNA did not. In subsequent flow cytometric analysis, C666-1 cells with control siRNA ex-

TABLE 1. Tumor incidence with AdAH cells transformed by LMP1 in BALB/c-nu/nu mice

Cells injected	No. of mice with tumors/total no.[a] injected with:	
	5×10^6 cells	5×10^5 cells
AdAH-pFB-Neo	0/8 (0)	0/8 (0)
AdAH-pFB-LMP1	5/8 (62.5)	0/8 (0)

[a] Tumor incidence, expressed as a percentage, is given in parentheses.

TABLE 2. Tumor incidence with AdAH cells transformed by LMP1 in NOD/SCID mice

Cells injected	No. of mice with tumors/total no. injected with the following no. of cells:			
	1×10^7	1×10^6	1×10^5	1×10^4
AdAH-pFB-Neo	3/4	0/0	0/0	ND[a]
AdAH-pFB-LMP1	2/2	4/4	4/4	1/4

[a] ND, not determined.

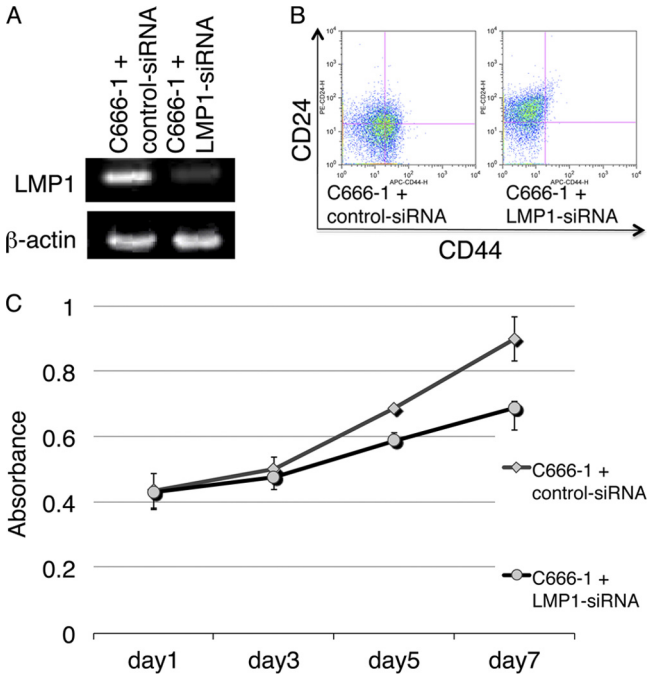

FIG. 4. LMP1 silencing reduced the $CD44^{high}$ $CD24^{low}$ profile and cellular proliferation in an EBV-positive NPC cell line. (A) Analysis of LMP1 transcripts by RT-PCR in C666-1 cells transfected with either control siRNA or LMP1 siRNA. β-Actin was used as a loading control. (B) FACS analysis of the cell surface markers CD44 and CD24 in C666-1 cells transfected with either control siRNA or LMP1 siRNA. (C) Quantification of the anchorage-independent growth of C666-1 cells with or without LMP1. The data are mean numbers of colonies per field ± standard deviations ($P < 0.001$).

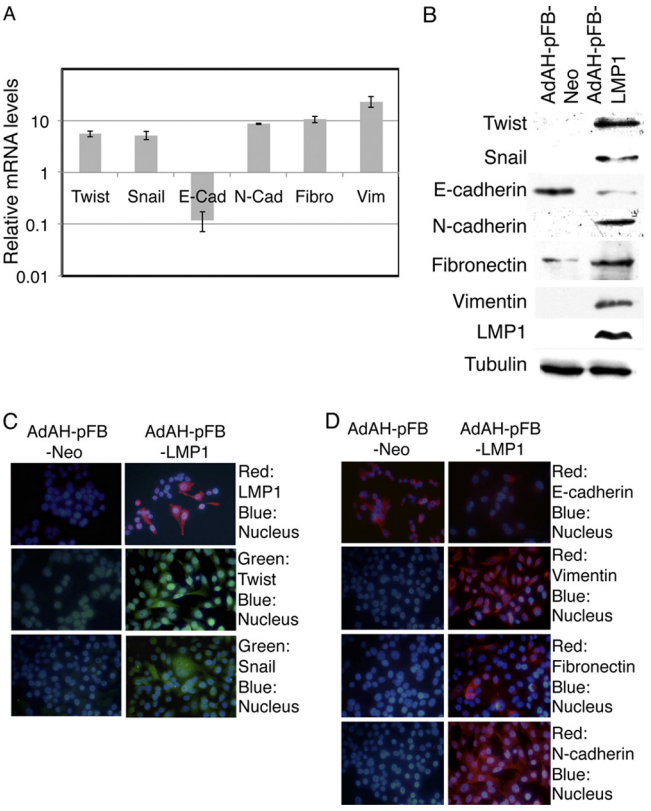

FIG. 5. LMP1 induces the epithelial-mesenchymal transition. (A) Expression levels of mRNAs encoding Twist, Snail, E-cadherin (E-Cad), N-cadherin (N-Cad), fibronectin (Fibro), and vimentin (Vim) in AdAH-pFB-LMP1 cells relative to those in AdAH-pFB-Neo cells as determined by real-time PCR. GAPDH mRNA was used to normalize the variability in template loading. The data are means ± standard deviations. (B) Western blot analysis of expression of Twist, Snail, E-cadherin, N-cadherin, fibronectin, and vimentin in AdAH cells expressing pFB-Neo or pFB-LMP1. Tubulin was used as a loading control. (C) Immunofluorescence images of mock-expressing (left) or LMP1-expressing (right) cells stained using antibodies against LMP1, Twist, or Snail. (D) Immunofluorescence images of mock-expressing (left) or LMP1-expressing (right) cells stained using antibodies against E-cadherin, vimentin, fibronectin, or N-cadherin.

pressed $CD44^{high}$ $CD24^{low}$ properties. However, C666-1 cells transfected with LMP1 siRNA revealed $CD44^{low}$ $CD24^{high}$ properties in comparison with control siRNA-transfected cells. Finally C666-1 cells with LMP1 siRNA showed slower cell proliferation than C666-1 cells transfected with control siRNA (Fig. 4C). These results suggest that LMP1 silencing reduces $CD44^{high}$ $CD24^{low}$ properties and is accompanied by repression of rapid cellular proliferation in this naturally occurring NPC cell line.

LMP1 induces EMT. Because EMT produces cells expressing the stem cell phenotype (20), it is of particular interest to test whether the LMP1-induced CSC/CPC show EMT characteristics. Representative EMT markers were thus analyzed by real-time RT-PCR. As shown in Fig. 5A, LMP1 induced higher levels of Twist and Snail mRNAs. Furthermore, as shown in Fig. 5B and C, the AdAH-pFB-LMP1 cell line expressed higher levels of the Twist and Snail proteins than the control cell line. Since Twist and Snail are known inducers of the EMT, we determined expression of EMT markers in the newly established cell lines at both the mRNA and protein levels.

LMP1 downregulated the level of an epithelial cell marker, E-cadherin (Fig. 5A, B, and D). In contrast, expression of the mesenchymal markers fibronectin, vimentin, and N-cadherin was induced in response to LMP1 expression (Fig. 5A, B, and D). Taken together, these results indicate that induction of CSC/CPC by LMP1 is accompanied by upregulation of EMT markers.

LMP1 expression upregulates other cell surface cancer stem cell markers. Since LMP1 induces the $CD44^{high}$ $CD24^{low}$ phenotype, which results in self-renewal ability and EMT-like cellular changes, we investigated whether LMP1 induces other markers of CSC/CPC. To examine the profile of CSC/CPC markers in LMP1-expressing cells, the expression of a panel of known stem cell markers, such as CD29, CD49b, CD49f, CD90, CD117, CD133, CD166, ABCG2, EpCAM, and CXCR4, was determined using flow cytometric analysis of nasopharyngeal epithelial cells with or without LMP1 expression (27). CD133, ABCG2, and EpCAM were expressed in LMP1-expressing cells (Fig. 6A). The expression of integrins that are CSC/CPC markers, such as CD29 (β1 integrin), CD49b (α2 integrin), and CD49f (α6 integrin), was also upregulated by LMP1 expression (Fig. 6A). However, LMP1 did not affect the expression of CD90, CD117, CD166, or CXCR4 (data not shown). These results suggest that LMP1 has heterogeneous

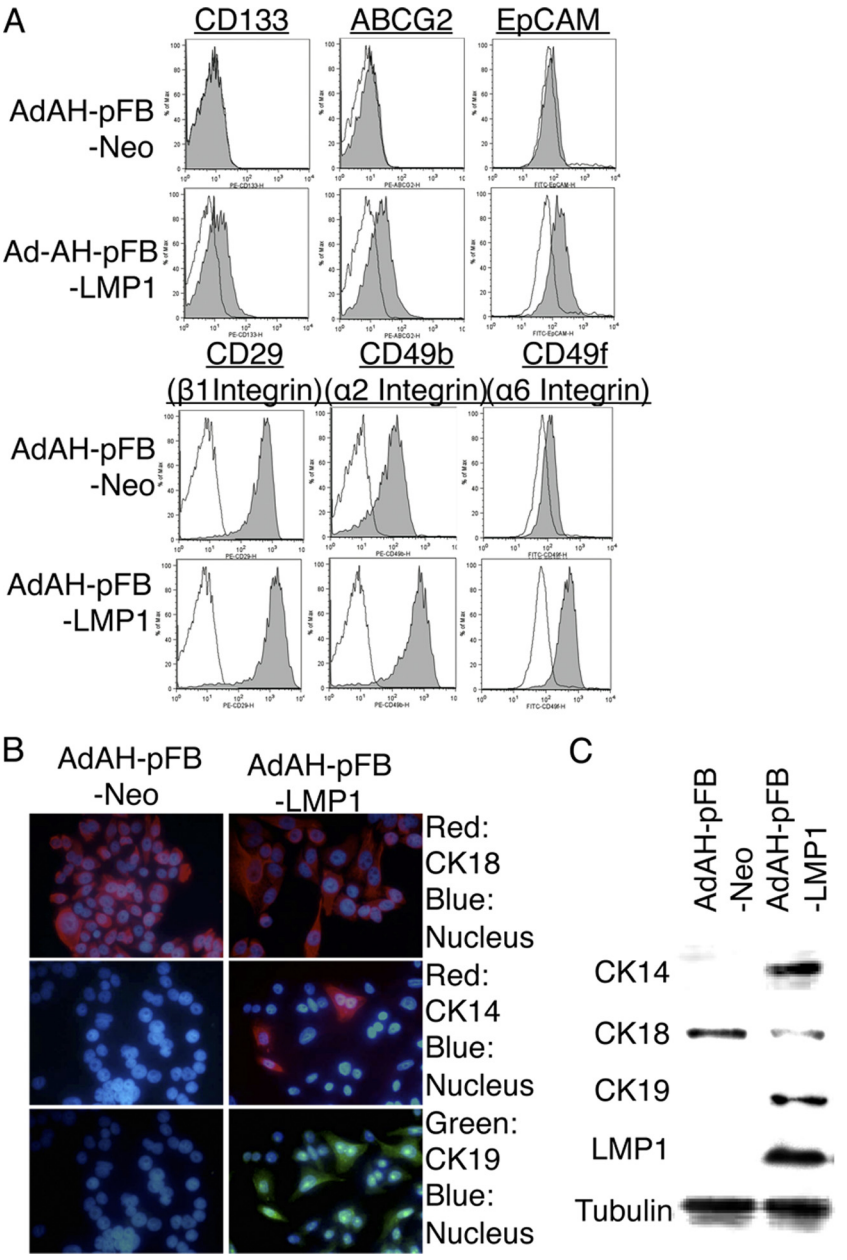

FIG. 6. LMP1 induces several CSC surface markers and undifferentiated cytokeratins (CKs). (A) Nasopharyngeal epithelial cells stably expressing LMP1 express several CSC surface markers. AdAH-pFB-Neo and AdAH-pFB-LMP1 cells were analyzed for the expression of cell surface markers. (B) LMP1 expression leads to changes in cytokeratin profiles to an undifferentiated status. Shown are images of AdAH cells with or without LMP1 immunostained with antibodies against CK14, -18, and -19. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). (C) Western blot analysis of AdAH-pFB-Neo and AdAH-pFB-LMP1 cells. CK14, CK18, CK19, and LMP1 were analyzed. Tubulin was used as a loading control.

effects on the expression of cell surface stem cell markers other than CD44 and CD24.

LMP1-induced cytokeratin profiles are consistent with an undifferentiated cell state. Cytokeratins (CKs) are indispensable markers for the classification of a cell's differentiation status. CK18 is expressed in differentiated cells, whereas CK14 is a basal cell keratin. CK19 is an undifferentiated keratin and is known as a normal stem cell marker (5). To further investigate the differentiation of LMP1-expressing cells, CK14, -18, and -19 were analyzed by immunofluorescence. As shown in Fig. 6B, LMP1 expression increased the levels of CK14 and -19, whereas CK18 was downregulated upon LMP1 expression (Fig. 6B). Immunoblot analysis revealed that LMP1 leads to increases in the levels of CK14 and CK19 and a decrease in that of CK18 (Fig. 6C). These findings suggest that LMP1 affects the CK profiles, shifting them toward an undifferentiated status.

Expression profiles of CPC-associated genes in LMP1-expressing cells. It has been reported that ABCG2$^+$ cells are CPC and have rapid cell cycling, whereas ABCG2$^-$ cells are

CSC and display little or slow cell cycling (25, 35). Previous reports showed that the expression of certain stem cell-like genes, such as *Oct4*, was decreased in ABCG2$^+$ cells (25, 26). Similarly, repression of pluripotent stem cell-like genes, such as *Oct4* and *Nanog*, is specific in CPC, whereas induction of such pluripotent stem cell-like genes is specific for the CSC phenotype in certain carcinoma cells (13, 32). For convenience, we defined the repression of several pluripotent stem cell-like genes or the induction of ABCG2 as CPC associated in this study.

According to the finding, as shown in Fig. 7A and B, that LMP1 induces ABCG2, we propose here that LMP1-expressing cells can be classified as having a CPC-like phenotype. To further analyze the characteristics of LMP1-expressing cells, levels of representative CPC-associated markers in AdAH cells with or without LMP1 expression were determined by real-time RT-PCR and immunoblotting (Fig. 7A and B). As expected, LMP1 downregulated levels of Nanog, nestin, and Oct4 compared with levels in the vector control. However, LMP1-expressing cells exhibited higher levels of KLF4 and c-Myc than the vector control.

A recent study showed that another EBV gene product, LMP2A, induces EMT as well as stem-like cancer cells by increasing side population (SP) cells, which can cause efflux of the DNA binding dye Hoechst 33342 from the cell membrane (9, 10, 16). SP cells may indicate primitive CSC, because SP cells normally exist in the quiescent phase, as do stem cells, and have stem cell characteristics (9, 10, 31, 36). To determine whether LMP1-expressing cells exhibit increased numbers of SP cells, we examined AdAH-pFB-LMP1 and AdAH-pFB-Neo cells. However, as shown in Fig. 7C, LMP1 does not affect the number of SP cells at different times of incubation (0.19% in AdAHpFB-Neo versus 0.2% in ADAH-PFB-LMP1 cells at 90 min; 0.33% in AdAH-PFB-Neo versus 0.42% in AdAH-pFB-LMP1 cells at 120 min). LMP1 does not increase the number of SP cells over that in AdAH-pFB-Neo cells. These results suggest that the CPC phenotype inducible by LMP1 has heterogeneous expression of CPC-associated markers.

TABLE 3. Hypothetical model: LMP1 induces cancer progenitor cells but not primitive cancer stem cells

Characteristic	Phenotype[a]		
	Cancer stem cells	Cancer progenitor cells	Cancer cells
Cell surface marker(s)	ABCG2$^-$ CD44$^+$	ABCG2$^+$ CD133$^+$? integrin $\alpha2\beta1^+$ CD44$^+$	ABCG2$^-$
Stem cell-like genes[b]	High level	Low level	None
Self-renewal capacity	High	Low	None
Proliferative potential	High	Low	None
Cell cycling	Slow	Rapid	None
Regulator	Other EBV products, LMP2A?	LMP1	

[a] CD133 is a potential marker of cancer progenitor cells.
[b] *Oct4* and *Nanog*, etc.

DISCUSSION

It has been reported that tumorigenic potential is exhibited almost exclusively by cells within the CD44$^+$ population (1). Thus, the identification of CD44 is the first step toward unambiguous characterization of cancer stem cells. However, whether a low level of CD24, such as that shown in our study, is essential for the phenotype of cancer stem cells is controversial. Al-Hajj et al. showed that a population of CD44^{high} CD24^{low} cells was highly tumorigenic (1). In contrast, Li et al. identified a highly tumorigenic subpopulation of CD44^{high} CD24^{high} pancreatic cancer cells (17). These discrepancies may be attributable to differences in cancer cell types, though our results are similar to those of Al-Hajj et al. (1). Thus, CD24 status is not essential for the identification of CSC/CPC.

Recent studies have proposed that solid tumors are organized as a hierarchy composed of a spectrum of CSC, CPC, and differentiated cancer cells (25, 26). According to these studies, certain markers, such as ABCG2 and integrin $\alpha2\beta1$, are putative markers that distinguish CSC from CPC (25, 26). Similarly, there are several reports that CD133 and integrin $\alpha2\beta1$ are enriched in CPC (6, 25). From these studies and the present study, we propose here that LMP1 is a potential inducer of the phenotype of CPC but not CSC (Table 3). CSC have greater self-renewal abilities and proliferative potentials but normally have slow cycling. In contrast, CPC proliferate more actively and exhibit rapid cycling but a lower self-renewal capacity. However, the question remains: if LMP1 can initiate CPC, what factors produce primitive CSC? A recent report from Kong et al. (16) showed that LMP2A induces EMT as well as stem-like cancer cells by increasing the number of SP cells, suggestive of primitive CSC, because SP cells normally exist in the quiescent phase, as do stem cells (9, 10, 31, 36). Burkert et al. proposed that the concept of the SP phenotype as a universal marker of stem cells does not apply to certain cancer cells (2). Whether the SP phenotype is a CSC marker is still controversial. In the present study, LMP1 did not affect the number of SP cells. Thus, we hypothesize here that LMP2A is a true inducer of primitive CSC, whereas LMP1 induces CPC. Both LMP1 and LMP2A induce CSC or CPC phenotypes; however, to clarify the differences between these two EBV products, further investigations are required.

In the present study, expression of some CPC-associated genes, such as *Oct4* and *Nanog*, was decreased with LMP1 expression. Since repression of certain pluripotent stem cell-like genes is a feature of CPC, these results support the hypothesis that LMP1 induces the CPC but not the CSC phenotype (25, 26). However, with LMP1 induction, other "stemness" genes, such as the c-Myc and KLF4 genes, are upregulated. These genes are closely related to oncogenic activity or malignant potential (18, 33). These results suggest that the LMP1-inducible CPC phenotype has heterogeneous effects on the expression of CPC-associated genes. Further investigations are required for the definition of CSC/CPC phenotypes.

During the process of cancer metastasis, which is enabled by EMT, the disseminated cancer cells seem to acquire self-renewal abilities, similar to those exhibited by stem cells. The present and previous studies have shown that LMP1 induces EMT via Twist and Snail (11, 12). In this study, we show that LMP1-induced human epithelial cells have both CSC/CPC and

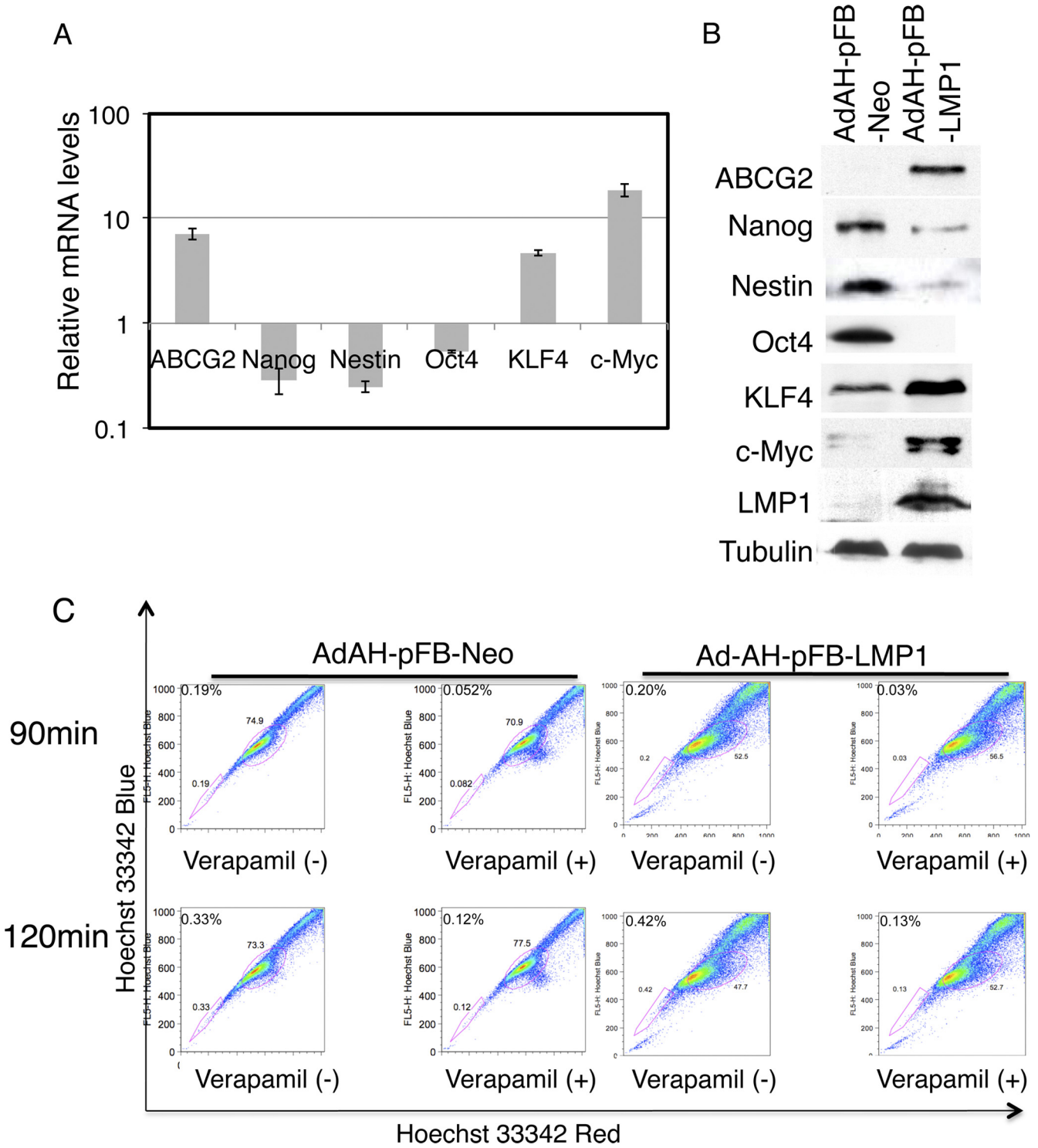

FIG. 7. LMP1 induces phenotypes of cancer stem/progenitor cells. (A) Expression levels of mRNAs encoding ABCG2, Nanog, nestin, Oct4, KLF4, and c-Myc in AdAH-pFB-LMP1 cells relative to those in AdAH-pFB-Neo cells as determined by real-time PCR. GAPDH mRNA was used to normalize variability in template loading. The data are means ± standard deviations. (B) Western blot analysis of AdAH-pFB-Neo and AdAH-pFB-LMP1 cells. ABCG2, Nanog, nestin, Oct4, KLF4, c-Myc, and LMP1 were analyzed. Tubulin was used as a loading control. (C) Flow cytometric profiles of SP cells in AdAH-pFB-LMP1 and AdAH-pFB-Neo cultures. SP cell profiles in the presence or absence of verapamil are shown. Percentages of SP cells are given.

EMT characteristics, confirming the close link between CSC/CPC and an EMT mechanism in LMP1-expressing cells. Keratin profiles in LMP1-expressing cell lines were also investigated. Here we show that LMP1 affects the CK profile, shifting it toward an undifferentiated status (5). Our results suggest that LMP1 is essential for maintaining the undifferentiated status in EBV-associated malignancies, such as NPC. These results suggest that LMP1 signaling is a key regulator of the pathobiological characteristics of NPC, not only its highly metastatic properties but also its undifferentiated status.

In conclusion, LMP1 is the first identified initiator that induces CPC but not CSC. Understanding the role of EBV infection in the generation of CSC/CPC in NPC can be an important step toward further clarification of the biological significance of general CSC and CPC theories.

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