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Cyclin D1 overexpression supports stable EBV infection in nasopharyngeal epithelial cells

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Undifferentiated nasopharyngeal carcinomas (NPCs) are commonly present with latent EBV infection. However, events regulating EBV infection at early stages of the disease and the role of EBV in disease pathogenesis are largely undefined. Genetic alterations leading to activation of cyclin D1 signaling in premalignant nasopharyngeal epithelial (NPE) cells have been postulated to predispose cells to EBV infection. We previously reported that loss of p16, a negative regulator of cyclin D1 signaling, is a frequent feature of NPC tumors. Here, we report that early premalignant lesions of nasopharyngeal epithelium overexpress cyclin D1. Furthermore, overexpression of cyclin D1 is closely associated with EBV infection. Therefore we investigated the potential role of cyclin D1 overexpression in dysplastic NPE cells in vitro. In human telomerase reverse transcriptase-immortalized NPE cells, overexpression of cyclin D1 or a p16-resistant form of CDK4 (CDK4^{R24C}) suppressed differentiation. This suppression may have implications for the close association of EBV infection with undifferentiated NPC. In these in vitro models, we found that cellular growth arrest and senescence occurred in EBV-infected cell populations immediately after infection. Nevertheless, overexpression of cyclin D1 or a p16-resistant form of CDK4 or knockdown of p16 in the human telomerase reverse transcriptase-immortalized NPE cell lines could counteract the EBV-induced growth arrest and senescence. We conclude that dysregulated expression of cyclin D1 in NPE cells may contribute to NPC pathogenesis by enabling persistent infection of EBV.

Epstein-Barr virus | episome | viral persistence

EBV infection is detected in nearly all undifferentiated or poorly differentiated nasopharyngeal carcinoma (NPC) in endemic regions, such as southern China, including Hong Kong (1). Establishment of persistent infection in nasopharyngeal epithelial (NPE) cells is believed to be an early and essential step for NPC development (2, 3). However, events regulating EBV infection in premalignant NPE cells are largely undefined.

Although EBV infection in normal nasopharyngeal epithelium is uncommon, latent EBV infection could be detected ubiquitously in preinvasive lesions and is believed to play a crucial role in NPC pathogenesis (4). The detection of a single clonal EBV genome in these preinvasive lesions and NPC indicated that NPC pathogenesis involves clonal expansion of a single EBV-infected NPE cell (3, 4). EBV may be able to infect cells in premalignant nasopharyngeal epithelium, but it is likely that only specific clones with permissive cellular content can support long-term propagation of EBV. The current hypothesis is that genetic alterations or dysregulated cell-signaling pathways in premalignant NPE cells render them permissive for EBV persistence and malignant transformation (3, 5–7). We have established several primary and human telomerase reverse transcriptase (hTert)-immortalized cell lines to serve as cell models for EBV infection study (8). None of these cells was able to support stable EBV infection in prolonged culture, except for NP460hTert, which harbors a p16 deletion (8). We reported previously that allelic deletion of chromosome 9p (involving the loci of p16^{INK4A}) is a common feature of NPC and can be detected in the rare, low-grade dysplastic lesions of nasopharyngeal epithelium before EBV infection (5, 9). Furthermore, using high-resolution comparative genomic hybridization arrays, we mapped the gain of a 5.3-Mb amplicon including the cyclin D1 locus in chromosome 11q13.1 in NPC (10). Concordant amplification and/or overexpression of cyclin D1 is a common event in primary NPC and was detected in 35 of 38 primary NPC biopsies (92%). Deletion of p16 and overexpression of cyclin D1 lead to dysregulation and activation of the cyclin D1 signaling pathway, respectively. However, it remains to be determined if these genetic alterations predispose NPE cells to persistent EBV infection.

Cyclin D1 is a key cell-cycle regulatory protein promoting G_1/S transition in cells (11). It complexes with CDK4 or CDK6 to phosphorylate the retinoblastoma tumor suppressor protein, resulting in the release of the E2F transcription factor to promote cell-cycle progression (12). More importantly, ectopic expression of cyclin D1 has been shown to drive cell-cycle progression and to suppress terminal differentiation in several cellular systems, including keratinocytes, myotubes, adipocytes, and nerve cells (11, 13). An earlier in vitro study indicated that undifferentiated cells may be more permissive for latent EBV infection by supporting expression of EBNA1 (14). All these observations have implications for the close association of EBV infection with undifferentiated but not differentiated NPC.

We postulated that cyclin D1 may be involved directly in promoting cell-cycle progression and suppression of terminal differentiation of NPE cells, thus conferring a permissive cellular environment for persistent EBV infection and its propagation in NPE cells. In this study, we provide experimental evidence to support these roles of cyclin D1 in facilitating stable and latent EBV infection in premalignant NPE cells.

Results

Overexpression of Cyclin D1 is Common in EBV-Infected Dysplastic NPE Tissues. We previously reported that overexpression of cyclin D1 is a common event in NPC and can be detected in more than 90% of NPC specimens examined (35 of 38 cases) (10). To

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examine if aberrant expression of cyclin D1 is associated with EBV infection at early stage of disease development, expression of cyclin D1 and EBV-encoded RNA (EBER) was examined in consecutive histopathological sections of six rare archival specimens of dysplastic NPE tissues by immunohistochemistry and by in situ hybridization. Overexpression of cyclin D1 could be detected in all of the six cases examined. Overexpression of cyclin D1 coexisted with positive EBER staining in the epithelium of these dysplastic NPE tissues, supporting a close link between cyclin D1 overexpression and EBV infection in the early stage of NPC development (Fig. 1). In contrast, cyclin D1 was detected only in basal and suprabasal cell layers of the EBER-negative normal nasopharyngeal epithelium. Interestingly, LMP1 was expressed in one of the six dysplastic NPE tissues examined (Fig. 1). In our experience, LMP1 is rarely detected by immunocytochemistry in preneoplastic NPE tissues. The role of LMP1 in the early stage of NPC development remains to be determined.

Overexpression of Cyclin D1 Conferred Resistance to Serum-Induced Differentiation in hTert-Immortalized NPE Cells. EBV infection is closely associated with the histologically undifferentiated type of NPC. Overexpression of cyclin D1 is known to suppress cellular differentiation and can be detected readily in dysplastic NPE tissues (Fig. 1). We investigated a potential role of cyclin D1 overexpression or activation of the cyclin D1/CDK4 pathway in contributing to the undifferentiated property of dysplastic NPE cells using hTert-immortalized NPE cell lines derived from nonmalignant nasopharyngeal tissues (8). All these immortalized NPE cell lines have been propagated in serum-free medium for more than 100 passages and show no sign of growth cessation or differentiation. Serum is a potent agent commonly used to induce terminal differentiation in culturing epithelial cells. To examine if activation of the cyclin D1 pathway might influence the ability of NPE cells to resist differentiation, we overexpressed either cyclin D1 or a CDK4 mutant (CDK^{R24C}) in these immortalized NPE cell lines and examined their responses toward serum-induced differentiation. $CDK4^{R24C}$ is a dominantly active mutant of CDK4 insensitive to its inhibitor, p16. Overexpression of $CDK4^{R24C}$ resulted in constitutive activation of the cyclin D1/CDK4 complex in immortalized NPE cells. Although treatment with 10% (vol/vol) FBS effectively induced growth arrest in all the control hTert-immortalized NPE cell lines, cells overexpressing cyclin D1 or $CDK4^{R24C}$ were resistant to it (Fig. 2*A*). NPE cells overexpressing cyclin D1 or $CDK4^{R24C}$ continued to proliferate in the presence of serum (Fig. 2*A*). Furthermore, a terminally differentiated phenotype with enlarged and flattened cell morphology and with prominent intracellular keratin networks was observed in control NP550hTert or NP361Tert cells after treatment with serum but not after transduction of cyclin D1 or $CDK4^{R24C}$ (Fig. S1). In addition, the expression of involucrin, a marker for the terminal differentiation of squamous epithelium, was detected after FBS treatment in control NP550hTert cells but not in cells overexpressing cyclin D (Fig. 2*B*). A role for cyclin D1 overexpression and activation of the cyclin D1/CDK4 pathway in resisting differentiation in immortalized NPE cells was confirmed further by Western blots showing up-regulated expression of involucrin in the control immortalized NPE cells after FBS treatment but not in NPE cells overexpressing cyclin D1/$CDK4^{R24C}$ (Fig. 2*C*).

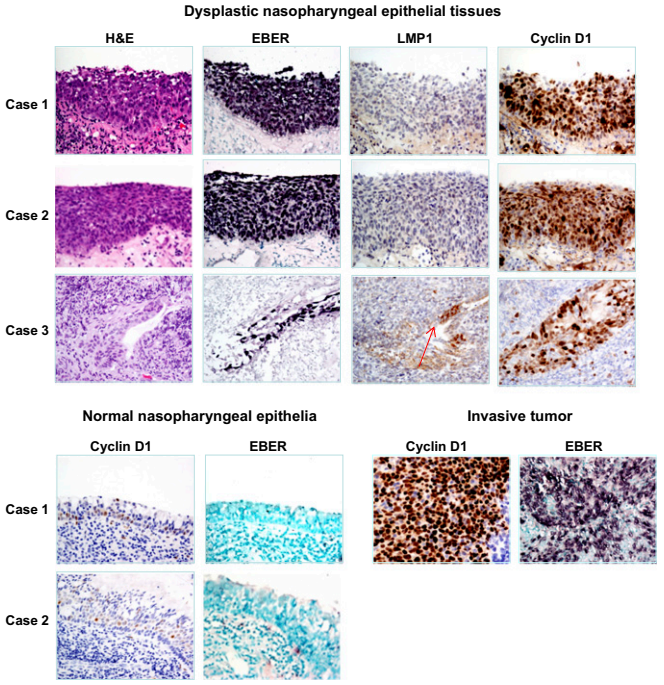

Fig. 1. Overexpression of cyclin D1 and EBV infection in dysplastic nasopharyngeal epithelium. (*Upper*) Representative images of H&E staining, EBER in situ hybridization, and immunohistochemical staining of cyclin D1 and LMP1 performed in consecutive sections of three specimens of dysplastic NPE tissue. In the first column from the left, H&E staining revealed a dysplastic epithelium. In the second column, the dysplastic epithelial cells were $EBER^+$ by in situ hybridization. In the third column, LMP1 expression (red arrow) was detected in one of the six dysplastic nasopharyngeal lesions. In the fourth column, overexpression of cyclin D1 was detected in dysplastic epithelium. (*Lower*) Images of two cases of normal epithelium and one invasive NPC tissue. (*Left*) Expression of cyclin D1 was detected in the basal and suprabasal layers in normal and $EBER^-$ nasopharyngeal epithelium. (*Right*) Overexpression of cyclin D1 in $EBER^+$ NPC.

Activation of the Cyclin D1 Pathway Supports Clonal Proliferation of EBV-infected Cells. Next, we examined whether activation of the cyclin D1 pathway could support persistent EBV infection in hTert-immortalized cells (NP550hTert and NP361hTert). Overexpression of cyclin D1 and $CDK4^{R24C}$ and p16 silencing were achieved in NP550hTert or NP361hTert cells (Fig. 3*A*). We then subjected these NPE cells to EBV infection using a previously published protocol (8). The immortalized NPE cells were plated at a low cell density after EBV infection to observe the appearance of proliferative EBV-infected (GFP^+) colonies. In our previous study, we had seen that the ability of EBV-infected NPE cells to form proliferative colonies is a reliable indication of stable EBV infection in NPE cells (8). Proliferative EBV-infected (GFP^+) colonies were readily observed in hTert-immortalized cells after cyclin D1 or $CDK4^{R24C}$ transducation or p16 silencing, but not in the respective control cells (Fig. 3*A*). These results suggest that activation of the cyclin D1/CDK4 pathway supports stable EBV infection in NPE cells. Moreover, susceptibility to EBV infection was not altered significantly in NPE cells overexpressing cyclin D1 and CDK^{R24C} or by knockdown of p16 (Fig. S2), suggesting that the mechanism for supporting stable EBV infection is dependent more on cellular content than on an enhanced rate of infection.

We have established additional immortalized NPE cells using various defined genetic components. When examined for their ability to support EBV infection, only NPE cells immortalized by hTert in combination with either cyclin D1 or $CDK4^{R24C}$ were able to support stable EBV infection. Proliferative EBV-infected clones were readily observed in these cells but not in control NPE cells immortalized by hTert alone (Fig. 3*B* and Table 1). The ectopic expression of cyclin D1 and $CDK4^{R24C}$ in these cells also was confirmed by Western blotting analysis (Fig. S3). Cyclin D1 and $CDK4^{R24C}$ levels were two- or threefold higher in the HA-cyclin D1/$CDK4^{R24C}$ transfectants. Apparently, these incremental levels of cyclin D1 and $CDK4^{R24C}$ are sufficient to support clonal proliferation of EBV-infected NPE cells. These observations provide further evidence supporting the hypothesis

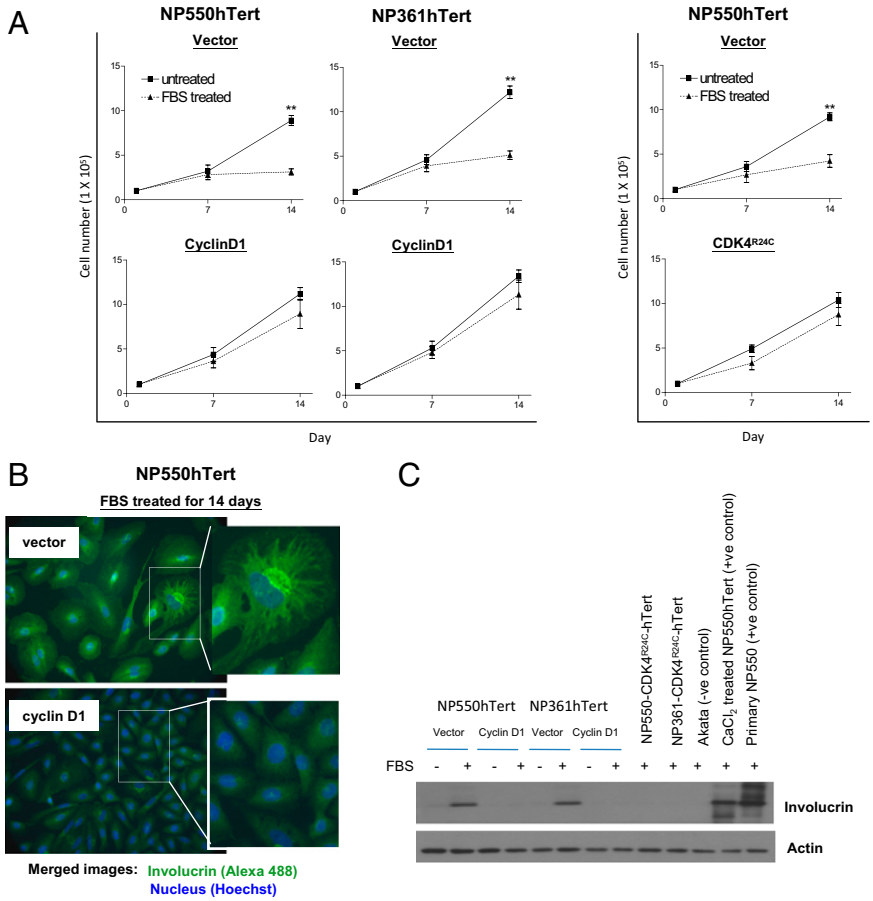

Fig. 2. Cells overexpressing cyclin D1 can resist differentiation induced by serum treatment. (*A*) (*Left*) Comparison of proliferation rates of control (infected with an empty retroviral vector) and cyclin D1-overexpressing NP550hTert and NP361hTert cells with or without FBS treatment. (*Right*) Growth curves of the control and $CDK4^{R24C}$-overexpressing NP550hTert cells treated with FBS. All the control cells eventually lost their proliferative potential after FBS treatment. The proliferative potentials after FBS treatment were much less affected in NP550hTert and NP361hTert cells overexpressing cyclin D1 than in control cells. Means and SDs were calculated from triplicate wells of two independent experiments. $^{**}P < 0.01$; two-tailed Student *t* test. (*B*) NP550hTert cells overexpressing cyclin D1 expressed much lower levels of involucrin (detected by immunofluorescence staining) than did control cells. (*C*) Western blot analysis shows that involucrin expression was induced in NP550hTert and NP361hTert control cells upon treatment with FBS but not in cyclin D1- or $CDK4^{R24C}$-transduced NP361hTert and NP550hTert cells. Akata cell lysate was used as the negative control for involucrin detection. High calcium content is known to induce robust differentiation in hTert-immortalized epithelial cells. Cell lysate of $CaCl_2$-treated NP550hTert cells was included as a positive control for involucrin detection. Primary NP550 cells, which are highly responsive to FBS-induced differentiation, were included as a positive control for involucrin detection.

that activation of the cyclin D1/CDK4 signaling pathway enables stable EBV infection in immortalized NPE cells.

EBV-Infection Induced Growth Inhibition and Senescence in NPE Cells Immortalized by hTert Alone. We then investigated the underlying cause prohibiting stable EBV infection in NP550hTert and NP361hTert cells that were immortalized by hTert alone. The rates of loss of EBV-infected NP550hTert and NP361hTert cells after infection were monitored when the cells were passaged at 7, 14, and 21 days postinfection (DPI) with a splitting ratio of 1:3 (Fig. 4*A*). The percentage of EBV-infected cells in hTert-immortalized NPE cell lines dropped rapidly at each passage (Fig. 4*A*). In our previous study (8) we had observed that the EBV-infected NPE cells have a lower growth rate than uninfected cells. The loss of EBV-infected cells may be a result of uninfected cells outgrowing the EBV-infected cells during prolonged culture. We proceeded to examine the effects of EBV infection on growth and cellular senescence in hTert-immortalized NPE cells. The expression of p16 and p21 (markers for cell-cycle arrest) in EBV-infected NP550hTert cells was examined at 14 DPI by immunofluorescence staining (Fig. 4*B*). A higher percentage of EBV-infected NP550hTert cells than of uninfected NP550hTert cells expressed p16 and p21. To confirm further the up-regulation of p16 in EBV-infected NP550hTert cells, the EBV-infected (GFP^+) and uninfected (GFP^-) cells were separated by FACS and were examined for p16 expression by immunostaining. Again,the EBV-infected NP550hTert cells expressed a higher level of p16 (Fig. 4*C*). We then separated the EBV-infected cells from uninfected cells by FACS at 3, 7, and 14 DPI and examined the percentage of cells undergoing cellular senescence by senescence-associated (SA)–β-galactosidase staining (an established biochemical marker for cellular senescence) (Fig. 4*D*). In both cell lines, a significantly higher percentage of cells expressed SA–β-galactosidase in the EBV-infected populations than in uninfected populations (Fig. 4*D*). All these observations showed that growth arrest and cellular senescence are common features of hTert-immortalized NPE cell lines after infection by EBV and may explain their slower growth rate compared with uninfected cells.

Overexpression of Cyclin D1 in NPE Cells Overrides Growth Inhibition and Cellular Senescence Induced by EBV Infection. To see if overexpression of cyclin D1 overrides the growth inhibition induced by EBV infection, we separated EBV-infected and uninfected cells (at 7 DPI) in both control and cyclin D1-overexpressing NP550hTert cells by FACS and compared their growth rates (Fig. 5*A*). The EBV-infected populations of the vector control NP550hTert cells failed to proliferate up to day 14 after sorting, whereas the NP550hTert cells overexpressing cyclin D1 continued to proliferate, albeit at a slower rate than uninfected cells (Fig. 5*A*). Using SA–β-galactosidase staining, we also showed that overexpression of cyclin D1 in NP550hTert cells suppressed the cellular senescence induced by EBV infection. EBV-induced cellular senescence is reduced almost fourfold in cells overexpressing cyclin D1 as compared with control NP550hTert cells (Fig. 5*B*). The EBV-infected NP550hTert cells overexpressing or not overexpressing cyclin D1 were examined further for proliferative potential by a BrdU incorporation assay. BrdU incorporation was 4.6-fold higher in EBV-infected NP550hTert cells overexpressing cyclin D1 than in control NP550hTert cells (Fig. 5*C*). Taken together, these observations strongly indicate that overexpression of cyclin D1 suppresses EBV-induced growth inhibition and cellular senescence in premalignant NPE cells.

Effects of Cyclin D1 Overexpression in Regulating Lytic and Latent EBV Gene Expression in Immortalized NPE Cells. Overexpression of cyclin D1 could suppress differentiation in immortalized NPE cells

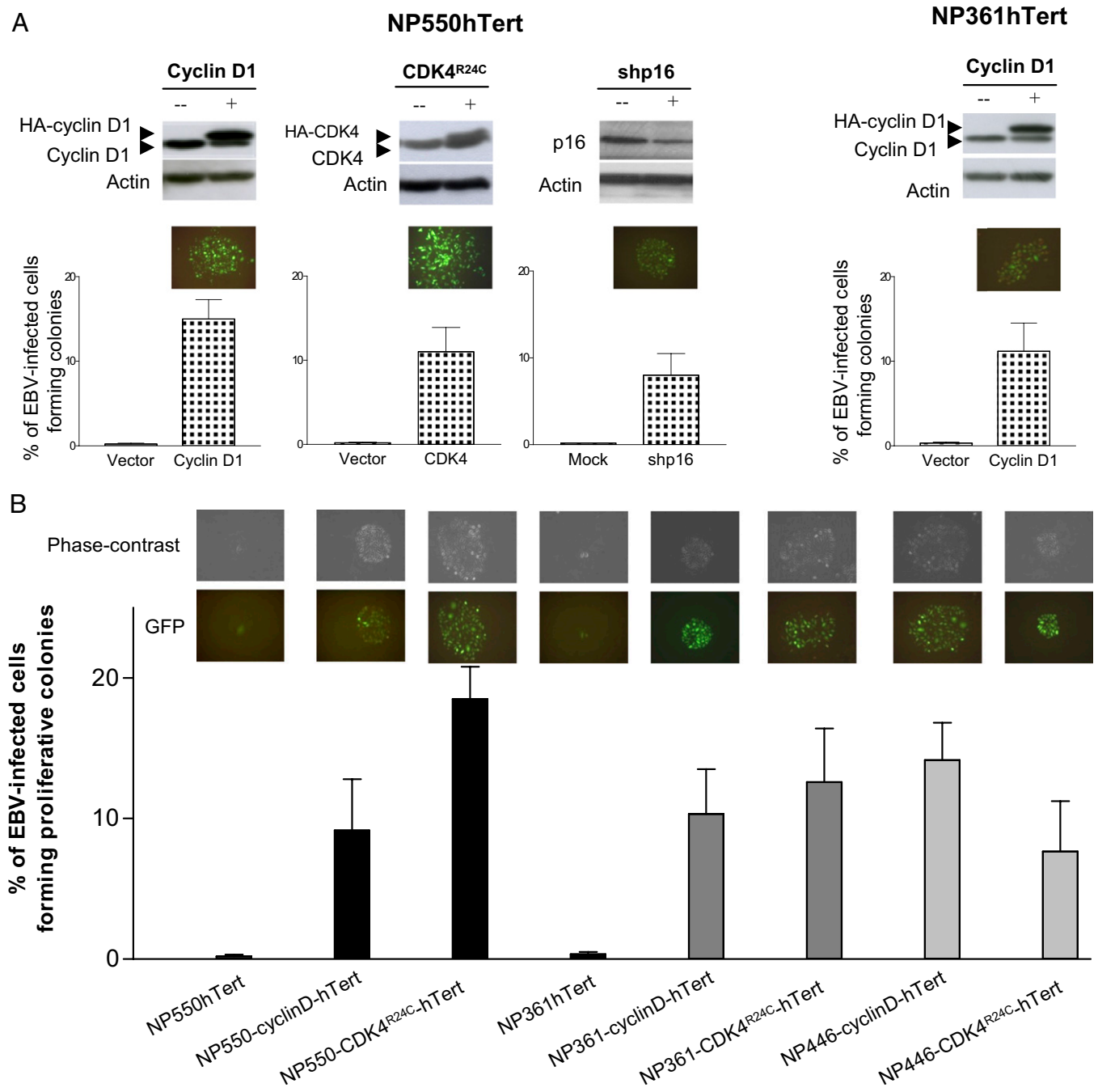

Fig. 3. Activation of the cyclin D1 pathway enhanced clonal proliferation of EBV-infected cells. (*A*) Western blots confirming the exogenous expression of cyclin D1 or CDK4^{R24C} (a p16-insensitive CDK4 mutant) or knockdown of p16 by shRNA in hTert-immortalized NPE cells. After infection with EBV, the immortalized NPE lines were sorted for GFP^{+} cells, were plated at a low cell density, and were inspected for the appearance of proliferative EBV-infected (GFP^{+}) colonies 21 d after sorting. Clonal proliferation was observed in all EBV-infected immortalized NPE cells overexpressing either cyclin D1 or CDK4^{R24} or after p16 expression was knocked down but not in their control parental lines infected with an empty vector. The morphologies of proliferative EBV-infected colonies (GFP^{+}) from immortalized NPE cells expressing cyclin D1 or CDK4^{R24C} or with p16 down-regulation are shown here. (*B*) Clonal proliferation of EBV-infected cells also was observed in NPE cell lines immortalized by the combined action of hTert and either cyclin D1 or CDK4^{R24C} but not in NPE cells immortalized by hTert alone. Phase-contrast and fluorescent images show the formation of colonies in cell lines overexpressing cyclin D1 or CDK4^{R24C} but not in cell lines immortalized by hTert alone.

(Fig. 2). Previous studies have suggested that cellular differentiation could induce lytic reactivation of EBV (14, 15). Therefore, we investigated the effect of overexpressing cyclin D1 on the expression of lytic and latent genes of EBV by real-time PCR, immunocytochemistry, and Western blotting analysis. Seven days after infection, the EBV-infected control and cyclin D1-overexpressing NP550hTert cells were live-sorted by FACS and examined for transcript levels of lytic and latent genes. The EBV-infected cells overexpressing cyclin D1 had higher levels of mRNA transcripts of latent EBV genes (*EBER1/2* and *EBNA1*) but lower transcript levels of EBV lytic genes (*BZLF1*, *BRLF1*, *BMRF1*, and *BGLF4*) than control NP550hTert cells (Fig. 6*A*). We further examined the expression of EBV-encoded proteins at the single-cell level using immunofluorescence staining. The

Table 1. Immortalized NPE cell lines with dysregulated components in the cyclin D1 pathway support proliferation of EBV-infected cells and their establishment in stable EBV-infected cell lines

Cell line	Dysregulated component in cyclin D1 pathway	Appearance of proliferative clones after EBV infection	Establishment of stable line with EBV infection?
NP460htert	p16 deletion	yes	yes
NP550hTert	—	No	No
NP361hTert	—	No	No
NP550hTert+cyclinD1	cyclinD1 overexpression	yes	yes
NP361hTert+cyclinD1	cyclinD1 overexpression	yes	yes
NP446-cyclinD1-hTert	cyclinD1 overexpression	yes	yes
NP550-CDK4^{R24C} -hTert	CDK4^{R24C} overexpression	yes	yes
NP361-CDK4^{R24C} -hTert	CDK4^{R24C} overexpression	yes	yes
NP446-CDK4^{R24C} -hTert	CDK4^{R24C} overexpression	yes	yes

EBV-infected populations of both control and cyclin D1-overexpressing cells were purified by FACS at 3, 7, and 14 DPI and were examined for expression of LMP1, BZLF1, and viral capsid antigen p18 (VCAp18) (Fig. 6*B*). Expression of LMP1 and BZLF1 was observed in only a small subset (3–6% at 3 DPI) of EBV-infected cells (Fig. 6*B*). We did not observe a significant difference in the percentage of LMP1-expressing cells between the control and cyclin D1-overexpressing NPE cells. The population of BZLF1^{+} cells ranged from 4–6% and appeared to be lower in cells overexpressing cyclin D1 than in control cells (Fig. 6*B*). The number of cells expressing BZLF1 diminished progressively at 7 DPI and 14 DPI. Another lytic EBV protein, VCAp18, which is expressed at a late stage of lytic replication, was barely detectable in EBV-infected cells by immunocytochemistry (Fig. 6*B*).

Western blot analysis also was performed to compare the protein levels of LMP1 and BZLF1 in various EBV-infected cell lines. EBV-transformed B-cell lines, including Akata cells before and after lytic activation, and a lymphoblastoid cell line (LCL) were included as positive controls for detection of LMP1 and BZLF1 expression by Western blotting (Fig. 6*C*). Cyclin D1 overexpression in immortalized NPE cells apparently had a minimal impact on levels of LMP1 expression but appeared to downregulate the expression of BZLF1 (Fig. 6*C*).

Western blot analysis revealed a gradual loss of LMP1 expression in the EBV-infected cells after several rounds of sorting for GFP^{+}, whereas BZLF1 expression rapidly diminished and became undetectable after the first round of sorting (Fig. 6*D*). The Cp promoter is involved in the transcription of EBNA3C and -3A in EBV-infected B cells to bypass the G1/S checkpoint for maintaining the growth of LCL (16). The Qp promoter usually is active in latent EBV infection in epithelial cells. Using semiquantitative RT-PCR, we examined the activity of both Cp and Qp in infected cells. We confirmed that the Cp promoter was inactive during EBV infection in epithelial cells, whereas Qp was substantially activated in EBV-infected NPE cells, particularly at 100 DPI (Fig. 6*E*).

Establishment of Stably EBV-Infected Cell Lines. After subsequent rounds of cell sorting by GFP fluorescence, stable EBV-infected cell lines were established in NP550hTert and NP361hTert cells overexpressing cyclin D1 (Table 1 and Fig. 7*A* and Fig. S4). Both lines were passaged for more than 18 mo, and more than 95% of cells retained the EBV genome (Fig. 7*A* and Fig. S4). Varying copy numbers of the EBV genomes were detected in the nuclei of EBV-infected cells, as demonstrated by FISH for EBV genomes (Fig. 7*B*). The high copy number of EBV genomes (the majority of cells contained 25–100 copies) (Fig. 7*B*) in the stably infected cell lines implies that the cells have amplified the episomal virus genome after infection. Most, if not all, the hybridization signals were obtained on only one chromatid in the paired-sister chromatids (Fig. 7*B*), indicating that the majority of EBV genomes in EBV-infected NPE cells were not integrated into host chromosomes.

Similarly, stable EBV-infected lines were established by sorting the EBV-infected cells from NPE cells immortalized by combined action of hTert with either cyclin D1 or CDK4^{R24C} (NP550-CDK4^{R24C}-hTert, NP361-CDK4^{R24C}-hTert, NP446-cyclinD1-hTert, and NP446- CDK4^{R24C}-hTert) (Table 1 and Fig. S5) but not in NPE cells immortalized by hTert alone, except for NP460hTert, which harbors the p16 deletion. These cell lines were able to retain more than 90% of EBV-infected cells over long period of propagation (Fig. S5). Real-time PCR and Western blots were performed to access various EBV-encoded genes in transcriptional and protein levels (Fig. 7*C* and Fig. S6). The expression levels of EBER1/2 were comparable among all the stably infected NPE cell lines and ranged from 0.2-fold to 0.8-fold that of Akata cells (Fig. S6). However, the number of EBNA1 and LMP1 transcripts was much lower in EBV-infected NPE cell lines than in EBV-infected Akata cells (Fig. S6). Interestingly, despite the low transcript level, EBNA1 protein levels in NPE cell lines were comparable to levels in Akata cells, as shown in the Western blot analysis (Fig. 7*C*). Western blots also showed that the stably EBV-infected NPE cell lines expressed low levels of LMP1 but not of BZLF1 (Fig. 7*C*).

Discussion

Cyclin D1 Expression Suppresses Growth Inhibition and Senescence Caused by EBV Infection. The current hypothesis of NPC pathogenesis is that infection of EBV in premalignant nasopharyngeal lesions harboring genetic alterations facilitates and promotes malignant transformation of NPE cells (3, 5, 7). We previously reported that cyclin D1 overexpression is a common event in NPC (10). In this study, we report that cyclin D1 overexpression also is common in dysplastic NPE tissues and coexists with EBV infection (Fig. 1). The close association of EBV infection and overexpression of cyclin D1 prompted us to investigate if there is a functional relationship between these two early events in NPC pathogenesis. During differentiation, cyclin D1 activity is inhibited, and cells exit the cell cycle. In contrast overexpression of cyclin D1 promotes cell-cycle progression and perturbs cellular differentiation (11, 13). A role of cyclin D1 in suppressing responses of immortalized NPE cells to serum-induced differentiation was observed in this study (Fig. 2) and may have implications for the close association of EBV infection with undifferentiated NPC.

In this study, we observed that EBV-infected NPE cells immortalized by hTert alone failed to propagate and were lost rapidly upon serial passages (Figs. 3 and 4*A*). We attributed the rapid loss of EBV-infected NPE cells to the induction of growth inhibition and cell senescence caused by EBV infection. Growth inhibition of EBV-infected hTert-immortalized cells was associated with the expression of high levels of p16 and p21, positive expression of SA–β-galactosidase, and lower rates of BrdU incorporation (Figs. 4 and 5). The induction of growth arrest in EBV-infected NPE cells may be a reason for the infrequent detection of EBV in normal nasopharyngeal epithelium in vivo (2). Additional factors may underlie the failure of EBV-infected normal NPE cells to propagate in vivo. For example, EBV infection per se may activate host immune-defense mechanisms to

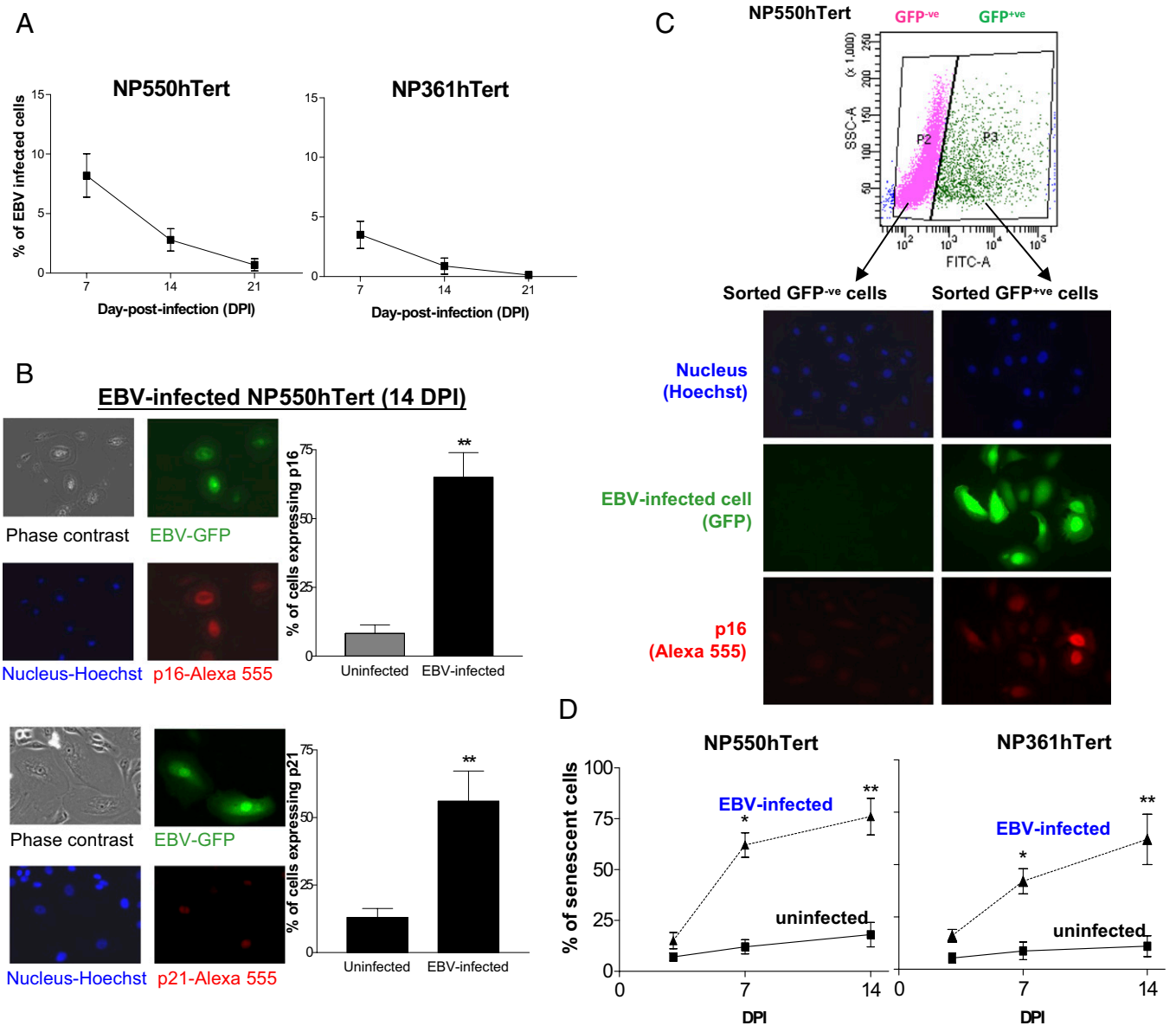

Fig. 4. EBV infection induced growth inhibition and senescence in hTert-immortalized NPE cells. (*A*) NP550hTert and NP361hTert cell lines were infected with EBV, and the percentage of EBV-infected cells was estimated at 7, 14, and 21 DPI. The percentage of EBV-infected NP550hTert and NP361hTert cells (GFP^+) dropped rapidly with each passage. (*B*) EBV infection induced expression of p16 and p21 in hTert-immortalized NPE cells. NP550hTert cells were infected with EBV, and the expression of p16 and p21 was detected by immunofluorescence staining. The EBV-infected cells showed significant up-regulation of p16 and p21 compared with uninfected cells. Means and SDs were calculated from triplicate wells. $^{**}P < 0.01$; two-tailed Student *t* test. (*C*) p16 expression was higher in FACS-enriched EBV-infected NP550hTert populations than in uninfected cells. (*D*) Induction of cellular senescence was observed in NP550hTert and NP361hTert cells after EBV infection. At 3, 7, and 14 DPI, cells were trypsinized and sorted into EBV-infected and uninfected populations based on GFP fluorescence. Higher SA–β-galactosidase activity was observed in the EBV-infected cells than in uninfected cells at 7 and 14 DPI. Means and SDs were calculated from triplicate wells. $^{*}P < 0.05$; $^{**}P < 0.01$; two-tailed Student *t* test.

suppress the proliferation of infected cells through the induction of type I interferons (17). It also is common for the invading virus to shut down the host's replication machinery to facilitate viral DNA replication (18, 19). For example, infection by influenza A virus, T-cell leukemia virus, and corona virus are all known to arrest the infected host cells at the G_0/G_1 phase (20–22). Expression of EBV lytic genes, e.g., *BGLF5*, *BZLF1*, and *BRLF1*, also can induce cell-cycle arrest in infected cells (19, 23, 24).

Importantly, we observed that overexpression of cyclin D1 in NPE cells can rescue the growth inhibition and senescence phenotypes caused by EBV infection (Fig. 5). In this study, we showed that immortalized NPE cell lines can be infected by EBV at variable infection rates (Fig. S2). However, stably EBV-infected NPE cell lines could be established only in immortalized NPE cells overexpressing cyclin D1 or in NPE cells with dysregulated cyclin D1-related pathways by expressing the p16-insensitive $CDK4^{R24C}$ or p16 silencing (Table 1). In addition, we reported previously that stable EBV infection can be achieved in a telomerase-immortalized NPE cell line, NP460hTert, harboring the p16 deletion (25). Furthermore, the *p16* gene, which is the key inhibitor of the cyclin D1/CDK4 activity, frequently is deleted or inactivated by methylation in premalignant nasopharyngeal epithelium before EBV infection (6, 26). We conclude that overexpression of cyclin D1 or aberrant activation of the cyclin D1 pathway may provide advantages supporting stable EBV infection in premalignant NPE cells.

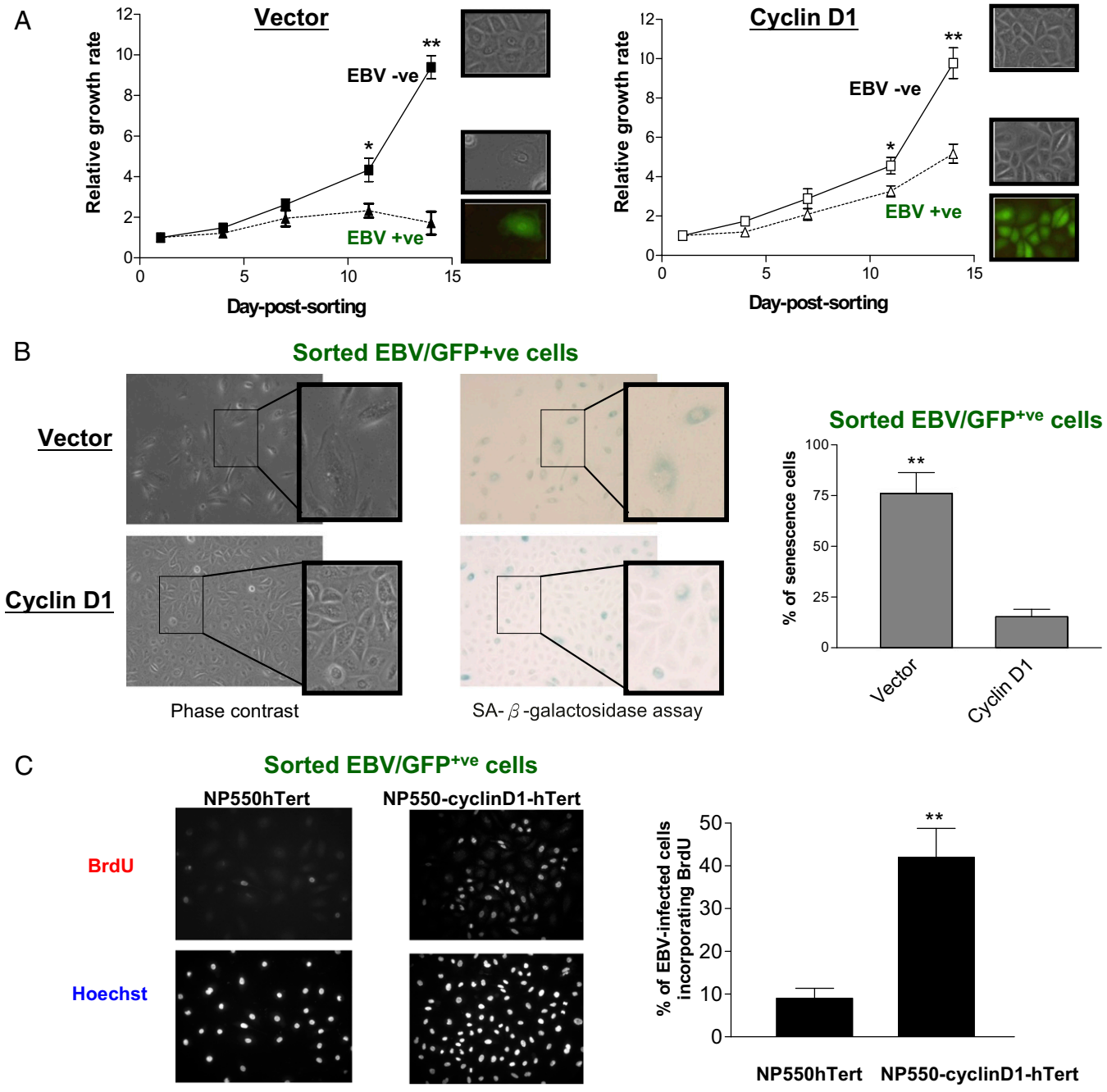

Fig. 5. Cyclin D1 overexpression rescued the hTert-immortalized NPE cells from the growth inhibition and cellular senescence induced by EBV infection. (*A*) Cyclin D1 overexpression was able to rescue NP550hTert cells from the growth-inhibiting effect of EBV infection. Growth curves were drawn for the sorted uninfected and infected populations of vector and cyclin D1-overexpressing cells. Means and SDs were calculated from triplicate wells. $^{**}P < 0.01$; $^{*}P < 0.05$ in a two-tailed Student *t* test. (*B*) Cyclin D1 overexpression in NP550hTert cells conferred resistance to the induction of cellular senescence upon EBV infection. The EBV-infected populations of both the control and cyclin D1-overexpressing NP550hTert cells were sorted by FACS. SA–β-galactosidase staining was performed, and phase-contrast images were taken at day 14 after sorting. Approximately 75% of the EBV-infected NP550hTert cells underwent senescence, whereas the EBV-infected NP550hTert cells overexpressing cyclin D1 sustained their proliferative ability over time, and only approximately 18% of cells underwent senescence. In the left images, the morphology of cells can be shown more clearly in the enlarged views. In the right images, enlarged views show perinuclear staining of SA–β-galactosidase. Means and SDs were calculated from triplicate wells. $^{**}P < 0.01$; in a two-tailed Student *t* test. (*C*) The rate of DNA synthesis (indicated by BrdU incorporation) was higher in EBV-infected populations of NP550-cyclinD1-hTert cells than in EBV-infected NP550hTert cells ($^{**}P < 0.01$; two-tailed Student *t* test). EBV-infected populations were live-sorted at 10 DPI. The cells then were allowed to incorporate BrdU for 24 h. Immunofluorescence staining was performed using an anti-BrdU antibody followed by an Alexa 555-tagged secondary antibody. For illustration purposes, white pseudocolor was chosen for both Hoechst and Alexa 555.

Effects of Cyclin D1 on EBV Gene Expression in EBV-Infected NPE Cells. We investigated the effects of cyclin D1 on the expression of representative latent and lytic EBV genes using real-time PCR. Up-regulation of EBNA1 and EBER1/2 was detected in EBV-infected hTert-immortalized NPE cells overexpressing cyclin D1 as compared with control cells (Fig. 6*A*). A main function of EBNA1 is to tether the EBV episome to the host chromosome to ensure proper segregation of EBV genomes during mitosis (27).

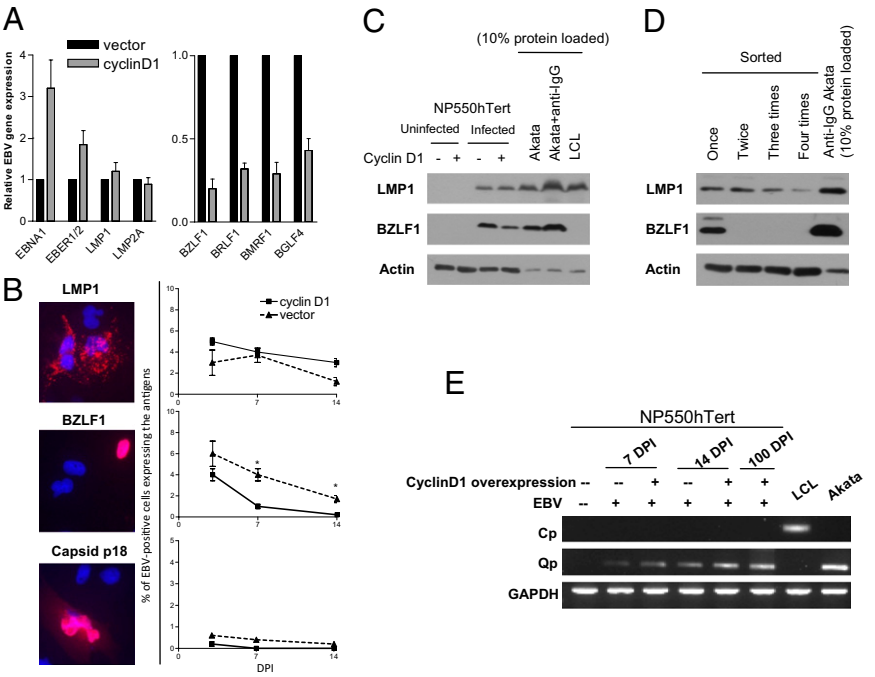

Fig. 6. Expression profiling of EBV genes in cells overexpressing cyclin D1 and in control cells. (*A*) Overexpression of cyclin D1 suppressed the mRNA transcription of lytic EBV genes in EBV-infected cells but up-regulated the mRNA transcripts of *EBNA1* and *EBER1/2*. Expression of individual EBV genes was normalized to GAPDH expression. The basal expression levels of lytic and latent genes in the control NP550hTert cells (infected with empty vector) are indicated as "1" for comparison with gene-expression levels in the cells overexpressing cyclin D1. (*B*) Expression of the EBV-encoded proteins including LMP1, BZLF1, and VCAp18 was examined in EBV-infected populations of control and cyclin D1-overexpressing NP550hTert cells. Means and SDs were calculated from triplicate wells. $*P < 0.05$ in a two-tailed Student *t* test. (*C*) Western blot analysis showing down-regulation of BZLF1 in cells overexpressing cyclin D1 vs. vector control cells. LMP1 expression levels were similar in control cells and in cells overexpressing cyclin D1. Protein lysates of Akata, anti–IgG-treated Akata, and LCL cells also were loaded for comparison. (*D*) Loss of LMP1 and BZLF1 expression in EBV-infected NP550hTert cells after prolonged culture. Several rounds of sorting of EBV/GFP$^+$ populations were performed in NP550hTert cells overexpressing cyclin D1. Proteins were extracted from cells after each sorting and were subjected to Western blot analysis for LMP1 and BZLF1 expression. (*E*) Promoter use in EBV-infected cell lines. LCL and Akata cells were used as positive controls for Cp and Qp use, respectively. Cyclin D1-overexpressing and vector control NP550hTert cells were infected with EBV. At the indicated day postinfection, RNA was extracted from the EBV$^+$ populations of these two cell lines purified by FACS. Promoter use was analyzed by semiquantitative PCR. Only transcripts derived from the Qp promoter, but not from the Cp promoter, were detected in the EBV-infected NP550hTert cells. GAPDH was used as loading control.

EBER expression has antiapoptotic and growth-stimulating effects on EBV-infected cells (28). Enhanced expression of EBNA1 and EBER1/2 in cells overexpressing cyclin D1 may support stable EBV infection in NPE cells. On the other hand, cyclin D1 appears to suppress lytic EBV infection, because all the lytic genes examined, including *BZLF1*, *BRLF1*, *BMRF1*, and *BGLF4*, were generally down-regulated in EBV-infected NPE cells overexpressing cyclin D1 (Fig. 6*A*). Previous reports have shown a close association of epithelial differentiation and lytic EBV gene expression (29, 30). The lytic promoter Zp, which is crucial in the initiation of EBV lytic reactivation, contains a differentiation-responsive element. Lytic gene expression could be detected in the upper differentiated layers of the EBV-infected epithelial cell epidermis of oral hairy leukoplakia in immunosuppressed patients (31). The suppressive effect of cyclin D1 on differentiation may suppress lytic reactivation of EBV in infected cells. However, activation of lytic replication of EBV may not be the main reason for the loss of EBV-infected hTert-immortalized cells in our study. Immunofluorescence staining reveals that only a small subset of EBV-infected cells expressing BZLF1 (Fig. 6*B*) and an even lower percentage of infected NPC cells (<1%) expressed the viral capsid protein VCAp18 (Fig. 6*B*). BZLF1 was detectable in only a small subpopulation of EBV-infected cells, making it unlikely that loss of EBV$^+$ cells in NPE cultures is a direct consequence of differentiation-induced EBV reactivation. However, the potential role of cyclin D1 in suppressing lytic replication of EBV in NPE cells in vivo remains to be defined by further investigation. Nonetheless, BZLF1-expressing cells in vitro were rapidly lost upon serial passages (Fig. 6*D*), suggesting that lytic infection may not support EBV persistence. It has been suggested that the expression of LMP1 contributes to the support of persistent EBV infection in NPC (32, 33). However, Western blot analysis indicated that cyclin D1 overexpression has minimal effects on LMP1 expression in EBV-infected NPE cells (Fig. 6 *B* and *C*). Besides, LMP1 is detectable in <5% of the cells in both infected cultures, ruling out a major contribution of LMP1 to enhancing clonal outgrowth of EBV-infected cells overexpressing cyclin D1 (Fig. 6*B*). LMP1 expression also diminished in EBV-infected cells upon serial passages (Fig. 6*D*).

We also confirmed that Cp is not activated in EBV-infected NP550hTert cells but is active in LCL cells (Fig. 6*E*). This result may explain why EBV can transform B cells into proliferative LCL cells but induces growth arrest in NPE cells (34). EBV infection in B cells initiates a growth/proliferative program. At the initial phase of infection, the Wp promoter of EBV is activated to transcribe the *EBNA2* gene, driving the infected B cell to enter the cell cycle from the resting phase (G_0) (16). EBNA2 also acts as a transcription factor that activates the viral Cp promoter to transcribe other genes, including *EBNA3C* and *-3A*, which have been shown to repress the expression of p16 and p14 to facilitate cell-cycle progression and growth of LCL (35). However, neither the Wp nor the Cp promoter is activated during EBV infection in epithelial cells (36). Clearly, the cellular content of NPE cells is different from that of B cells and is not permissive for the transcription of EBV genes, which are known to override the cell-cycle checkpoints of infected host cells. Hence, genetic alterations, such as overexpression of cyclin D1 and inactivation of p16, which dampen the growth arrest and senescence stimuli, may play an important role in facilitating the persistence of EBV infection in NPE cells.

Several rounds of sorting of EBV-infected populations in cell lines with deregulated cyclin D1 pathways led to the establishment of stably EBV-infected cell lines (Table 1, Fig. 7*A*, and Figs. S4 and S5). We determined the EBV genome load in NP550hTert+cyclinD1+EBV cells and NP361hTert+cyclinD1+EBV cells using in situ hybridization of fluorescent probes specific for EBV DNA (Fig. 7*B*). The high copy number of EBV in both cell lines suggests that the cells amplified the episomal virus genome after infection (36). This amplification may contribute to the persistence of EBV propagation. The examination of EBV gene expression profile revealed that all the stably infected cell lines express EBNA1, LMP1, LMP2A, and EBER1/2, as shown by Western blots or real-time PCR (Fig. 7*C* and Fig. S6), indicating that EBV infection manifested type II latency in these NPE cell lines, resembling the expression pattern in NPC tumors.

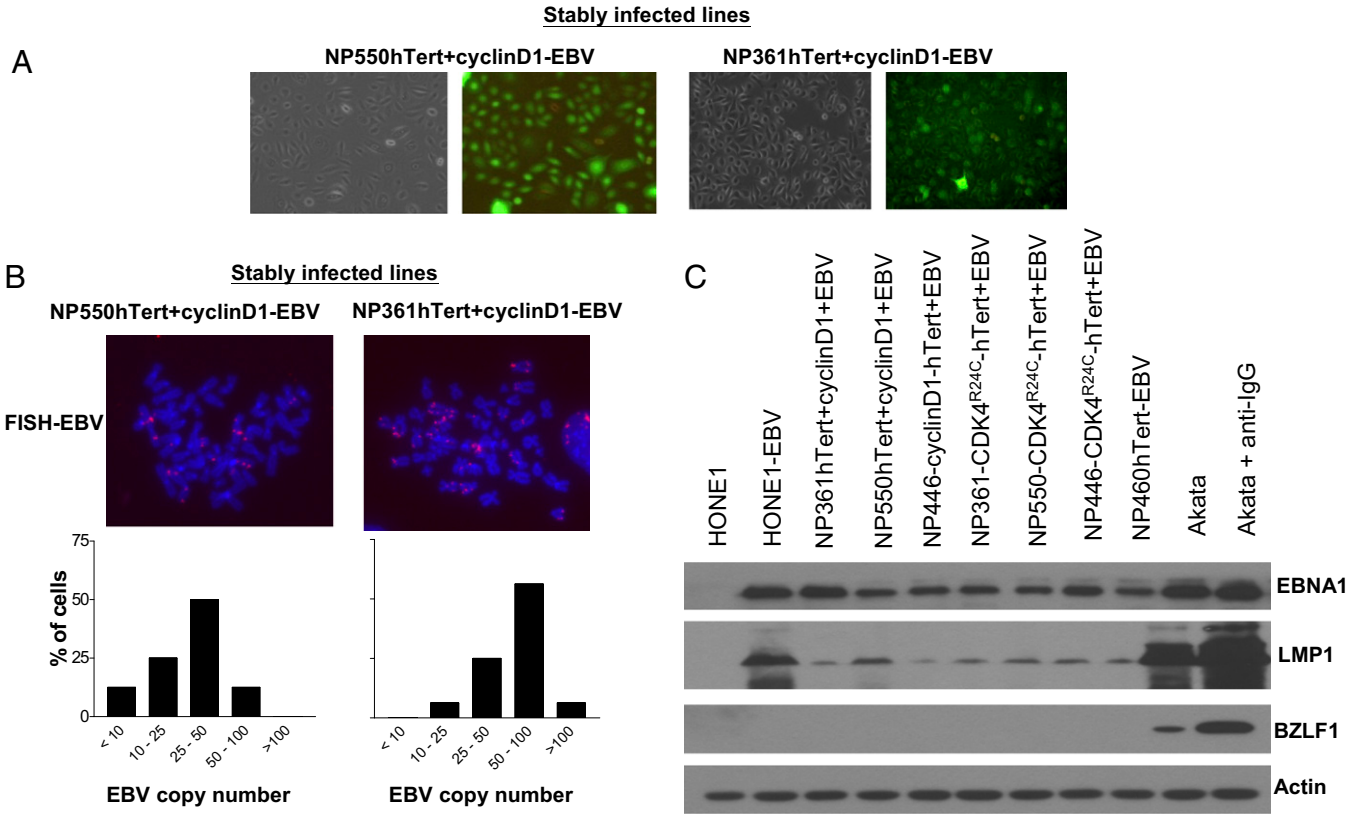

Fig. 7. Characterization of EBV status in various stably infected cell lines. (*A*) Phase-contrast and fluorescent images of NP550hTert+pBabe/cyclin D1 and NP361hTert+pBabe/cyclin D1 cells stably infected with EBV. More than 95% of these cells remained GFP$^+$ (i.e., retained the EBV genome) after prolonged cell culture (>100 DPI). (*B*) FISH analysis showing multiple copies of the EBV genome in the host genome of the NP550hTert+pBabe/cyclin D1 and NP361hTert+pBabe/cyclin D1 cells stably infected with EBV. (*Upper*) Fluorescent images showing the host genome (stained by Hoechst) and the presence of the EBV genome (indicated by red fluorescence). (*Lower*) Graphical presentation of EBV copy number in the cell lines. (*C*) Western blots showing the expression of LMP1 and EBNA1, but not BZLF1, in all the stably EBV-infected NPE cell lines. HONE1 is a NPC cell line that has lost EBV and contains no EBV genome. EBV-reinfected HONE1 (HONE1-EBV), Akata, and lytically activated Akata cells were included as positive controls for the detection of EBV-encoded genes.

Conclusion. In this study, we show that premalignant nasopharyngeal epithelium commonly overexpresses cyclin D1 and is closely associated with EBV infection. We further demonstrate that overexpression of cyclin D1 can suppress differentiation. EBV infection in NPE cells induces growth arrest and cellular senescence that can be suppressed by the overexpression of cyclin D1 or by dysregulation of the p16/CDK4 pathway. Overexpression of cyclin D1 or CDK4^{R24C} in immortalized NPE cells supports stable EBV infection, leading to the establishment of stable EBV-infected cell lines expressing latent EBV genes. Our study provides evidence that preexisting genetic events, notably cyclin D1 overexpression, and related molecular events support the establishment of stable EBV infection in premalignant nasopharyngeal epithelium and play a role in NPC pathogenesis.

Materials and Methods

Please refer to *SI Materials and Methods* for detailed descriptions of experimental procedures and reagents used in this study. The immortalized NPE cell lines were established and were maintained in a serum-free medium with growth factor supplements. Expression of *hTert, cyclin D1*, or *CDK4^{R24C}* in primary NPE cells was achieved by retrovirus infection. Immortalized cell lines with p16 knockdown were generated using lentiviral-mediated expression of shRNA.

The NPE cells were infected with EBV using a previously published protocol (8). The detailed protocol of the EBV infection and the experimental procedures of FACS analysis, live-cell sorting, SA–β-galactosidase assay, proliferation and colony formation assays of EBV-infected cells, the BrdU incorporation assay, and FISH are described in *SI Materials and Methods*.

Western blotting, immunofluorescence staining, real-time PCR, and semiquantitative PCR were used to assess protein or gene expression in EBV-infected cells. The probes or primers used in real-time PCR or PCR are listed in Table S1. Detailed experimental procedures are described in *SI Materials and Methods*.

In this study, H&E staining, cyclin D1 immunostaining, and EBER in situ hybridization were performed on consecutive sections of six biopsies of dysplastic nasopharyngeal tissue.

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