Published OnlineFirst February 26, 2013; DOI: 10.1158/1078-0432.CCR-12-2537

Clinical Cancer Research

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Clin Cancer Res 2013;19:2036-2047. Published OnlineFirst February 26, 2013.

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Cancer Therapy: Preclinical

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hTERT Inhibition Triggers Epstein–Barr Virus Lytic Cycle and Apoptosis in Immortalized and Transformed B Cells: A Basis for New Therapies

Silvia Giunco[1], Riccardo Dolcetti[3], Sonia Keppel[2], Andrea Celeghin[1], Stefano Indraccolo[2], Jessica Dal Col[3], Katy Mastorci[3], and Anita De Rossi[1,2]

Abstract

Purpose: Induction of viral lytic cycle, which induces death of host cells, may constitute a useful adjunct to current therapeutic regimens for Epstein–Barr virus (EBV)-driven malignancies. Human telomerase reverse transcriptase (hTERT), essential for the oncogenic process, may modulate the switch from latent to lytic infection. The possible therapeutic role of hTERT inhibition combined with antiviral drugs was investigated.

Experimental Design: EBV-negative BL41 and convertant EBV-positive BL41/B95.8 Burkitt's lymphoma cell lines and lymphoblastoid cell lines (LCL) were infected with retroviral vector encoding short hairpin RNA (shRNA) anti-hTERT and cultured with or without the prodrug ganciclovir. The effects on EBV lytic replication, cell proliferation, and apoptosis were characterized.

Results: hTERT silencing by shRNA induced the expression of BZLF1, EA-D, and gp350 EBV lytic proteins and triggered a complete lytic cycle. This effect was associated with downregulation of BATF, a negative regulator of BZLF1 transcription. hTERT silencing also resulted in antiproliferative and proapoptotic effects. In particular, hTERT inhibition induced an accumulation of cells in the S-phase, an effect likely due to the dephosphorylation of 4E-BP1, an AKT1-dependent substrate, which results in a decreased availability of proteins needed for cell-cycle progression. Besides inducing cell death through activation of complete EBV lytic replication, hTERT inhibition triggered AKT1/FOXO3/NOXA–dependent apoptosis in EBV-positive and -negative Burkitt's lymphoma cells. Finally, ganciclovir enhanced the apoptotic effect induced by hTERT inhibition in EBV-positive Burkitt's lymphomas and LCLs.

Conclusions: These results suggest that combination of antiviral drugs with strategies able to inhibit hTERT expression may result in therapeutically relevant effects in patients with EBV-related malignancies. *Clin Cancer Res; 19(8); 2036–47. ©2013 AACR.*

Introduction

Epstein–Barr virus (EBV) is a γ-herpesvirus that has elegantly evolved the ability to establish a persistent infection in more than 90% of the world's population. The latency reservoir is composed of memory B lymphocytes in which only a few viral genes are expressed. Occasionally, the EBV lytic program is activated allowing for local spreading and host-to-host transmission of the virus. EBV is causally associated with both B-cell and epithelial cell malignancies (1), in which the virus expresses different sets of latency-associated proteins with transforming properties. Although latency programs predominate in EBV-driven tumors, recent evidence suggests that lytic EBV replication may also be of pathogenic importance, at least in the early phases of cell transformation (2–4). Consistent with a tumorigenic role of lytic infection, prophylactic treatment of transplant patients with antiviral drugs that inhibit EBV lytic replication decreased the occurrence of EBV-associated lymphoproliferative disease (5, 6). On the other hand, there is increasing interest in developing strategies that reactivate EBV lytic gene expression in latently infected tumor cells to treat overt EBV-associated malignancies, as lytic infection promotes the death of EBV-positive tumor cells both *in vitro* and *in vivo* (7–10). Triggering EBV lytic replication *in vivo* may be particularly effective and therapeutically important as it promotes immune recognition of viral antigens that further enhances the killing of tumor cells. Several

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

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Translational Relevance

Induction of the viral lytic cycle is a promising strategy for treatment of Epstein–Barr virus (EBV)–driven malignancies, as viral lytic replication is associated with death of EBV-positive tumor cells. This study indicates that inhibition of human telomerase reverse transcriptase (hTERT), the catalytic component of the telomerase complex, by short hairpin RNA promotes induction of the viral lytic cycle and death of EBV-immortalized and fully transformed B cells. Besides inducing cell death through activation of complete viral lytic replication, hTERT inhibition triggered AKT1/FOXO3/NOXA–dependent apoptosis in both EBV-positive and -negative lymphoma cells. Notably, antiviral drug ganciclovir markedly enhanced the death of EBV-positive tumor cells induced by hTERT inhibition. These results provide the rationale to activate clinical studies based on the combination of antiviral drugs with inhibitors of hTERT expression/activity for the treatment of EBV-driven malignancies.

chemotherapeutic drugs are known to trigger EBV replication, including 5-fluorouracil, methotrexate, doxorubicin, and several histone deacetylase inhibitors (9–12). EBV-carrying tumor cells may also be killed by prodrugs, such as ganciclovir or radiolabeled nucleoside analogs, which are activated by EBV lytic proteins, such as the viral thymidine kinase (9, 13, 14). Notably, combined treatment with arginine butyrate and ganciclovir induced a significant rate of clinical responses in patients with refractory EBV-associated lymphoid malignancies (15). The combination of antivirals with lytic cycle inducers is thus emerging as a highly promising strategy to treat EBV-driven tumors.

As for other oncogenic viruses, EBV-induced cell immortalization requires induction of telomerase activity to overcome cell senescence and maintain replicative potential (16–18). We have previously shown that, in early-passage EBV-infected B lymphocytes, activation of human telomerase reverse transcriptase (hTERT), the rate-limiting component of the telomerase complex, occurs concomitantly with induction of latent EBV genes and downregulation of lytic EBV gene expression (19). Interestingly, our findings also suggested a possible role of hTERT in regulating EBV lytic replication (19). Elucidation of this issue may allow us to target hTERT for therapeutic strategies by exploiting the induction of the EBV lytic cycle. As hTERT is frequently overexpressed by tumor cells and sustained telomerase activity is required for their unlimited proliferative capacity, telomerase is a particularly attractive target for cancer therapy. Several strategies targeting telomerase are being explored at the preclinical level, and both pharmacologic and immunologic approaches are currently under clinical investigation (20, 21).

On these grounds, we sought to characterize the interplay between hTERT and the EBV lytic cycle with the goal of providing a basis for hTERT inhibition in treating EBV-driven malignancies. In view of the emerging interest in approaches combining antiviral agents with various lytic cycle inducers, we also investigated the effects of combining hTERT inhibition and ganciclovir in both immortalized and fully transformed EBV-positive B-cell lines.

Materials and Methods

Cell lines

Lymphoblastoid cell lines (LCL) 4141 and 4134 were obtained by infecting peripheral blood mononuclear cells (PBMC) from normal donors with EBV strain B95.8. Establishment and characterization of these cell lines have been previously described (19). 4134 hTERT+ cells were obtained by infecting 4134 cells with a retroviral vector containing hTERT cDNA (19). BL41 is an EBV-negative Burkitt's lymphoma cell line, with translocated *MYC* gene. BL41/B95.8 is the counterpart cell line infected *in vitro* with EBV strain B95.8. Raji is an EBV-positive Burkitt's lymphoma cell line. Raji and BL41 were kindly provided by Martin Rowe (Cancer Center, University of Birmingham, Birmingham, United Kingdom). BL41/B95.8 cells were kindly provided by Martin Allday (Ludwig Institute for Cancer Research, London, United Kingdom). HBL-1, an EBV-positive Burkitt's lymphoma cell line derived from an HIV-1 seropositive patient, was a kind gift from Dr. Riccardo Dalla Favera (Columbia University, New York, NY). Cell lines were checked and controlled by cytogenetic analyses. Cell lines were maintained in culture at 37°C and 5% CO_2. LCLs, BL41, Raji, and HBL-1 cells were cultured in RPMI-1640 medium (Euroclone), supplemented with 2% glutamine, 50 μg/mL gentamycin (Sigma), and 10% heat-inactivated FBS (Gibco; standard medium). BL41/B95.8 cells were grown in standard medium supplemented with 1 mmol/L sodium pyruvate, 1% nonessential amino acids (Sigma), and 50 μmol/L β-mercaptoethanol.

Retroviral vectors and viral infection

Vectors pGFP-V-RS-shTERT 3 (pRSshTERT3) and pGFP-V-RS-shTERT 4 (pRSshTERT4), which contain the packaging sequences of the Moloney leukemia virus (MLV), the sequence encoding the short hairpin RNA (shRNA) hTERT under the U6 promoter, the puromycin resistance gene, and the *GFP* gene (Origen) were used. Vector pGFP-V-RS (pRS; Origen), lacking shRNA hTERT, was used as a control. Infectious retroviruses were produced in packaging 293T cells by cotransfecting one of the above plasmids with the gag-polgpt plasmid, which encodes the *gag/pol* gene of the MLV and the HCMV-G plasmid, which encodes the G protein of the vesicular stomatitis virus. Forty hours after transfection, supernatants containing viral particles were harvested, filtered to remove cells and cell debris, and centrifuged at 24,000 rpm for 2 hours at 4°C. The pellet was resuspended at 1 of 30 of the original volume, and ×30 concentrated viral stocks were stored at −80°C. For infection, an equal volume of pRSshTERT3 and pRSshTERT4 was used (shTERT3.4). Cell infection was repeated twice on alternate days. Cells were then short-term cultured in RPMI

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medium with or without the prodrug ganciclovir (Sigma). Cell viability was determined by Trypan blue exclusion using a Countess automated cell counter (Invitrogen).

Real-time PCR for quantification of hTERT transcripts

Cellular RNA was extracted and retro-transcribed into cDNA, as previously detailed (19). Both all hTERT transcript (hTERT-AT) and the full-length hTERT transcript (hTERT-FL), which encodes the functional protein (22), were quantified by real-time PCR, using the AT1/AT2 and FL1/FL2 primer pairs, respectively, as previously described (19).

Reverse-transcriptase PCR

Transcripts of viral *BZLF1* and cellular housekeeping *GAPDH* genes were detected, as previously reported (23). The PCR reaction for quantification of BATF transcript was carried out on LightCycler 480 Real-Time PCR System (Roche). Each PCR was conducted in 50 μL of mixture containing 25 μL Lightcycler 480 probe master (Roche), 200 nmol/L of fluorogenic probe, 900 nmol/L of each primer, and 10 μL of cDNA samples. After 2 minutes at 50°C to allow the uracil N-glycosylase to act, and after a denaturation step of 10 minutes at 95°C, 45 cycles were run, each for 15 seconds at 95°C and 1 minute at 60°C. Samples were run in triplicate. Primers and probes for PCR analysis were: BATF-forward: 5′-GACAAGAGAGCCCAGAGGTG-3′; BATF-reverse: 5′-GTAGAGCCGCGTTCTGTTTC-3′; BATF-probe: 5′-Cy5-TGGCAAACAGGACTCATCTG-BBQ-3′. To normalize BATF levels for the amount of RNA, 10 μL of cDNA from each sample were amplified for the *HPRT1* housekeeping gene, as described previously (19, 24). Relative quantification was conducted using mean C_t values according to the $2^{-\Delta C_t}$ formula, where $\Delta C_t = C_{t\ BATF} - C_{t\ HPRT1}$.

EBV genome quantification

Total EBV genomes and DNA from virus particles released in culture supernatants were quantified by real-time PCR (25), before and after ultracentrifugation and DNase treatment (26), as previously reported (19).

Assessment of telomerase activity

Telomerase activity was assessed using a PCR-based telomeric repeats amplification protocol (TRAP), as previously reported (27, 28).

Western blotting

Western blot analyses were prepared as previously reported (29). The expression of EA-D was detected with the monoclonal antibody clone 6D1 (Abcam). Cellular basic leucine zipper transcription factor ATF-like (BATF), hTERT, and α-tubulin were evaluated by monoclonal anti-BATF (1G4; Novus Biologicals), polyclonal anti-hTERT (sc-7212; Santa Cruz Biotechnology), and clone B-512 (Sigma), respectively. The AKT1 pathway activation was analyzed using phospho-AKT1 (Ser473), phospho-RPS6 (Ser235/236), phospho-4E–binding protein 1 (4E-BP1; T37/46), phospho-FOXO3 (S318/321), AKT1, 4E-BP1, BAD antibodies (Cell Signaling Technology), and NOXA antibody (Alexis Biochemicals). Apoptotic effects of the inhibition of the AKT1 pathway, obtained by exposing cells to 25 μmol/L SH5 inhibitor (Alexis Biochemicals) for 24 and 48 hours, were evaluated by immunoblotting analysis of PARP-1 cleavage and caspase-3 using polyclonal anti-PARP-1 clone F2 (Sigma) and caspase-3 (D175) antibodies (Cell Signaling Technology). Blots were incubated with an appropriate peroxidase-conjugated secondary antibody (Sigma) and stained with chemiluminescence detection kit (Super-Signal West Pico Chemiluminescent Substrate; Pierce).

Cell cycle and apoptosis analysis

To evaluate cell-cycle distribution, cells were harvested and processed as previously described (29). The samples were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson) and cell-cycle analyses were conducted with ModFit LT™ Cell Cycle Analysis software (version 2.0,Verity Software House). Apoptosis was evaluated by staining cells with annexin V and propidium iodide (PI; Sigma), as previously detailed (29), and analyzed by flow cytometry. At least 50,000 events were acquired, data were processed using CellQuestPro software (Becton-Dickinson), and analyzed with Summit software, version 4.3. Annexin V–positive/PI-negative and annexin V–positive/PI-positive samples were classified as early and late apoptotic cells, respectively, and both fractions were included in the percentages of apoptotic cells.

Immunohistochemistry

Cytospins were fixed in cold acetone (4°C) for 10 minutes. Expression of gp350 protein was analyzed using the monoclonal antibody clone 0221 (Abcam). After incubation for 1 hour with the primary antibody, immunostaining was carried out using the avidin–biotin peroxidase complex (ABC kit; Vector Laboratories), and 3,3′ diaminobenzidine chromogen as substrate (Dako). The cells were lightly counterstained with Mayer's hematoxylin.

Statistical analysis

For statistical comparison, the Mann–Whitney *U* test was conducted using SPSS software version 18 (SPSS Inc.). *P* value less than 0.05 was considered to be statistically significant.

Results

hTERT silencing in B-cell lines

Preliminary experiments indicated that the shTERT3 and shTERT4 retroviral vectors were the most effective in inhibiting h*TERT* mRNA expression when used individually and induced even stronger inhibition when used together (not shown). Therefore, all the following shRNA experiments were carried out using the combination of these 2 vectors (shTERT3.4). Infection of Burkitt's lymphoma cell lines with shTERT3.4 induced a 60% reduction in the levels of both hTERT-AT and hTERT-FL transcripts after 48 hours, with a 90% decrease in all cell lines after 72 hours (Fig. 1A). Inhibition of h*TERT* mRNA expression was paralleled by a

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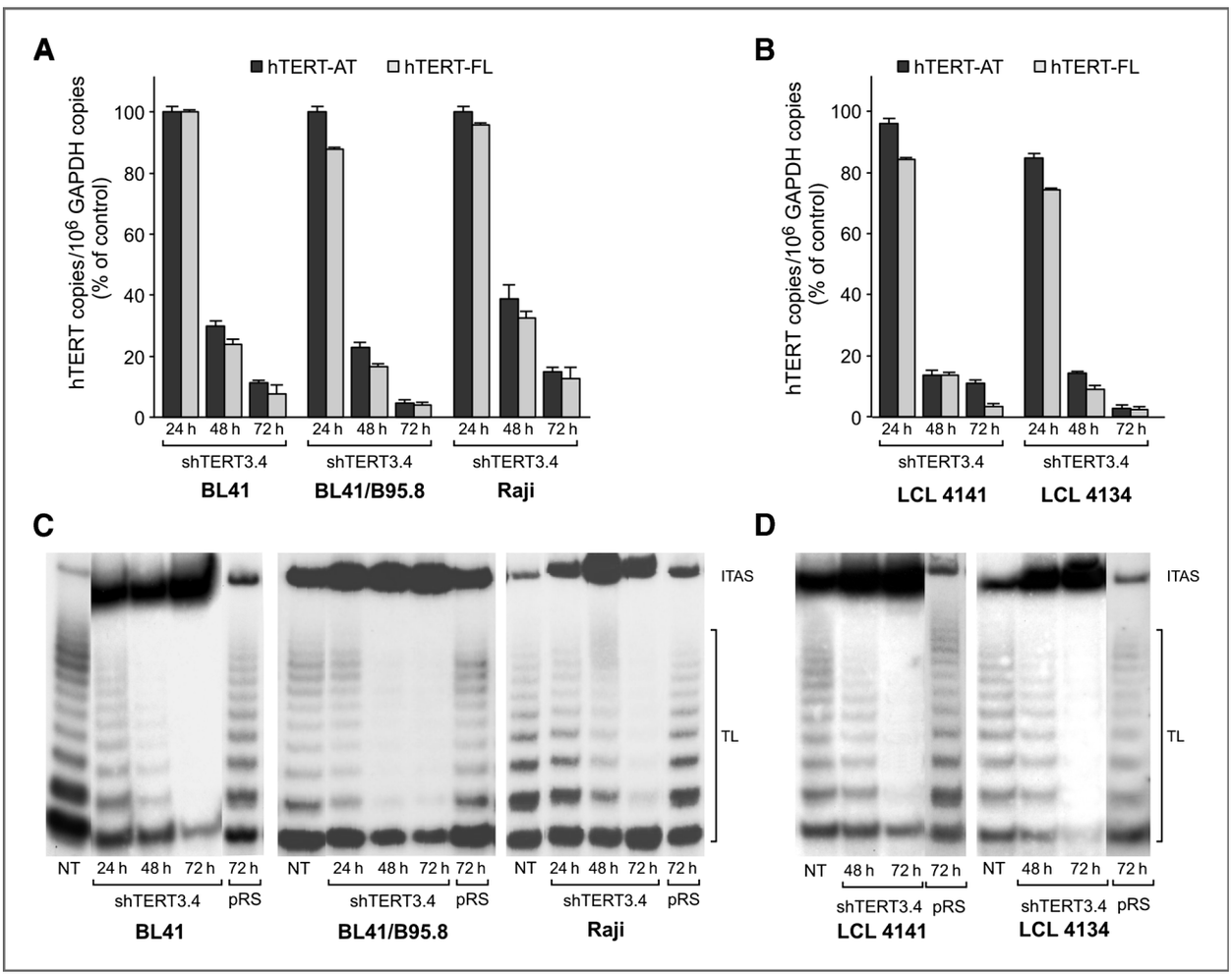

Figure 1. shRNA hTERT decreases h*TERT* transcripts and telomerase activity. A and C, Burkitt's lymphoma cell lines, and (B and D) LCLs were infected with the shTERT3.4 vectors and analyzed at 24, 48, and 72 hours after infection. A and B, values of hTERT-AT and hTERT-FL transcripts are reported as percentages of hTERT levels quantified in corresponding cells infected with control pRS vector. Mean and SD (bar) of values from 3 separate experiments are shown. C and D, telomerase activity was tested by TRAP assay in uninfected (NT) and shTERT3.4 or pRS-infected cells. Panels from one representative experiment are shown. TL, telomerase ladder; ITAS, internal telomerase assay standard.

concomitant time-dependent decrease in telomerase activity (Fig. 1C). Notably, BL41 cells showed markedly higher levels of hTERT transcripts compared with EBV convertant BL41/B95.8 cells [14,950 ± 2,996 vs. 2,672 ± 538 hTERT copies/10^6 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) copies], thus confirming that the shRNA-based approach can effectively inhibit hTERT even in cells with high h*TERT* mRNA expression. Similar findings were observed in different LCLs, which showed an early decrease in the levels of hTERT-AT and hTERT-FL transcripts, detectable after 24 hours of infection, with inhibition of more than 90% of h*TERT* mRNA expression and telomerase activity at 72 hours (Fig. 1B and D). Downregulation of hTERT transcripts and telomerase activity induced by shTERT3.4 were observed also in the HBL-1 cell line (not shown). All cell lines infected with the pRS control vector showed activity comparable with untreated cells.

Induction of EBV lytic replication by shRNA-mediated hTERT silencing

Our preliminary findings showed that hTERT inhibition by siRNA in LCLs resulted in expression of *BZLF1* mRNA and EA-D lytic protein, suggesting induction of the EBV lytic cycle (19). To characterize this phenomenon and to assess whether it also occurs in other B-cell backgrounds, we investigated the effects of shRNA-mediated hTERT silencing on activation of EBV lytic replication in a larger panel of EBV-positive B-cell lines. The decrease in hTERT transcripts induced by shTERT3.4 infection was associated with upregulation of *BZLF1* mRNA in all EBV-positive cell lines (Fig. 2A). Immunoblot analysis revealed induction of EA-D viral lytic protein as early as 48 hours after shTERT3.4 infection in Burkitt's lymphoma cells and 72 hours in LCLs (Fig. 2B). Similar findings were also observed in shTERT3.4-infected HBL-1 cells (not shown). hTERT inhibition also

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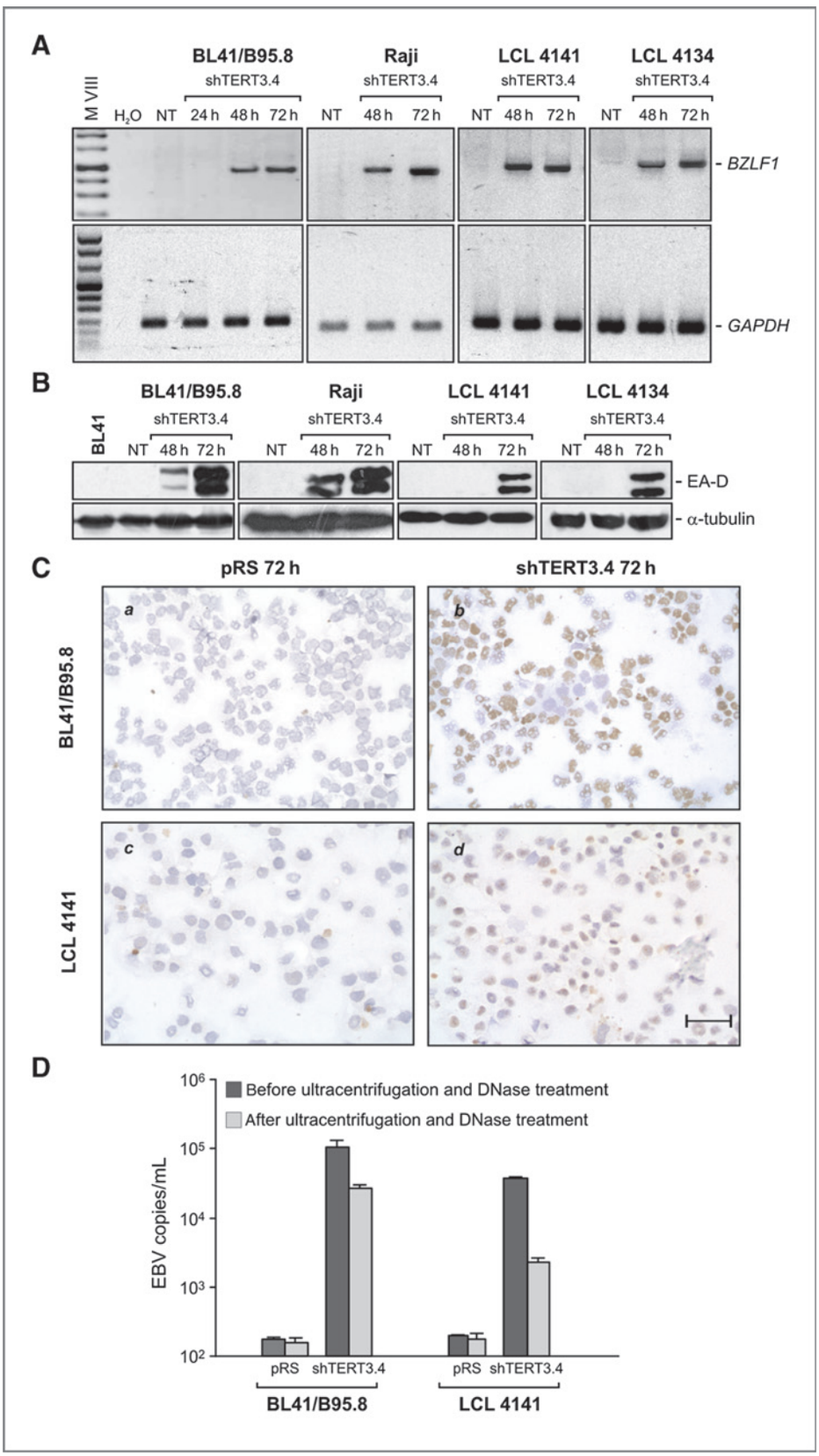

Figure 2. shRNA hTERT induces EBV lytic cycle. Cells were analyzed before (NT) and at 48 and 72 hours after infection with shTERT3.4 vectors. A, *BZLF1* (top) and *GAPDH* (bottom) mRNA were analyzed by reverse transcriptase PCR. Analysis of *GAPDH* was used for sample comparison. B, expression of lytic EA-D viral protein and cellular α-tubulin was assessed by Western blotting; α-tubulin expression was used for sample comparison. C, gp350 protein expression in BL41/B95.8 (a and b) and in 4141 (c and d) cells at 72 hours after infection with shTERT3.4 (b and d) or pRS vector (a and c) (×20). Scale bar, 100 μm. D, quantification of EBV-DNA in cell culture supernatants before (black column) and after (gray column) ultracentrifugation and DNase treatment after 72 hours of infection with shTERT3.4 or pRS vector. Values are means and SD (bar) of 3 replicates.

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gave rise to a remarkable increase in the number of positive cells expressing the late viral lytic gp350 protein (<1% and >30% in cell cultures at 72 hours after infection with control pRS or shTERT3.4, respectively) in both BL41/B95.8 and 4141 cell cultures (Fig. 2C). To assess whether shRNA-mediated hTERT silencing resulted in the induction of complete EBV lytic replication, the level of EBV genomes released into culture supernatants was quantified in BL41/B95.8 and LCLs. pRS-infected cells showed very low levels of spontaneous EBV-DNA release, whereas an increase in the level of EBV-DNA was detected in the supernatant of all cell cultures 72 hours after shTERT3.4 infection. Persistence of EBV-DNA in DNase-treated samples confirmed the presence of virions in these supernatants (Fig. 2D).

hTERT modulation of BATF

To shed light on possible mechanisms underlying the activation of EBV lytic replication induced by hTERT inhibition, we investigated the involvement of BATF, a transcription factor, which negatively regulates AP-1 activity (30, 31). BATF has been shown to inhibit the expression of BZLF1, thus reducing EBV lytic replication in latently infected B cells (32). The ectopic expression of hTERT in 4134 cells (4134/hTERT+ cells) increased by 5-fold the mRNA levels and protein expression of BATF, whereas hTERT silencing by shTERT3.4 infection decreased by more than 80% the levels of BATF mRNA and protein expression, decreased hTERT expression and induced the expression of lytic EA-D protein (Supplementary Fig. S1A and S1B).

Antiproliferative effects of hTERT silencing in EBV-positive and -negative cells

BL41 cells infected with shTERT3.4 or pRS vector showed a similar distribution of cells in the different phases of the cell cycle during the first 72 hours of infection (Fig. 3A). Conversely, at 96 hours, shTERT3.4-infected BL41 cells showed a marked accumulation of cells in G_0–G_1, which was not evident in cells exposed to control pRS vector (Fig. 3A). In the EBV convertant BL41/B95.8 cells, shTERT3.4 infection induced alterations in cell-cycle distribution at

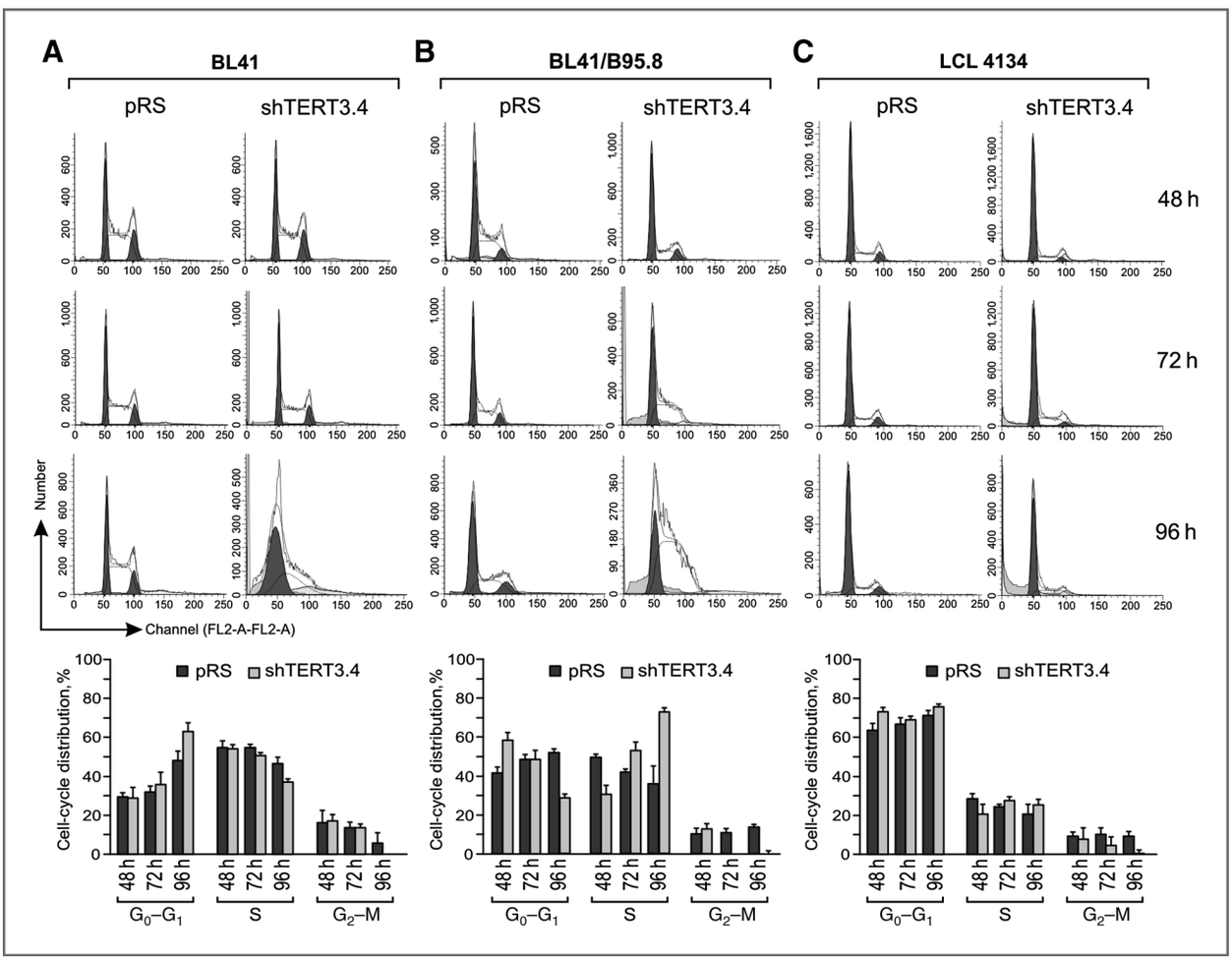

Figure 3. shRNA hTERT modifies cell-cycle distribution. A–C, cells were infected with shTERT3.4 or pRS vector and analyzed at 48, 72, and 96 hours after infection. Cells were labeled with PI and analyzed by flow cytometry. Panels from 1 representative experiment are shown. Percentages of cells in G_0–G_1, S, and G_2–M are reported in the graphs on the bottom. Values are means and SD (bar) of 3 separate experiments.

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earlier time point (i.e., 72 hours) with a virtual disappearance of the G_2–M phase and a marked accumulation of cells in the S-phase (Fig. 3B). A similar increase in the number of cells in the S-phase and decrease of the G_2–M phase were also observed in Raji and HBL-1 cells infected with shTERT3.4 compared with cells treated with pRS control vector (not shown). hTERT silencing also induced cell-cycle perturbations in LCL 4134, with a progressive decrease of cells in the G_2–M phase (Fig. 3C).

Proapoptotic effects of hTERT silencing in EBV-positive and -negative cells

As inhibition of hTERT may affect cell survival, we investigated the possible proapoptotic effects of hTERT silencing in the EBV-negative BL41 cell line. An increase in the number of apoptotic cells (30%) was observed at 96 hours after shTERT3.4 infection (Fig. 4A). Greater numbers of apoptotic cells were already detectable at 72 hours after shTERT3.4 infection in the EBV-positive BL41/B95.8 cell line (Fig. 4B). The extent of apoptosis was progressively more pronounced at later time points, with about 50% of apoptotic cells at 96 hours (Fig. 4B). hTERT inhibition also induced comparable levels of apoptosis in LCL 4134 (Fig. 4C). Notably, silencing of hTERT induced a significantly higher extent of apoptosis in EBV-positive BL41/B95.8 cells than in EBV-negative BL41 cells both at 72 (28 ± 2 vs. 10 ± 3; $P = 0.019$) and 96 hours (48 ± 4 vs. 29 ± 2; $P = 0.020$), thus indicating that activation of EBV lytic replication enhances the cytotoxic effect induced by hTERT inhibition in the EBV-negative background. Induction of apoptosis in both BL41 and BL41/B95.8 cells was confirmed by the detection of cleaved caspase-3 and PARP-1 (Fig. 5A). In all cell lines tested, infection with pRS control vector did not affect cell viability.

hTERT silencing inhibits the AKT1 signaling cascade

Considering the critical role of the AKT1 pathway in the control of B-cell proliferation and survival, we investigated the effects of hTERT silencing on the activation of AKT1 and its downstream substrates in both EBV-negative and EBV-

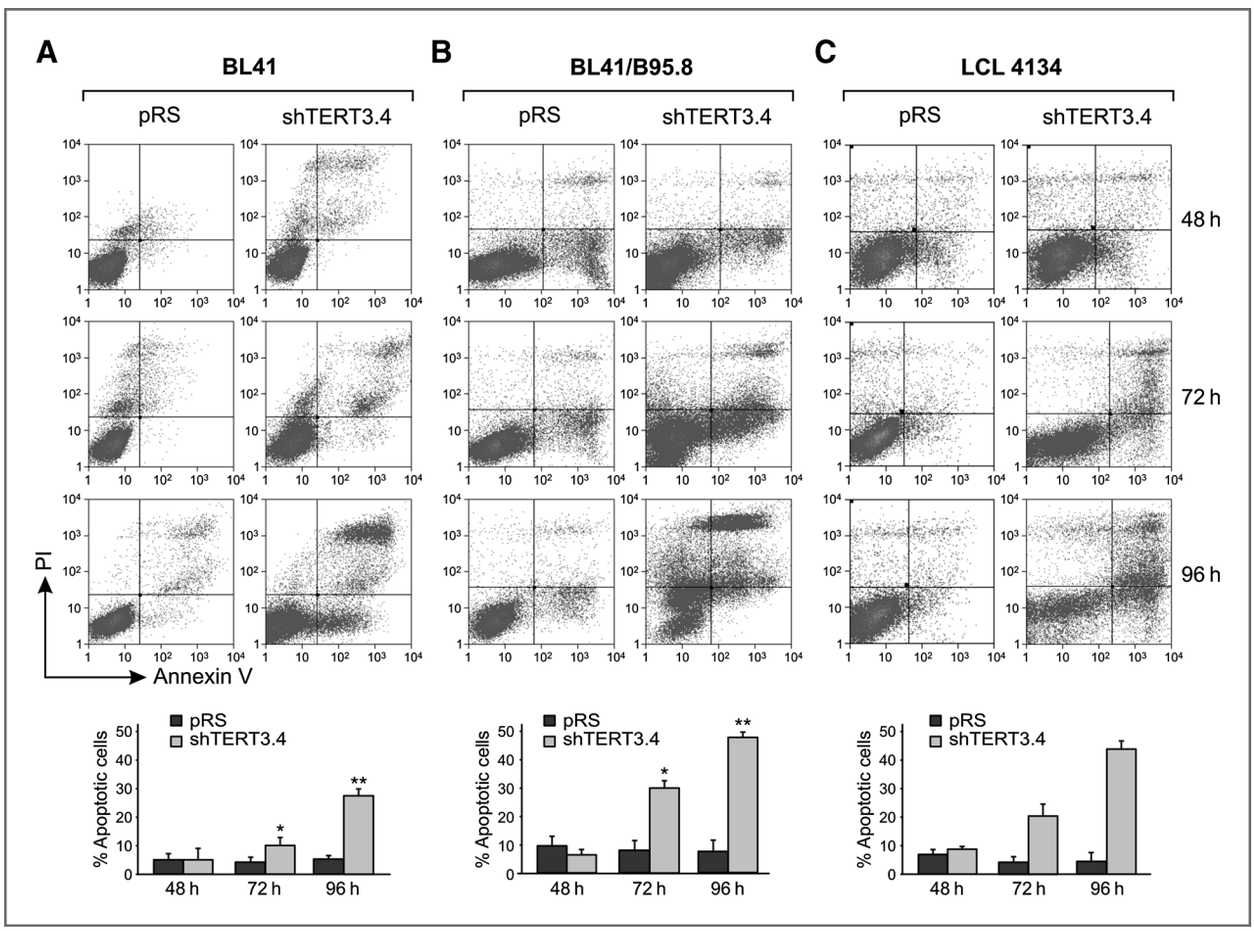

Figure 4. shRNA hTERT induces apoptosis. A–C, cells were infected with shTERT3.4 or pRS vector and analyzed at 48, 72, and 96 hours after infection. The apoptotic cells were quantified by annexin V/PI staining and cytofluorimetric analysis. Panels from 1 representative experiment are shown. Percentages of apoptotic cells are reported in the graphs on the bottom. Values are mean and SD (bar) of 3 separate experiments. Percentages of apoptotic cells were significantly higher ($P < 0.05$) in BL41/B95.8 than in BL41 cells after 72 (*) and 96 hours (**) of infection.

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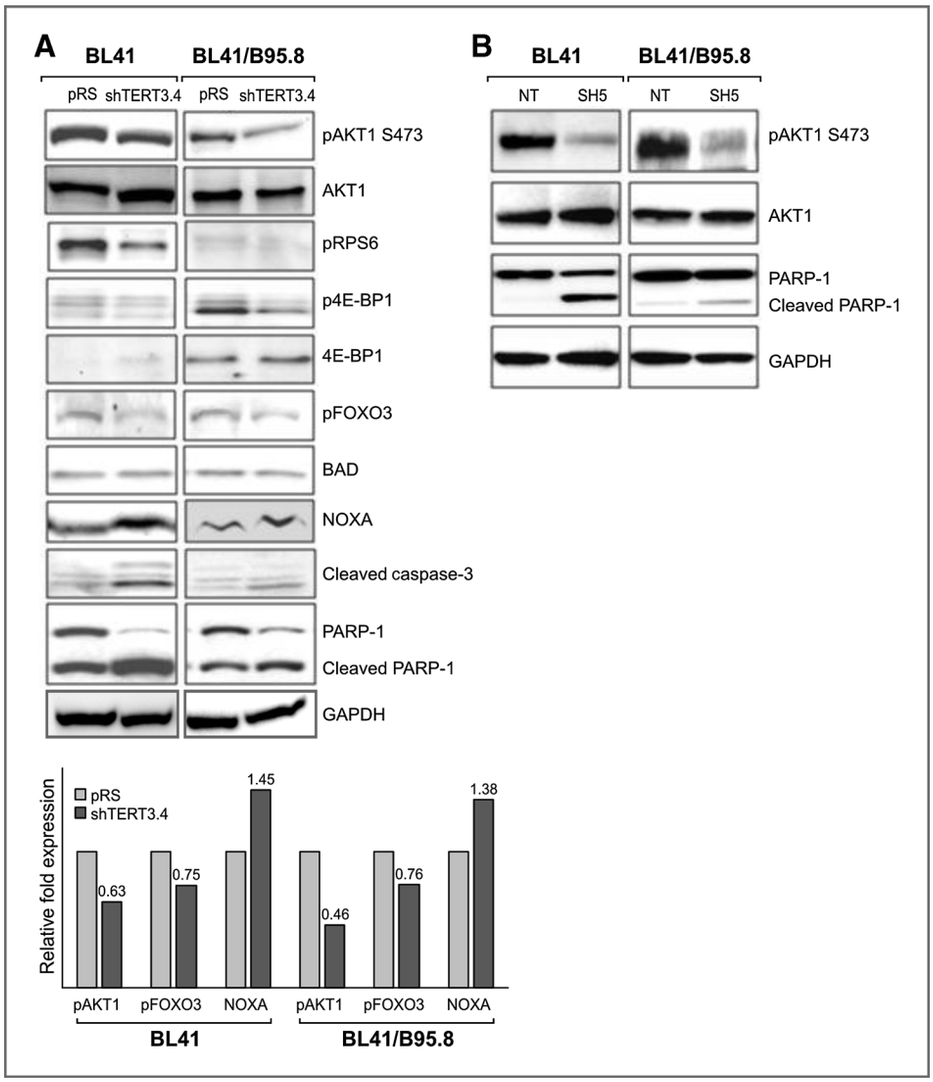

Figure 5. shRNA hTERT results in AKT1 pathway inhibition in BL41 and BL41/B95.8 cells. A, Western blotting shows decreased levels of phosphorylated/active form of AKT1 kinase after hTERT silencing by shTERT3.4 in BL41 (96 hours) and BL41/B95.8 (72 hours) cells. Phosphorylation/dephosphorylation status of AKT1 substrates was evaluated using phospho-specific antibodies and levels of the AKT1-dependent proapoptotic proteins BAD and NOXA were analyzed. Apoptotic extent was evaluated by PARP-1 and caspase-3 cleavage. In the graph on the bottom, densitometry analysis in arbitrary units assigning to the corresponding pRS-infected sample the value of 1 is reported. B, pharmacologic inhibition of AKT1 induces apoptosis in both BL/41 and BL41/B95.8 cells. Cells were treated for 48 hours with AKT1 inhibitor SH5 (25 μmol/L) and apoptotic extent was evaluated by PARP-1 cleavage.

positive B-cell lines. shTERT3.4 infection resulted in decreased levels of the phosphorylated active form of AKT1 in both BL41 and BL41/B95.8 cells, with a more pronounced effect in the EBV convertant cell line (Fig. 5A). In particular, in BL41 cells AKT1 inhibition was associated with hypophosphorylation of the S6 ribosomal protein, which is not inherently active in BL41/B95.8 cells. Analysis of 4E-BP1, another AKT1 substrate activated by EBV (33), revealed marked inhibition of phosphorylated 4E-BP1 in BL41/B95.8 cells (Fig. 5A). Hypophosphorylation of these AKT1 substrates inhibits mRNA translation and cell-cycle progression (34, 35). AKT1 pathway inhibition induced by hTERT silencing in both BL41 and BL41/B95.8 cells was also associated with dephosphorylation/activation of the transcription factor FOXO3, which is an effector of AKT1 kinase functioning in several cellular activities, including regulation of survival (Fig. 5A; refs. 36, 37). Notably, in both BL41 and BL41/B95.8 cells, hTERT inhibition was also associated with increased levels of the proapoptotic NOXA protein, which is known to be upregulated by the AKT1/FOXO3 axis (37). Conversely, no change in BAD protein level was observed (Fig. 5A). Pharmacologic inhibition of the AKT1 kinase induced apoptosis in both BL41 and BL41/B95.8 cells (Fig. 5B), thus confirming the critical role of AKT1 in controlling B-cell survival. Pharmacologic inhibition of AKT1 did not induce any evidence of EBV lytic replication in BL41/B95.8 cells (not shown).

Ganciclovir enhances the antiproliferative and proapoptotic effects of hTERT silencing in EBV-positive cells

Ganciclovir is activated by EBV lytic proteins, such as viral protein kinase (9, 13, 14). Treatment of shTERT3.4-infected BL41/B95.8 cells with 100 μmol/L ganciclovir further increased the accumulation of cells in the S-phase (up to 40%–80%; Fig. 6A) compared with that observed in cell cultures exposed to shTERT only (Fig. 3A). This effect was also evident at 96 hours in LCL culture (Fig. 6A). The

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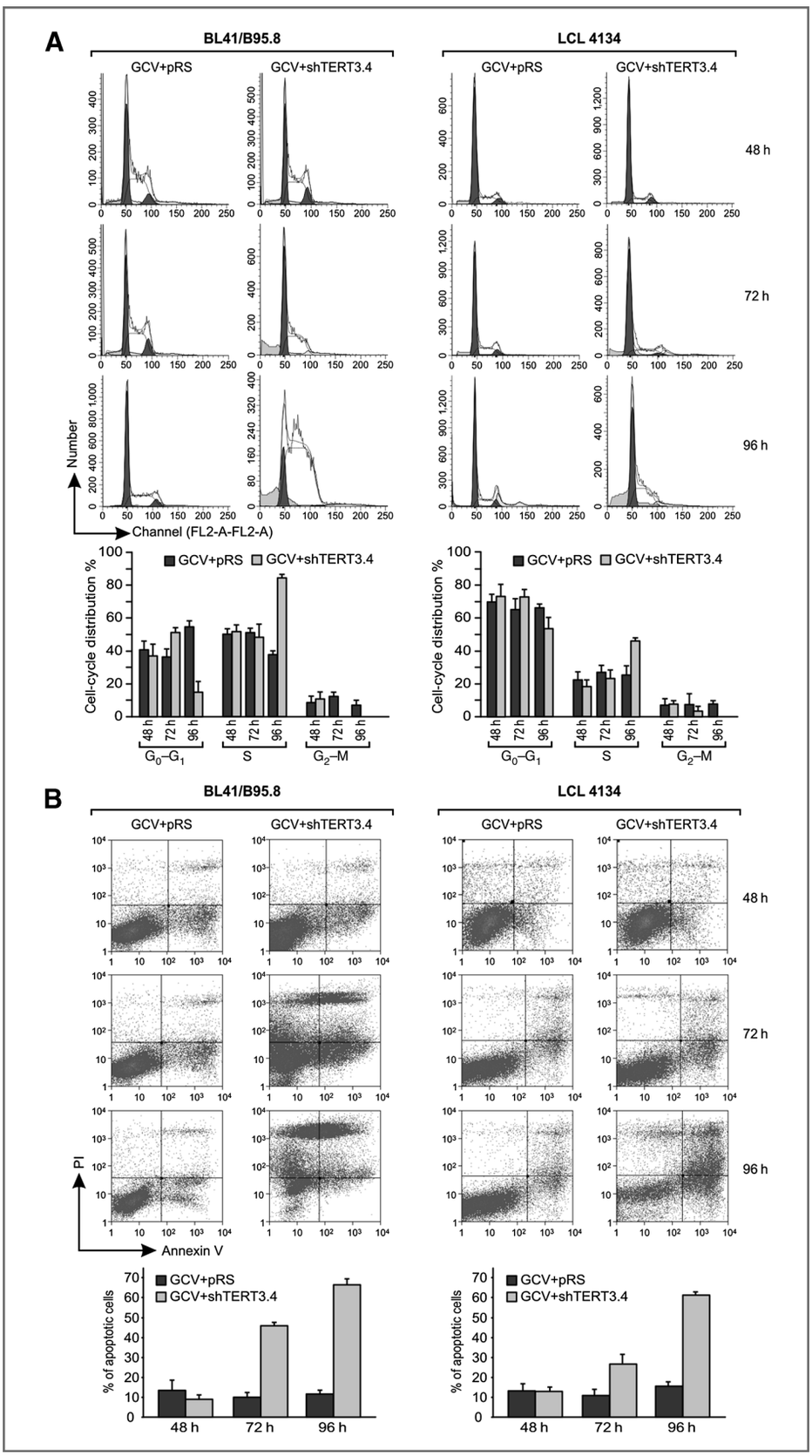

Figure 6. Effects of shRNA hTERT and ganciclovir on cell cycle and cell viability. Cells were infected with shTERT3.4 or pRS I vector and treated with ganciclovir (GVC). Cells were analyzed at 48, 72, and 96 hours after infection. A, cells were labeled with PI and analyzed by flow cytometry. Panels from 1 representative experiment are shown. Percentages of cells in G_0–G_1, S, and G_2–M are reported in the graphs on the bottom. Values are mean and SD (bar) of 3 separate experiments. B, cells were labeled with annexin V/PI and analyzed by flow cytometry. Panels from 1 representative experiment are shown. Percentages of apoptotic cells are reported in the graphs on the bottom. Values are mean and SD (bar) of 3 separate experiments.

Published OnlineFirst February 26, 2013; DOI: 10.1158/1078-0432.CCR-12-2537

combined treatment with ganciclovir and shTERT3.4 of BL41/B95.8 cells and LCL 4134 resulted in enhanced rates of apoptotic cells (Fig. 6B), significantly higher ($P < 0.05$) than those observed in corresponding cell cultures treated with shTERT3.4 only (Fig. 4). Similar antiproliferative and proapoptotic effects were also observed in Raji and HBL-1 Burkitt's lymphoma cell lines exposed to combined treatment with shTERT3.4 and ganciclovir (Supplementary Fig. S2). Conversely, in EBV-negative BL41 cells the shTERT3.4 and ganciclovir combination did not significantly modify the effects induced by shTERT3.4 alone, with respect to both cell-cycle perturbation and apoptosis (not shown). Treatment of all cell lines with ganciclovir alone (100 μmol/L) did not alter cell viability and cell-cycle distribution compared with untreated cells or cells treated with pRS control vector. Moreover, Burkitt's lymphoma and LCL cells treated with ganciglovir alone showed no evidence of EBV lytic reactivation (not shown).

Discussion

Strategies exploiting the induction of viral lytic cycle may constitute a useful adjunct to current therapeutic regimens for EBV-driven malignancies. In the present study, we show that inhibition of hTERT by shRNA triggers virus replication in both EBV-immortalized and fully transformed B cells, indicating that this is a general phenomenon for EBV-carrying B lymphocytes although EBV reactivation occurs earlier in Burkitt's lymphoma cell lines than in LCLs. These effects were also observed in Burkitt's lymphoma cells with high basal levels of h*TERT* mRNA expression and telomerase activity, features associated with malignant behavior and resistance to conventional treatments (27). hTERT inhibition invariably resulted in the induction of a complete lytic cycle with the production of EBV virions, as shown by gp350 expression and the persistence of increased EBV-DNA levels in DNase-treated culture supernatants. These findings extend our previous observations (19) and support evidence that hTERT is a critical regulator of the balance between viral latency and lytic replication in EBV-immortalized and transformed B lymphocytes.

Activation of EBV lytic cycle after hTERT inhibition might depend on modulation of BATF, a member of the AP-1/ATF transcription factors (31), which seems to play a critical role in promoting and sustaining EBV latency. BATF forms heterodimers with the JUN protein and binds preferentially to AP-1 consensus sites (30, 31), BATF/JUN heterodimers have lower transcriptional activity than FOS/JUN heterodimers and inhibit the expression of AP-1 target genes, including *BZLF1* (31). BATF expression, upregulated after EBV infection, negatively affects the expression of the *BZLF1* gene, thus reducing the frequency of the EBV lytic cycle in infected cells (32). Our results suggest that hTERT silencing promotes the activation of EBV replication by reducing inhibition of BATF-driven *BZLF1* transcription. Further studies are required to elucidate in depth the mechanisms, whereby hTERT modulates BATF expression.

Consistent with the notion that telomerase is involved in more cell processes than mere maintenance of telomere length (38), this study provides evidence that, besides inducing EBV lytic cycle, hTERT inhibition has antiproliferative and proapoptotic effects that may be of therapeutic importance. While hTERT inhibition in EBV-negative Burkitt's lymphoma cells induced accumulation of cells in G_0–G_1, as shown in other cell systems (39–41), infection of EBV-positive Burkitt's lymphoma cells with shTERT3.4 resulted in a marked increase of cells in the S-phase. This peculiar cell-cycle deregulation is associated with the presence of EBV infection and is probably related to the induction of EBV lytic replication. Indeed, as seen for other herpesviruses, the productive cycle of EBV occurs in an S-phase–like cellular environment and activates DNA damage responses (42). Recent evidence indicates that the conserved cysteine protease encoded in the amino terminus of major tegument protein BPLF1 is a viral deneddylase responsible for deregulation of cell cycle during EBV replication (43). In particular, BPLF1 has been shown to bind cullins and regulate culling-RING ligases, thereby establishing the S-phase–like background that is required for efficient replication of the viral genome (43). Our results indicate that accumulation of cells in the S-phase may be also favored by AKT1-dependent dephosphorylation of 4E-BP1 induced by hTERT inhibition. Inactivation of this translation initiation factor, which is involved in the control of cap-dependent mRNA translation (44), may in fact decrease the level of many proteins needed for cell-cycle progression.

The inhibition of the AKT1 kinase induced by hTERT silencing was also involved in the apoptotic responses observed in both EBV-negative BL41 cells and in the EBV convertant BL41/B.95.8 cell line. This is consistent with the finding that EBV infection can activate the AKT1 pathway, which is critical for the growth and survival of both EBV-negative and EBV-transformed B lymphocytes (35, 45, 46). The results of the present study indicate that hTERT silencing induces apoptosis through dephosphorylation/activation of the FOXO3 transcription factor, a downstream target of AKT1 kinase (37), which in turn induces upregulation of *NOXA* (see Supplementary Fig. S3 for a proposed mechanistic model). This proapoptotic protein is a critical mediator of lymphoma cell apoptosis triggered by several classes of drugs (47, 48). Notably, recent evidence also indicates that latent EBV infection prevents B-cell apoptosis by blocking the induction of *NOXA* expression (49), although the viral gene product(s) responsible for this phenomenon remains elusive. The observation that hTERT inhibition can overcome the block of *NOXA* upregulation induced by EBV, thus favoring B-cell apoptosis, is particularly important in a therapeutic perspective and further supports the role of the AKT1/FOXO3/NOXA axis as critical target for B-cell malignancies. Taken together, our results suggest that hTERT inhibition promotes the induction of EBV-infected cell death through the combined effect of AKT1/FOXO3/NOXA–dependent apoptosis and activation of complete EBV lytic replication.

Our results also show that ganciclovir markedly enhances the antiproliferative and proapoptotic effects induced by

Published OnlineFirst February 26, 2013; DOI: 10.1158/1078-0432.CCR-12-2537

hTERT inhibition in both EBV-positive Burkitt's lymphomas and LCLs. This may be the result of prodrug activation by EBV lytic protein kinase (9, 13, 14). Activation of ganciclovir does require phosphorylation; because its affinity for eukaryotic thymidine kinases is 1,000 times lower, ganciclovir is essentially phosphorylated by viral thymidine kinase (14). Phosphorylated ganciclovir competitively inhibits DNA synthesis catalyzed by both viral and cellular DNA polymerases, thus resulting in cell death and reduction of viral replication (7). In this respect, drugs that inhibit hTERT may be regarded as sensitizers of antiviral activity. The combination of antiviral drugs with inhibitors of hTERT expression/activity may thus result in therapeutically important effects in patients with EBV-related malignancies. This possibility seems particularly promising in light of the recent development of specific telomerase inhibitors, including imetelstat (GRN163L), which is currently under evaluation in several clinical trials (20, 50).

In conclusion, our findings indicate that compounds that inhibit hTERT may be useful agents for inducing viral lytic cycle and provide a rationale for conducting further research to assess the effects of combination therapies with inhibitors of hTERT and antiviral drugs for treatment of EBV-associated malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Giunco, R. Dolcetti, J. Dal Col, A. De Rossi
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Giunco, R. Dolcetti, S. Keppel, A. Celeghin, A. De Rossi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Giunco, R. Dolcetti, A. De Rossi
Writing, review, and/or revision of the manuscript: S. Giunco, R. Dolcetti, A. De Rossi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Giunco, S. Keppel, A. Celeghin, S. Indraccolo, A. De Rossi
Study supervision: A. De Rossi
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Acknowledgments

The authors thank Liliana Terrin for help in preparing retroviral vectors, Gianni Esposito for immunohistochemistry, Pierantonio Gallo for artwork, and Lisa Smith for editorial assistance.

Grant Support

This study was supported by Program Integrato Oncologia, Contract RO4/2007; Italian Ministry of University and Research, Prin Prot. 2007CH4K9T_002; Associazione Italiana per la Ricerca sul Cancro (AIRC; contract IG5326 and IG10301). S. Giunco is supported by a fellowship from AIRC.

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Received August 6, 2012; revised January 30, 2013; accepted February 18, 2013; published OnlineFirst February 26, 2013.

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