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ORIGINAL PAPER

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Suppression of Epstein-Barr nuclear antigen 1 (EBNA1) by RNA interference inhibits proliferation of EBV-positive Burkitt's lymphoma cells

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Abstract *Purpose*: Epstein-Barr virus (EBV) is associated with the development of several lymphoid and epithelial malignancies, including Burkitt's lymphoma. The EBV latent protein, EBV Nuclear Antigen 1 (EBNA1), is detectable in almost all types of EBV-associated tumors and is essential for replication and maintenance of the latent episome of EBV. We here examined whether the RNA interference (RNAi) technique could be employed to suppress expression of EBNA1 in EBV-positive Burkitt's lymphoma cells. *Methods*: A Raji cell line expressing small hairpin RNAs (shRNAs) against EBNA1 was established and EBNA1 mRNA level was determined by real-time RT-PCR analysis. We investigated the effects of EBNA1 silence on lymphoma cell growth and cell cycle progression. *Results*: Transfection of an EBNA1 RNAi plasmid resulted in substantial loss of EBNA1 mRNA and significantly inhibited proliferation of Raji cells relative to the control plasmid case. Suppression of EBNA1 was also associated with downregulation of EBV oncogene EBNA2, a decreased PCNA labeling index and increased G0/G1 fraction in cell cycle analysis. *Conclusions*: These findings point to potential therapeutic applications for vector-mediated siRNA delivery to control EBV-associated malignant disorders.

Keywords EBV · EBNA1 · RNA interference · Burkitt's lymphoma · Cell growth

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Introduction

The Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus implicated in the pathogenesis of several lymphoid and epithelial malignancies, including Burkitt's lymphoma and nasopharyngeal carcinoma, as well as Hodgkin's disease, lymphomas occurring in immune-deficient patients and gastric carcinomas (Young and Murray 2003). Infection with EBV in vitro easily transforms resting B cells from human peripheral blood to permanently growing lymphoblastoid cell lines (LCLs) (Sugimoto et al. 2004; Middeldorp et al. 2003). Most EBV diseases are associated with EBV latency, during which the viral gene expression is limited to the six members of the EBNA family of nuclear proteins, two membrane proteins, and two small polymerase III transcripts (Kieff 1996). EBV can establish three types of latency (I, II, and III), each of which is characterized by differential expression of a group of latency proteins.

During latency, the EBV genome exists predominantly as a multicopy circular episome that is replicated once per cell cycle and partitioned at mitosis (Adams 1987; Yates and Guan 1991). Dimers of EBNA1 bind with high affinity to two elements within the origin of plasmid replication (OriP), namely, the family of repeats (FR) and the dyad symmetry (DS) (Yates et al. 1984, 1985; Reisman et al. 1985; Chen et al. 1993). At the same time EBNA1 associates with host-cell chromosomes through its chromosome-binding domains (Marechal et al. 1999). The exact mechanisms by which EBNA1 facilitates replication and maintains viral episome are incompletely understood but possible functions include

the recruitment of cellular replication machinery during S phase (Schepers et al. 2001; Zhang et al. 1998) and segregation of episomes to daughter cells during mitosis (Hung et al. 2001).

EBNA1 furthermore participates in regulation of latency gene transcription. Binding of EBNA1 to a site downstream of the *Bam*HI Q promoter (Qp) represses Qp activity, but this repression can be overcome by E2F that displaces EBNA1 (Sung et al. 1994). EBNA1 has also been shown to upregulate two key EBV latency promoters, the *Bam*HI C promoter (Cp) (Sugden and Warren 1989) and the LMP1 promotor (Gahn and Sugden 1995). Although EBNA1 is exogenous to the host, EBV-infected cells expressing EBNA1 are barely recognized by cytotoxic T lymphocytes (CTLs) and the Gly-Ala repeat domain of EBNA1 has been shown to prevent the presentation of CTL epitopes via the Major Histocompatibility Complex (MHC) class I pathway by blocking ubiquitin/proteasome-dependent processing (Levitskaya et al. 1995). This evasion mechanism has been implicated in the pathogenesis of EBV-associated diseases.

RNA interference is a new powerful tool for selective suppression of gene expression at the post-transcriptional level (Dykxhoorn et al. 2003). Small interfering RNAs (siRNAs) are short, double stranded RNAs (dsRNAs) that target mRNA with complementary sequences for endonucleolytic degradation. The Dicer family of RNase III enzymes cleaves long dsRNA to produce siRNAs, which become incorporated into a multiprotein RNA-inducing silencing complex (RISC). Introduction of chemically synthesized 21-nucleotide siRNAs is sufficient to initiate RNA interference in mammalian cells (Elbashir et al. 2001) and DNA vectors expressing small hairpin RNAs (shRNAs) using RNA polymerase III (RNA pol III) promoters have been developed for effective and persistent gene silencing (Brummelkamp et al. 2002; Tomar et al. 2003). This approach is finding increasing employment for analysis of gene function and gene therapy (Hall and Alexander 2003; Butz et al. 2003; Li et al. 2004).

We here aimed to suppress EBNA1 expression in Raji Burkitt's lymphoma cells using the RNA interference strategy. A plasmid expressing shRNAs against EBNA1 was therefore constructed and transfected to Raji cells. Effective suppression at the mRNA level could be confirmed by real-time RT-PCR analysis post transient transfection. One month after antibiotics selection, the EBNA1 shRNA expressing Raji cells displayed a significantly lower cell growth as compared with control cells. Furthermore, we observed decreased expression of the EBV oncogenic protein EBV Nuclear Antigen 2 (EBNA2) as well as a lower labeling index for proliferating cell nuclear antigen (PCNA). Finally, we provided evidence that the growth inhibitive effect is associated with elevated frequency of G0–G1 phase cells but not increased apoptosis. Our findings support the possibility that RNA interference could be an efficacious novel therapy for EBV-related diseases.

Materials and methods

Cell culture Raji is an EBV-positive human Burkitt's lymphoma cell line whose cells contain 50–60 copies of EBV genome per cell (Adams 1987; Polack et al. 1984; Hatfull et al. 1988). Raji cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C.

Plasmid construction The sequence of EBNA1 hairpin was designed by iGENE Therapeutics (Ibaraki, Japan). The shRNA-encoding DNA template comprised the 19-base sense sequence followed by the hairpin loop and antisense sequences and five consecutive Ts as an RNA pol III terminator sequence. This template was subcloned downstream of the human U6 promotor in the RNAi-Ready pSIREN-RetroQ vector (BD Biosciences Clontech), between the *Bam*H I and *Eco*R I restriction sites. A control vector was generated by utilizing the Negative Control siRNA Annealed Oligonucleotide supplied with the vector.

Transfection Transfection was performed with Nucleofector™ Technology (Amaxa), with 2×10^6 logarithmically growing cells nucleofected using a Cell Line Nucleofector™ Kit T, program O-17 and 2 μg of plasmid. Raji cells transfected with EBNA1 RNAi or control plasmid were plated in 100-mm dishes and 24–48 hours post transfection, puromycin (BD Biosciences Clontech) was added at a concentration of 5 μg/ml. The cells were maintained under these conditions with medium change twice a week.

RNA extraction and reverse transcription (RT) Total cellular RNA was isolated using an RNeasy Midi Kit (QIAGEN) as recommended by the supplier. Samples were digested with an RNase-Free DNase Set (QIAGEN) during purification to remove residual DNA. Twenty micro litres of cDNA was synthesized using 200 ng of total RNA as the template and random hexamers as primers, as described in the manufacturer's protocol for Taqman Reverse Transcription Reagents (Applied Biosystems).

Real-time PCR PCR was carried out using 1×of TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer, 250 nM of TaqMan probe and cDNA template in a 25 μl reaction mixture. The PCR primers used for the quantitation of EBNA1 mRNA were 5′-CGCATCATAGACCGCCAGTA-3′ (forward) and 5′-CTGGCCCCTCGTCAGACAT-3′

(reverse). A TaqMan probe (5′-[FAM]-CCGCGGCCG TCTCCTTTAAGTGTG-[TAMRA]-3′) located between the PCR primers was synthesized by Nippon EGT (Toyama, Japan). The Pre-Developed TaqMan Assay Reagent for GAPDH (20×primers and probe mixture) was obtained from Applied Biosystems. The reaction conditions were as follows: 50°C for 2 min (activation of the AmpErase uracil N-glycosylase [UNG]) and then 95°C for 10 min (activation of the AmpliTaq Gold DNA polymerase), followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. All reactions were performed in duplicate. A standard curve for threshold cycle (C_T) values was run in parallel using serial twofold dilutions of 50 ng cDNA of untreated Raji cells. Amplification data were collected using an ABI PRISM 7700 Sequence Detector system (Applied Biosystems) and analyzed using Sequence Detector software version 1.7 (Applied Biosystems).

Western blot analysis Total cellular protein was prepared as described in previous report (Davenport and Pagano 1999). Briefly, cells were washed once with 1×phosphate-buffered saline (PBS) and then resuspended in lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5 mM dithiothreitol [DTT], 10 mM NaF, protease inhibitor cocktail [Sigma]). After freezing and thawing three times, debris was spun down at 4°C for 30 min and the protein concentration was determined by the Bradford protein assay. Twenty microgram aliquots of lysates were separated on 8% sodium dodecyl sulfate—polyacrylamide gels (SDS-PAGE) and proteins were transferred to Immobilon-P (Millipore). Expression of EBNA1 and EBNA2 proteins was examined using mouse monoclonal antibodies to EBNA1 (Advanced Biotechnologies) and PE2 (Dako), respectively. Signals were detected with either anti-mouse or anti-rabbit immunoglobulin (Ig)—horseradish peroxidase (Daco) and Western Lightening Plus Chemiluminescence Reagent (PerkinElmer Life Sciences).

Immunocytochemistry Immunocytochemical analysis was performed on 4 μm thick sections obtained from formalin-fixed and paraffin-embedded fibrin-clot preparations as described previously (Kumada et al. 2004). After antigen-retrieval using microwave heating, sections were inactivated for endogenous peroxidase in 3% H_2O_2 and then blocked with 5% BSA. Subsequently, slides were stained with mouse monoclonal antibodies against Ki-67 (Clone MIB-1, 1:50 dilution, Dako) or PCNA (Clone PC10, 1:100 dilution, Dako) followed by treatment with EnVision+, Peroxidase, Mouse (Dako). Finally, Peroxidase activity was determined with diaminobenzidine (DAB) solution and counterstaining was achieved with hematoxylin.

Cell proliferation and apoptosis assays For growth curve analysis, stably transfected Raji cells cultured to mid-log phase were diluted in standard growth medium with 5 μg/ml puromycin to 0.25×10^5/ml and seeded at 2 ml/well in 6-well plates. Cell numbers of control or EBNA1 RNAi plasmid-transfected cells were determined on four consecutive days by trypan blue exclusion. The cell cycle distribution and numbers of apoptotic cells were determined using a BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol. Logarithmically growing Raji cells were pulsed with 10 μM bromodeoxyuridine (BrdU) for 30 min and stained with FITC-conjugated anti-BrdU antibody in combination with a DNA-specific dye, 7-amino-actinomycin D (7-AAD). Two-color analyses were then conducted on a flow cytometer FACSCaliber (Becton Dickinson).

Results

Choice of RNAi target for EBNA1 silencing in Raji cells

Suppression of gene expression by RNA interference is highly target sequence-specific so that only one base pair mutation will greatly disturb the inhibition efficiency (Elbashir et al. 2001). The size of EBNA1 protein varies greatly among latently infected cell lines due to the different lengths of the Gly-Ala repeat (Heller et al. 1982; Grasser et al. 1994). Besides, although EBNA1 does not display any obvious polymorphism between the two major types of EBV, types 1 and 2, a degree of inter-strain sequence variation, especially in the region between residues 466–527, has been reported from the analysis of EBV-associated tumors and samples from asymptomatic carriers (Habeshaw et al. 1999).

In order to block EBNA1 expression selectively in Raji cells by RNA interference, four potential 19-nt sequences within the EBNA1 open reading frame (ORF) were chosen as siRNA targets. Blast searching against the human genome was performed to ensure that no host cell gene was targeted. The corresponding oligonucleotides were then introduced into the pSIREN-RetroQ vector, which contains a puromycin resistance gene for selection of stable transfectants. Treatment with one plasmid targeting 5′-GTTCCAACCCGAAATTTGA-3′ (+1418 to +1436 of the EBNA1 ORF) reproducibly suppressed EBNA1 expression with high efficiency and was used in all subsequent experiments. The predicted secondary structure of the shRNA against EBNA1 is indicated in Fig. 1a.

Quantitation of EBNA1 transcripts by real-time RT-PCR

Previous reports indicated that EBNA1 protein has a long half-life in excess of 36–48 h, which is in agreement with the finding that the Gly-Ala repeat inhibits processing by the ubiquitin/proteasome pathway (Davenport and Pagano 1999; Levitskaya et al. 1997). In

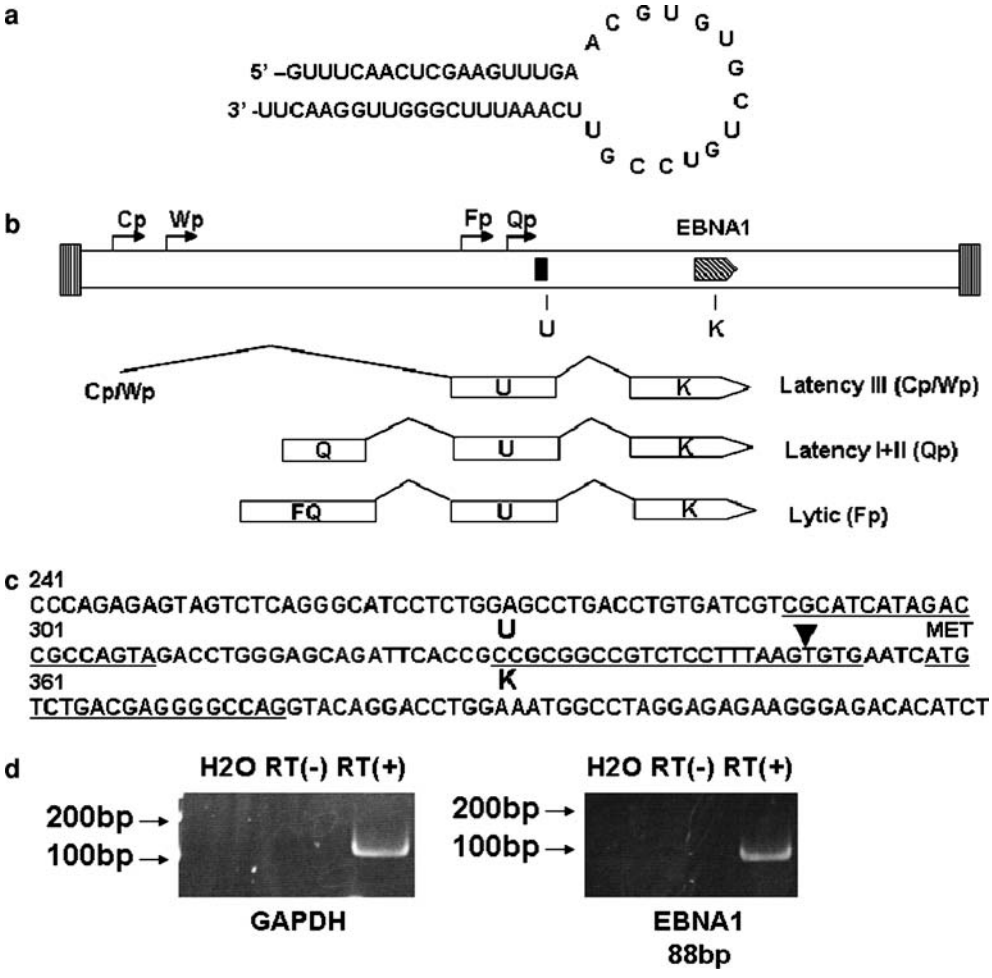

Fig. 1 Designs of the EBNA1 shRNA template and real-time PCR Primers. **a** The predicted secondary structure of the shRNA against EBNA1. Since hairpin sequences are difficult to sequence, three nucleotides in the target sense sequence were substituted to facilitate insert verification. **b** Model for transcription and splicing of EBNA1 mRNAs. The *top bar* illustrates the linear form of the EBV genome and the positions of four different promoters (*bent arrows*). The *lower panel* shows three classes of EBNA1 transcripts and their corresponding infection types. Coding regions are indicated by boxes and excised intron sequences by *thin lines*. **c** Partial cDNA sequence of the 5′ end of the EBNA1 mRNA (GenBank accession number M13941). The sequences of the PCR primers and TaqMan probe used for EBNA1 quantitation are underlined. The splice site between exons U and K is indicated with an *arrowhead*. The start codon of EBNA1 ORF is shown translated. **d** Specificity and efficiency of PCR primers designed for real-time PCR analysis. GAPDH is amplified as an internal standard. Negative water blanks were included in each analysis

order to investigate the effect of candidate RNAi sequences on EBNA1 transcription, the mRNA level was analyzed post transient transfection by performing real-time RT-PCR assays. Since TaqMan RT-PCR analysis with a low-abundance target is sensitive to very small amounts of DNA, we designed primers that anneal at intron splice junctions so that genomic DNA would not be amplified.

Although EBNA1 expression is common to the three types of latency, the promoter used for its expression differs (Fig. 1b). The open reading frame for EBNA1 is located in *Bam*HI K exon at the 3′ end of the message and is preceded by a long 5′ untranslated region (5′ UTR) derived from several short exons (Fig. 1b). The U leader exon is common to all four transcripts and spliced directly to the K exon (Isaksson et al. 2003; Sample et al. 1986, 1991). Raji cells exhibit the type III latency program in which long primary transcripts, containing coding sequences for EBNA1-6, are alternatively spliced into bi- or monocistronic transcripts (Davenport and Pagano 1999).

For amplification of the EBNA1 transcript, primers flanking the U/K intron splice junctions were utilized (Fig. 1c). Figure 1d shows that EBNA1 primers amplified an 88 bp fragment corresponding to the EBNA1 coding region, nucleotides −69 to +19. No DNA contamination was detected following control RT-PCR experiments in which no reverse transcriptase was added to the RT step.

RNAi selectively reduces expression of the EBNA1 viral gene

To determine efficient siRNA sequence for EBNA1 targeting, Raji cells were transiently transfected with siRNA-generating plasmids and selected for successfully transfected cells by puromycin resistance (de la Luna and Ortin 1992). After 2 days of exposure to puromycin, total cellular RNA was prepared and EBNA1 transcripts were assessed by real-time RT-PCR. We observed marked reduction in the level of EBNA1 mRNA in cells transfected with one RNAi vector compared with that in control cells, when the levels were normalized to GAPDH expression (Fig. 2a). In two separate experiments the mRNA levels were consistently diminished by no less than 50% relative to the control (51.3 and 75.0%).

After 1 month's puromycin selection, two Raji cell populations, stably transfected with either EBNA1

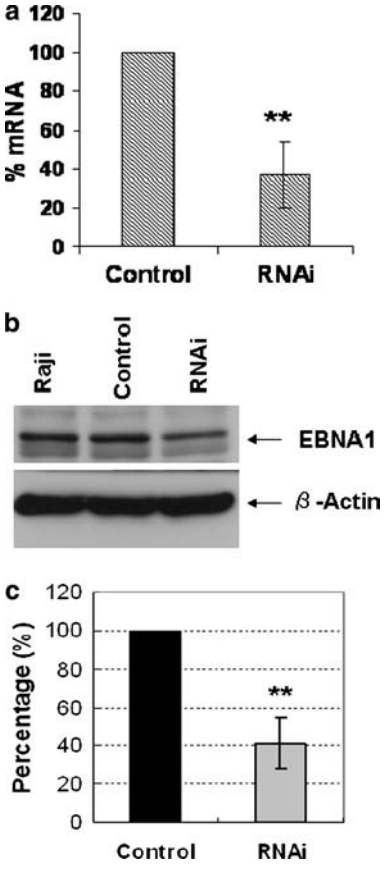

Fig. 2 Suppression of EBNA1 expression in Raji cells by RNA interference. **a** Quantitation of EBNA1 mRNA by real-time RT-PCR analysis. Values represent the mean ± standard deviation (SD) of two separate experiments performed in duplicate. $^{**}P < 0.01$ versus the control cells. Significance was determined using the Student's *t*-test. **b** Cells stably transfected with EBNA1 RNAi or control vector were extracted and analyzed by Western blotting for EBNA1 and actin as a loading control. **c** Relative amounts of EBNA1 protein in Fig. 2b as determined by densitometry. Data are mean ± SD from three separate experiments. $^{**}P < 0.01$ versus the control cells. Significance was determined by using Student's *t*-test

RNAi or control plasmid, were obtained. To test the effects of siRNA delivery on expression of the EBNA1 protein, we separated equal amounts of protein by SDS-PAGE and hybridized the blots with antibodies to EBNA1 and β-actin, respectively. As shown in Fig. 2b and c, Western blots confirmed partial (about 60%) decrease in EBNA1 expression in the cells stably transfected with the RNAi plasmid. Importantly, β-actin protein levels appeared unaffected, suggesting that RNAi against EBNA1 did not activate global suppression of protein expression.

RNA interference against EBNA1 inhibits proliferation of Raji cells

Having demonstrated the selective silencing of the viral gene, we next studied the cellular consequences of EBNA1 depletion. In cell growth curve assays, as illustrated in Fig. 3a, the EBNA1 shRNA-expressing Raji cells displayed a significantly lower growth rate as compared with control cells.

We also observed substantial reduction in the level of EBNA2 protein in Raji cells transfected with EBNA1 RNAi vector relative to the control cells (Fig. 3b). EBNA2 transcriptionally activates certain cellular proto-oncogenes as well as viral oncogenes and is involved in G0–G1 transition (Middeldorp et al. 2003). This finding suggested the possibility that suppression of EBNA1 might attenuate proliferation through down-regulating the expression of other EBV transforming genes.

The inhibitory effect on cell growth was further corroborated by immunostaining studies of proliferative antigens. PCNA is an auxiliary protein of DNA polymerase δ, whose synthesis reaches a peak during the S phase of the cell cycle (Mathews et al. 1984). Immunocytochemistry revealed a marked decrease of PCNA-positive Raji cells stably transfected with EBNA1 RNAi vector as compared with those transfected with the control vector (Fig. 3c). In contrast, no apparent changes were detected in the immunoreactivity of Ki-67, which is known to be preferentially expressed during all active phases of cell cycle (G1, S, G2 and M phases) but absent in resting cells (G_0 phase) (Gerdes et al. 1984).

To further elucidate whether the inhibited cell growth is associated with cell cycle arrest, log-phase Raji cells were analyzed using a BrdU Flow Kit. Compared with the control cells, the frequency of G0–G1 phase cells significantly increased in EBNA1 RNAi vector-transfected cells, while there was a marked reduction of the frequencies of S and G2/M phase cells (Fig. 4). Monitoring cell death showed that inhibition of cell proliferation by EBNA1 RNAi was not due to increased apoptosis under standard growth conditions (Fig. 4).

Discussion

Of the EBV-encoded products, EBNA1 is the only latent protein that is found to be consistently expressed in EBV-associated tumor cells. EBNA1 ensures episomal replication and genome maintenance to establish EBV latent infection efficiently. Furthermore, EBNA1 transactivates the enhancers of a number of other viral genes and is implicated in the evasion from host immune response. A previous report has also suggested that EBNA1 itself may have tumorigenic potential (Wilson et al. 1996). The present study demonstrated that the shRNA approach is effective for suppression of EBNA1 expression in Raji cells and that this is associated with decreased cell growth and increased frequency of G0–G1 phase cells.

A variety of different EBV-based therapies are currently being developed for the treatment of EBV-positive cancers, including prevention of viral oncogene expression, causing loss of the EBV episome, targeted induction of the lytic form of EBV infection, and enhancing host immune responses to virally encoded antigens

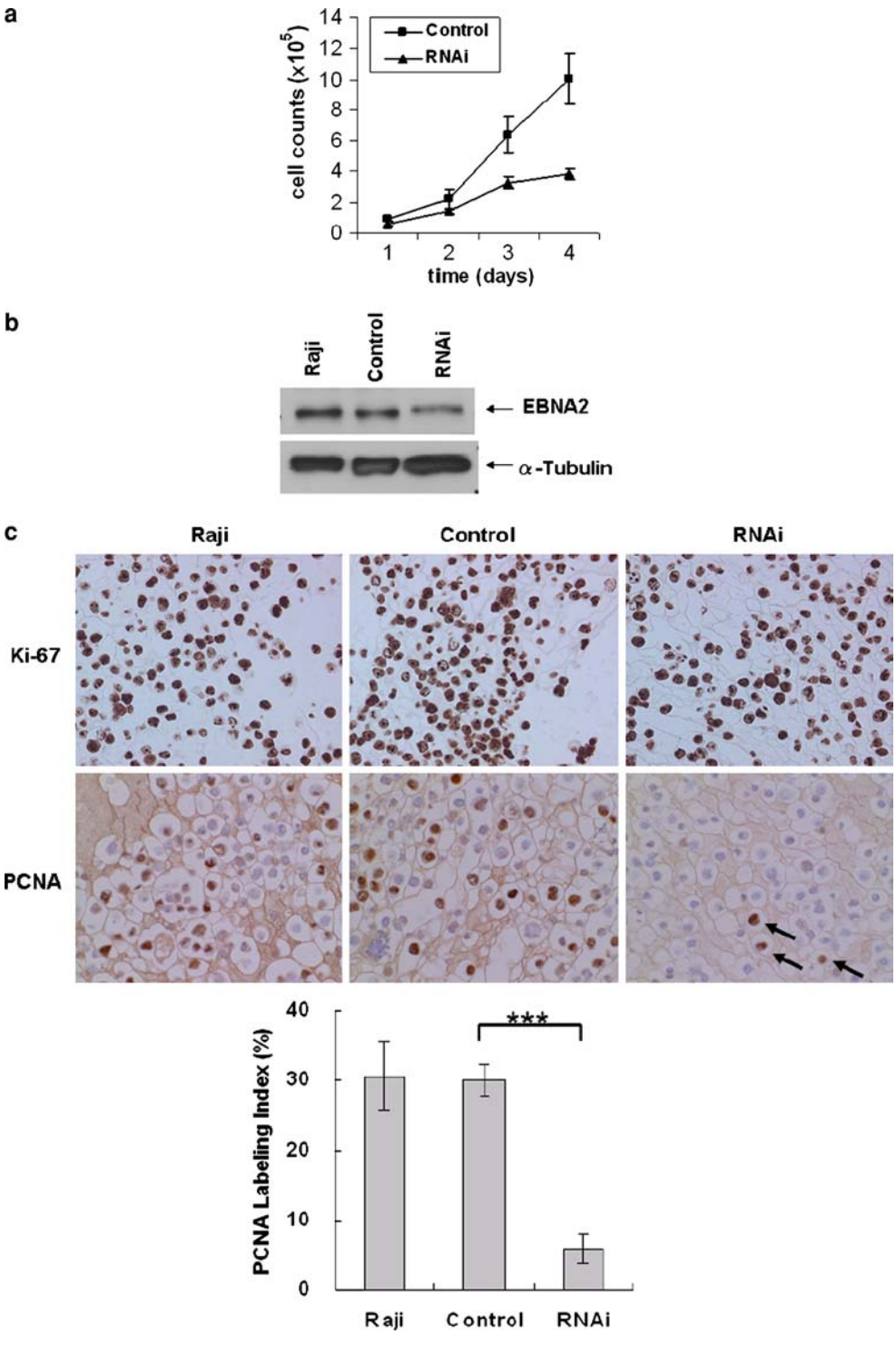

Fig. 3 Effects of EBNA1 RNAi on proliferation of Raji cells. **a** Cell growth curve assays. EBNA1-targeted RNA interference significantly inhibited the proliferation of Raji cells over four days (time×treatment interaction: $P = 0.0001$). Data are mean ± SD from triplicate assays and are representative of two independent experiments. Significance was determined using repeated ANOVA (analysis of variance). **b** Downregulation of EBNA2 protein. Cells stably transfected with EBNA1 RNAi or control vector were extracted and analyzed by Western blotting for EBNA2 and α-Tubulin as a loading control. **c** Ki-67 immunoreactivity and PCNA labeling index determined by immunocytochemistry. Raji cells in three random fields of each slide were counted and the percentage of PCNA-positive cells was calculated and expressed as the mean ± SD. ***$P < 0.001$. Significance was determined using the Student's *t*-test

(Israel and Kenney 2003). Several antisense-based strategies have previously been used in order to target EBV transcription (Roth et al. 1994; Mattia et al. 1997; Kenney et al. 1998) and studies have already shown that antisense oligodeoxynucleotides (Roth et al. 1994) or adenovirus-delivered ribozymes (Huang et al. 1997) directed to EBNA1 can suppress EBNA1 expression and inhibit proliferation of EBV-immortalized B lymphocytes. Anti-sense RNA, ribozymes and RNA interference all operate at the post-transcriptional level to suppress gene expression. However, the process of RNA interference is several orders of magnitude more efficient than anti-sense or ribozyme strategies (Jiang and Milner 2002). We therefore sought to down-regulate the EBNA1 gene by devising a vector-borne siRNA that, by binding to a target sequence on mRNA, would lead to the degradation of EBNA1 transcripts via endonucleolytic cleavage. We here screened several po-

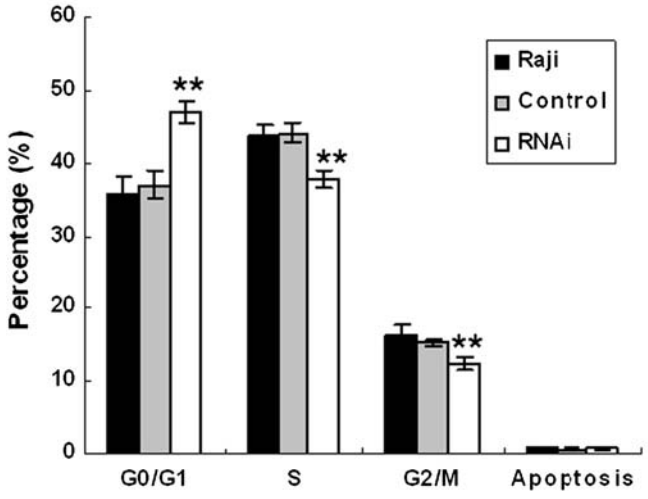

Fig. 4 Suppression of EBNA1 expression in Raji cells results in increased frequency of G0–G1 phase cells. FACS analysis of the cell cycle distribution using a BrdU Flow Kit. Data are mean ± SD from three separate experiments. $^{**}P < 0.01$ versus the control cells. Significance was determined using the Student's *t*-test

tential RNAi sequences directed against EBNA1 and established that one sequence significantly decreases the intracellular concentration of EBNA1 mRNA, and effectively inhibits cell proliferation of EBV-positive Raji Burkitt's lymphoma cells.

However, the molecular mechanisms underlying inhibition of proliferation due to EBNA1 silencing remain obscure. Based on previous reports (Sugden and Warren 1989; Wilson et al. 1996; Huang et al. 1997), we propose that RNAi-mediated EBNA1 suppression inhibits cell growth through three potential mechanisms. First, given the indispensable role of EBNA1 in the replication of latent viral genome, it is possible that the viral episome might be eliminated over a number of cell divisions in the absence of EBNA1. Thus, suppression of EBNA1 expression would be expected to disrupt the latent infection and thereby reverse the transformed phenotype. This hypothesis is supported by a previous observation that suppression of EBNA1 by an adenovirus-delivered specific ribozyme reduced the number of EBV genomes in EBV-immortalized B lymphocytes (Huang et al. 1997). Second, suppression of EBNA1 may retard cell proliferation by down-regulating the expression of other viral genes directly involved in growth transformation, such as EBNA2. As inhibition of EBNA1 expression was shown to correlate with loss of EBV episomes (Huang et al. 1997), the lower level of EBNA2 detected in Western blotting may reflect the presence of cells that have reduced number of EBV plasmids. Reduction of EBNA2 protein level might also be explained by impaired transcription activation due to a possible role for EBNA1 as an enhancer protein (Sugden and Warren 1989). Finally, EBNA1 is the only viral protein expressed in the Burkitt's lymphoma cells in vivo and in early passage in vitro, raising the possibility that EBNA1 may be directly linked to the oncogenic transformation of these cells.

For the first time, we here selectively silenced EBV latent gene EBNA1 in human Burkitt's lymphoma cells by exploiting RNAi technology. Our results indicate that targeting of EBNA1 by RNA interference represents a promising novel tool for fundamental research into the pathogenesis of EBV-induced diseases, and for the development of specific treatment strategies against EBV-positive cancers. Two key challenges in developing RNAi as a therapy are avoiding off-target effects and ensuring efficient delivery (Hannon and Rossi 2004). Transfection of cells with double-stranded (ds)RNAs can activate innate immune pathways, resulting in sequence-independent destruction of RNAs. In an in vivo study, hydrodynamically injected siRNAs targeting Fas protected mice from fulminant hepatitis (Randall et al. 2003). Despite the considerable obstacles to overcome, we anticipate the development of therapeutics based on RNA interference in the not-too-distant future.

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