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Molecules and Cells

Sustained Viral Activity of Epstein-Barr Virus Contributes to Cellular Immortalization of Lymphoblastoid Cell Lines

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EBV-transformed lymphoblastoid cell lines (LCLs) are used as a resource for human genetic, immunological, and pharmacogenomic studies. We investigated the biological activity of 20 LCL strains during continuous long-term subculture up to a passage number of 160. Out of 20 LCL strains, 17 proliferated up to a passage number of 160, at which point LCLs are generally considered as "immortalized". The other three LCL strains lost the ability to proliferate at an average passage number of 41, during which these LCLs may have undergone cellular crisis. These non-immortal LCL strains exhibited no telomerase activity, decreased EBV gene expression, and a lower copy number of the EBV genome and mitochondrial DNA when compared with immortal LCLs. Thus, this study suggests that sustained EBV viral activity as well as telomerase activity may be required for complete LCL immortalization.

INTRODUCTION

Primary cells normally proliferate for a very limited life span in culture. Viral infection or ectopic expression of oncogenes is widely used to immortalize primary cells (Hahn, 2002). When infected with Epstein-Barr virus (EBV), primary B cells are transformed into continuously proliferating lymphoblastoid cell lines (LCLs) *in vitro*, enabling LCLs to be used as a genome resource for human genetics and immunological studies (Young and Rickinson, 2004). With respect to cell growth, EBV infection mimics B cell activation signals or factors such as CD40-CD40L, IgM, and LPS. Thus, EBV-infected B cells exhibit higher proliferative activity than resting primary B cells (Kilger et al., 1998).

EBV infection induces gene expression of the latent EBV genes in primary B cells, while EBV-associated B cell malignancies express only particular subsets of latent genes (Kieff and Rickinson, 2001). EBV infection also induces changes in cellular gene expression through the viral genes EBNA1, EBNA2, EBNA3a, EBNA3c, and LMP1. For example, EBNA1 induces the expression of the recombination-activating genes, RAG1 and RAG2. Aberrant activity of these RAGs has been implicated in chromosomal translocations in B cell neoplasms, and chromosomal integration of EBV genome (Srinivas and Sixbey, 1995). EBNA2 and EBNA3 regulate the expression of cellular genes as well as the viral LMP1 gene by interacting with RBP-Jk/CBF1 (Johannsen et al., 1996). The cellular targets of EBNA2 include TNF-α, LTβ, G-CSF, cyclin D2, c-Myc, IL-16, and the AML family (Carter et al., 2002; McClain et al., 1997; Spender et al., 2002). LMP1 activates the NF-κB/Rel, JKN (c-Jun N-terminal kinase), p38/MAPK, and JAK/STAT signaling pathways, and it promotes tumorigenesis by inhibiting apoptosis, in part, by inhibiting BAX (Cahir-McFarland et al., 2004; Grimm et al., 2005; Saito et al., 2003; Sylla et al., 1998; Zhang et al., 2004). During B cell immortalization, LMP1 expression is essential for the continuous proliferation of EBV-infected B cells (Eliopoulos and Young, 2001; Mei et al., 2006). In addition, LCLs frequently acquire structural genomic aberrations during initial transformation and further long-term culture through 160-180 passages, at which point LCLs are considered as "terminally immortalized" (Redon et al., 2006; Sugimoto et al., 2004). Such genetic alterations do not affect the results of genome-wide association studies.

On the other hand, LCLs are currently used in pharmacogenomic studies to investigate variation in human gene expression as well as drug responses among individuals. Indeed, the effects of gene expression on the impact of nucleotide and copy number variation have been investigated using LCLs (Rodriguez-Revenga et al., 2007). In an effort to expand the utility of our LCL collection, we have characterized LCLs using genomic and proteomic approaches (Jeon et al., 2007; 2008). In this study, we propagated 20 LCL strains for long-term subculture and investigated the biological activities of immortal and non-immortal LCLs. Our results suggest that EBV viral activities and telomerase activity require to be sustained to complete the LCL immortalization.

MATERIALS AND METHODS

Cell culture

We used 20 LCL strains from the LCL collection of the Korean HapMap project which included 90 unrelated healthy Koreans

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with an equal sex ratio and age (40-69 years) for the Korean Health and Genome Epidemiology Study (http://cdc.go.kr), as described in previous reports (Kim et al., 2006; Yoo et al., 2006). For LCL generation, peripheral blood was subjected to Ficoll-Hypaque gradient centrifugation to obtain peripheral blood mononuclear cells (PBMCs), according to the manufacturer's instructions (Amersham Biosciences). An EBV viral stock was collected from a culture of an EBV-transformed B95-8 marmoset cell line, which was maintained at 37°C and 5% CO_2 for 4-7 d, and then stored at -80°C until needed.

The PBMCs were infected with the EBV viral stock (B95-8) in complete RPMI-1640 medium supplemented with 10% FBS and penicillin-streptomycin (100 μg/100 unit) as described previously (Hur et al., 2005). Following incubation for 2 h at 37°C, an equal volume of complete medium containing cyclosporin A (0.5 μg/mL) was added to kill the T lymphocytes. The culture was incubated for 10-20 d until clumps of EBV-infected B cells were visible. The culture was maintained in complete medium without cyclosporin A. When EBV-transformed lymphoblastoid cells grew to 1-2 $\times$ 10^6/ml, the cells were collected and washed with PBS prior to subsequent subculturing. Cells were then seeded at 0.1-0.2 $\times$ 10^6/ml after the remaining cells were frozen in freezing medium (RPMI-1640 in 10% FBS : FBS : DMSO = 50%: 40%: 10%) to preserve cell stocks at every passage.

LCLs were propagated up to 160 times during long-term subculture. The maximal passage number of each LCL strain was determined as the passage number at which the cell number did not increase 4 weeks after subculture. Such LCL strains were collected at the maximal passage number for freezing stocks. These LCL strains were referred to as non-immortal LCLs. The other LCL strains were grown to a passage number of 160, which took about more than 2 years. The biological activities of non-immortal LCL strains were assayed in cultures of the cell stocks that were obtained at one passage number prior to the final passage number (N-1 passage). LCLs at the passage number of 6 (p6) were used to examine early events during long-term subculture. All cell lines were free of *Mycoplasma*, as determined using the Mycoplasma Primer Set (Stratagene, USA).

Telomerase repeat amplification protocol for telomerase activity

Telomerase activity of three non-immortal LCL strains was determined using cell cultures at the N-1 passage. Among 17 immortal LCL strains, three were randomly chosen as reference controls. Telomerase activity of the control LCL strains was assayed using cell cultures with a passage number of 41, which corresponded to the average maximal passage number of three non-immortal LCL strains. Cell numbers were counted every 24 h after thawing to estimate the cell growth rate. Telomerase activity was determined from 2 $\times$ 10^5 cells using the TeloTAGGG telomerase PCR ELISA kit (Roche, Basel, Switzerland), which is a photometric enzyme immunoassay.

Real-time PCR

The same cell cultures used for the telomerase activity assays were also used for expression analysis. Cells from the cultured LCLs were harvested either 3 days (for K4 strain) or 4 days (all other strains) after thawing. Total RNA was isolated using an RNeasy kit (QIAGEN, Germany), treated with RNase-free DNase I (Roche, Basel, Switzerland), and then used to synthesize first-strand cDNA with random primers using the Superscript III first-strand synthesis system (Invitrogen, USA). RT-PCR amplification mixtures (20 ul) contained 100 ng of first-strand cDNA template, 2× SYBR Green Master Mix buffer (Invitrogen, USA), and forward and reverse primers for the genes of interest: EBNA1, forward 5′-GAGCGGGGAGATAATGTACA-3′ and reverse 5′-TAAAAGATGGCCGGACAAGG-3′; EBNA2, forward 5′-AACCCTCTAAGACTCAAGGC-3′ and reverse 5′-ACTTTCGTCTAAGTCTGCGG-3′; LMP1, forward 5′-ACTGATGAACACCACCACGA-3′ and reverse 5′-GTGCGCCTAGGTTTTGAGAG-3′; and RBP-Jkappa, forward 5′-TTCAAAAACCCCGTTGTCTC-3′ and reverse 5′-AAAAACCAACCAACCAACCA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control; forward 5′-CAGGGCTGCTTTTAACTCTGGTAA-3′ and reverse 5′-GTGGAATCATATTGGAACATGTAAACC-3′. The PCR cycles were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Reactions were run on an ABI HT 7900 (Applied Biosystems, USA). The average fold change was calculated from the difference of threshold cycles (Ct) between GAPDH and test genes from three experiments. Each reaction was performed in triplicate.

The relative copy number of the EBV genome and mitochondrial DNA was analyzed with real-time quantitative PCR, as previously described (Jeon et al., 2007). EBNA1 and ND-1 primers were used for targeting the EBV genome and mitochondrial DNA, respectively. EBNA1, forward 5′-GAGCGGGGAGATAATGTACA-3′, reverse 5′-TAAAAGATGGCCGGACAAGG-3′; ND-1, forward; 5′-ACTACAACCCTTCGCTGACG-3′, reverse; 5′-GCCTAGGTT-GAGGTTGACCA-3′. The Factor VIII gene was used as a reference gene for the normalization of input DNA. The primers of Factor VIII are forward 5′-TACCATCCAGGCTGAGGTTTAT-3′, and reverse 5′-AAAGAGTTGTAACGCCACCATT-3′. Reaction mixtures in triplicate were prepared in a master mix containing 8 uM each of forward and reverse primer, 2X SYBR Green PCR master mix, and 50 ng of genomic DNA.

Statistical analysis

Data were presented as mean ± standard deviation from at least three replications. Comparison of results between two groups was statistically assessed by Student's *t*-test.

RESULTS AND DISCUSSION

Continuous long-term subculture of LCLs

In order to determine the maximal end passage number of EBV-transformed B lymphocytes, 20 LCL strains were randomly selected for continuous subculture from an LCL collection at the Korean HapMap project (Table 1). Culture medium was exchanged with fresh complete medium at every passage during long-term subculture. Under our culture conditions, most of LCL strains (n = 17) proliferated up to a passage number of 160, at which time an LCL is generally considered as "immortalized" (Sugimoto et al., 2004; Toda and Sugimoto, 2003). These LCL strains are thus referred to as immortal LCLs (Fig. 1A). The other LCL strains (n = 3) stopped proliferating at passage numbers 33, 44, and 48, and thus referred to as non-immortal LCLs. The average life span of these non-immortal LCL strains was 41 ± 8 passages. Most of the LCL strains (85%) were immortalized, whereas only a small proportion (15%) proliferated with a limited life span after EBV transformation. This finding suggests that a proliferative crisis event might occur around passage number 41. This observation contrasts with a previous report stating that the majority of EBV-transformed LCLs finished their life span before passage number 160 (Sugimoto et al., 1999). This discrepancy is possibly due to the different culture conditions used in these studies. According to the culture condition described by the Sugimoto group, continuous cellular

Table 1. Demographic data of 20 LCL strains

LCL No.	Gender	age	Disease history[a]	Immortalization[b]
A-1	F	65		Immortal
A-2	M	61		Immortal
A-3	M	45	Stomach ulcer (20)	Immortal
A-4	M	57		Immortal
A-5	M	57		Immortal
A-6	M	47		Immortal
A-7	F	45	Hepatitis (35)	Non-immortal (p48)
A-8	F	53		Immortal
A-9	F	58		Non-immortal (p33)
A-10	F	68		Immortal
K-1	F	61		Immortal
K-2	F	46		Immortal
K-3	M	64		Immortal
K-4	M	41		Non-immortal (p44)
K-5	M	65		Immortal
K-6	M	58		Immortal
K-7	F	55	Cerebrovascular disease (48)	Immortal
K-8	M	47		Immortal
K-9	M	69		Immortal
K-10	F	43		Immortal

[a]Numbers in parenthesis indicate disease-diagnosed ages
[b]Numbers in parenthesis indicate maximal passage numbers of each LCL strain.

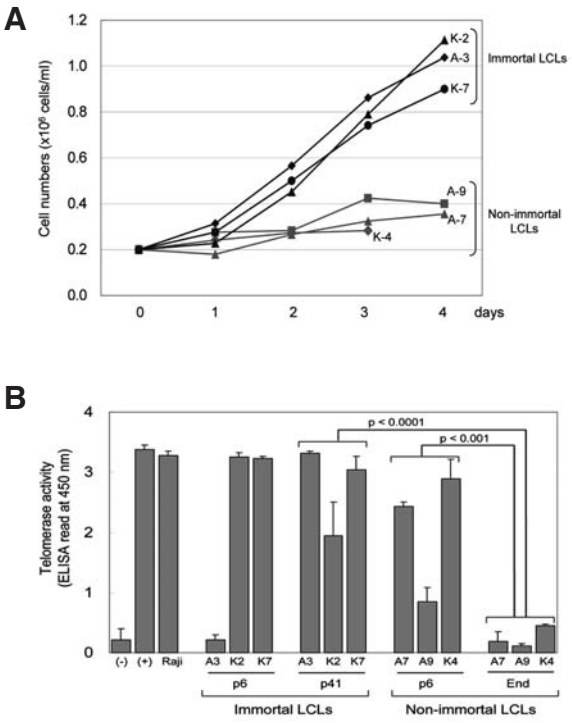

Fig. 1. Characteristics of non-immortal LCL strains. (A) Growth properties of immortal and non-immortal LCL strains at the passage number of 41 and the maximal end passage numbers, respectively. Among 20 LCL strains, three non-immortal LCL strains (A7, A9, and K4) stop proliferation at their maximal end passage number of 48, 33, and 44, respectively, whereas the remaining 17 immortal LCL strains proliferated exceeding the passage number of 160. (B) Telomerase activities of immortal and non-immortal LCL strains. TRAP assay was performed using cell extracts of LCL strains at the early passage (p6) or middle passages (p41 for immortal strains, and maximal end passages for non-immortal strains). (+) and (-) indicate a positive control (293T) and a negative control (heat-inactivated LCL extract), respectively. Raji, EBV-positive lymphoma cell line; End, maximal end passage number of each non-immortal LCL strain.

passages were made every 3 or 4 days by exchanging a half volume of cultivating cell suspension with fresh medium. In contrast, we washed cells with PBS prior to subsequent subculturing, and then fresh complete medium was added to adjust the seeding density to 0.1-0.2 $\times$ 10^6/ml. Thus, it is likely that our culture conditions were more favorable for long-term LCL subculturing and eventually complete immortalization. The PBS washing and subsequent medium exchanging steps may reduce an exposure to environmental conditions containing cytotoxic signals secreted from dying immortal cells, as in the case of radiation induced bystander effects. The bystander effect has been described in studies of radiation-induced cells in which radiated cells send cell-to cell communication related biological detrimental signals (e.g., free radicals) to neighboring cells (Hei et al., 2004). Therefore, this result suggests that culture conditions may be an important factor to increase success rate of LCL immortalization during the LCL immortalization process.

Growth properties and telomerase activity of LCLs

Cellular immortalization is a multi-step process involving oncogene activation, telomerase activation, aneuploidy, and ultimately immortalization. Telomerase activation is required for cells to overcome a proliferative crisis (Belair et al., 1997). More recently, different EBV proteins have been shown to activate or inhibit the human telomerase reverse transcriptase gene by modulating intracellular signaling pathways (Liu et al., 2006). Among 20 LCL strains tested in the present study, three LCL strains failed to complete cellular immortalization, and therefore were determined to be non-immortal LCLs. The results from our long-term subculture experiments implied that LCLs might undergo a proliferative crisis at a relatively early passage number (average passage number of 41). Thus, we next examined the growth properties and telomerase activity of non-immortal LCL strains (n = 3) at their maximal passage number. Immortal LCL strains (n = 3) at a passage number of 41 were included as controls. As expected, non-immortal LCL strains had lost nearly all proliferative activity at the maximal passage number, whereas immortal LCL strains exhibited normal growth properties (Fig. 1A). Furthermore, little telomerase activity was detected in non-immortal LCLs at the maximal passage number, whereas immortal LCLs exhibited strong telomerase activity at a passage number of 41 which was comparable to that of Raji (Fig. 1B). Interestingly, non-immortal LCL strains also exhibited telomerase activity even in the early passage number of 6 (p6), as also observed in two (K2 and K7) of three immortal LCL strains. This finding indicated that telomerase activation occurred at very early passage numbers irrespective of immortal and non-immortal LCL strains. Thus, this result suggests that telomerase activity may require to be sustained during long-term subculture in order for LCLs to overcome cellular crisis.

EBV infection induces cellular immortalization through telomerase activation; however, high telomerase activity was not detected in all EBV-transformed cells (Liu et al., 2006). For

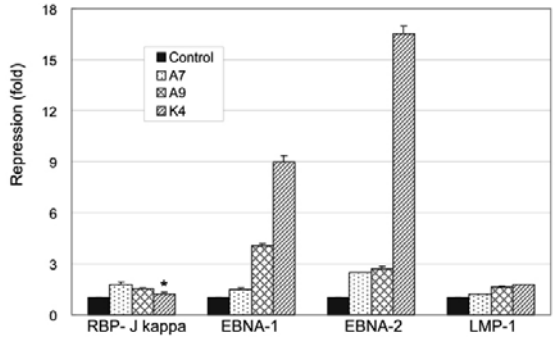

Fig. 2. Decreased expression of EBV-related genes in non-immortal LCL strains. Real-time PCR was performed to obtain expression levels of target genes in relative to GAPDH which was based on the difference of threshold cycles (Ct) between target genes and GAPDH. Control was comprised of three immortal LCL strains (A3, K2, K7) for which the equal numbers of cells were pooled from each of three LCL strains. A7, A9 and K4 indicate individual non-immortal LCL strains. Error bars indicate the standard deviations of three real-time PCR reactions. The expression levels of EBV-related genes were significantly decreased in non-immortal LCL strains when compared with control LCL strains ($p < 0.05$) except RBP-J kappa in K4 indicated as a star (*).

example, about 85% of primary NPC cells displayed high telomerase activity by mechanisms involving EBV infection (Liu et al., 2006), which was consistent with our observed proportion of immortalized LCLs. Thus, only a small fraction of LCLs seemed to be resistant to sustain telomerase activity during EBV-mediated cellular immortalization.

EBV viral activity in LCLs

We next investigated the differential gene expression of EBNAs between immortal and non-immortal LCL strains in order to identify which factors, in addition to telomerase activation, affect the mortality of LCLs. The same cell cultures used in the growth and telomerase assays were also used in these gene expression studies. Quantitative RT-PCR showed that EBNA1, EBNA2, and LMP1 were downregulated in all non-immortal LCL strains when compared with a reference of pooled three immortal LCL strains (Fig. 2). Among the EBNAs, EBNA1 regulates DNA replication of the episomal EBV genome. EBNA2, which is the first protein expressed in EBV-infected B cells, plays a crucial role in EBV-mediated B cell transformation and immortalization. EBNA2 is associated with the sequence-specific DNA binding protein RBP-Jk/CBF-1/CSL during transcription of its target genes, including CD21, CD23, c-fgr, c-myc, EBNAs, and LMP1 (Cooper et al., 2003; Henkel et al., 1994; Johannsen et al., 1995; Zhao et al., 2006). RBP-Jk is a shared binding partner of multiple EBNA proteins, including EBNA2, 3A, 3B, and 3C (Robertson et al., 1996). LMP1 has an important role in the Nk-κB and TNF signaling pathways. We also observed downregulation of RBP-Jk gene expression in non-immortal LCL strains when compared with a reference of pooled immortal LCL strains. Thus, these results suggest that the decreased expression of EBNA genes may be unfavorable for LCL immortalization.

To gain further insight into EBV's influence on LCL immortalization, the copy number of the EBV genome was examined in three non-immortal LCL strains at their maximal passage number, and in three randomly selected immortal LCL strains at passage number 41 as a reference. Quantitative real-time PCR

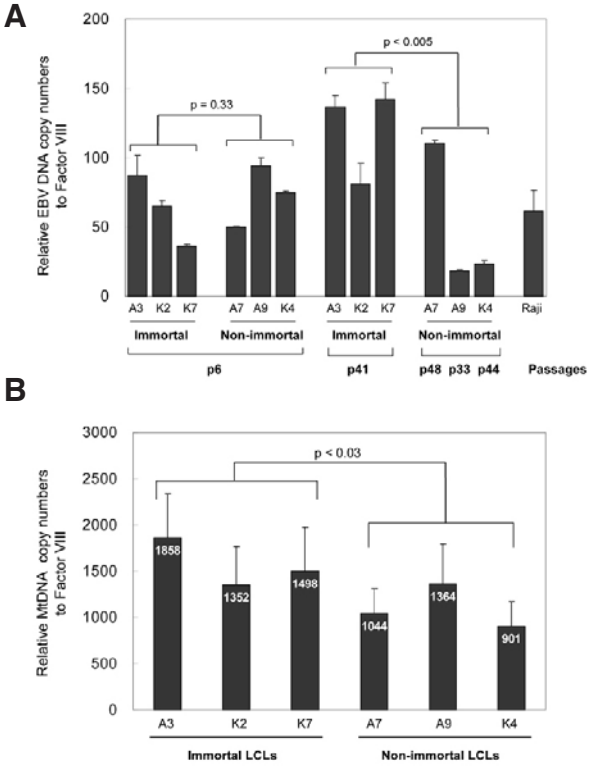

Fig. 3. Copy numbers of the EBV genome and mitochondrial DNA in immortal and non-immortal LCL strains. EBNA-1 and ND-1 genes were used to calculate copy numbers of EBV viral and mitochondrial DNA (mtDNA), respectively. The copy numbers of EBV DNA (A) and mtDNA (B) were represented in relative to that of Factor VIII gene. Error bars indicate the standard deviation of results from three experiments.

detected a substantial decrease in the copy number of the EBV genome in non-immortal LCL strains at the maximal end passage numbers, when compared with the immortal LCL strains which contained comparable high copy numbers of the EBV genome to Raji (Fig. 3A). However, the copy numbers of the EBV genome at the early passage number of 6 were not significantly different between immortal and non-immortal LCL strains. Among non-immortal LCL strains, A7 strain shared characteristics of EBV copy numbers with immortal LCLs which represented more copy numbers of the EBV genome at the passage 41 than the passage 6. Therefore, the A7 strain did not succeed in overcoming cellular crisis despite elevated EBV viral load. Thus, the A7 strain may require different pathways from sustained EBV viral load to complete cellular immortalization. Possibly, fatal loss of genomic components for cell growth might occur in the A7 strain due to chromosomal alterations during long-term subculture.

In addition, we observed that non-immortal LCLs maintained relatively lower copy numbers of mitochondrial DNA (mtDNA) than immortal LCLs (Fig. 3B). Our previous report showed that the mitochondrial DNA number was related to mitochondrial biogenesis and cell proliferation activity in EBV-infected B cells (Jeon et al., 2007). Thus, reduced mtDNA copy numbers may be indicative of diminished EBV-mediated proliferative activity. Together with the gene expression patterns, this copy number difference of the EBV genome and mtDNA between immortal and non-immortal LCLs suggests that the biological influence of

EBV may be required for complete LCL immortalization. However, it is likely that sustained viral activity of EBV was not sufficient for LCL immortalization, given that LCL A7, which lacked telomerase activity, was not immortalized.

EBV-positive tumors and EBV-transformed B lymphoblastoid cells harbor multiple copies of circularized episomal and/or integrated EBV genomes. The malignant phenotypes of Burkitt's lymphoma, such as growth in low serum, anchorage-independent growth in soft agar, and tumorigenicity in nude mice, are dependent on the presence of EBV genomes (Shimizu et al., 1994). A recent report showed that EBV-positive Burkitt's lymphoma cell lines displayed increased genomic instability and telomere length (Kamranvar et al., 2007; Mochida et al., 2005). Therefore, besides sustained telomerase activity, sustained viral activity of EBV may be one of the important factors to complete cellular immortalization of EBV-infected B-cells. To precisely assess immortalization effects of EBV viral load, it would be interesting to examine whether a second round of EBV infection would convert non-immortal LCLs into immortal LCLs.

In conclusion, our long-term subculture analysis of LCLs revealed that a high proportion of LCL strains survive to become immortalized. These LCLs may overcome a proliferative crisis during early rounds of subculturing, prior to passage number 41. Non-immortal LCLs exhibited no telomerase activity, decreased EBV gene expression, and lower copy numbers of the EBV genome and mtDNA compared to immortal LCLs. Thus, in addition to telomerase activity, sustained EBV viral activities may contribute to the completion of LCL immortalization.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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