Differential gene regulation by Epstein-Barr virus Type 1 and Type 2 EBNA2

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Abstract

A transfection assay in a lymphoblastoid cell line infected with Epstein-Barr virus was used to compare the ability of type 1 and type 2 EBNA2 to sustain cell proliferation. The reduced proliferation in cells expressing type 2 EBNA2 correlated with loss of expression of some cell genes that are known to be targets of type 1 EBNA2. Microarray analysis of EBNA2 target genes identified a small number of genes that are more strongly induced by type 1 than type 2 EBNA2 and one of these genes (CXCR7) was shown to be required for proliferation of lymphoblastoid cell lines. The Epstein-Barr virus LMP1 gene was also more strongly induced by type 1 EBNA2 than type 2 but this effect was transient. Type 1 and type 2 EBNA2 were equally effective at arresting cell proliferation of Burkitt's lymphoma cell lines lacking Epstein-Barr virus and were also shown to cause apoptosis in these cells. The results indicate that differential gene regulation by Epstein-Barr virus type 1 and type 2 EBNA2 may be the basis for the much weaker B cell transformation activity of type 2 Epstein-Barr virus strains compared to type 1 strains.

Introduction

Epstein-Barr virus (EBV) infects human B cells, giving rise in cell culture to lymphoblastoid cell lines (LCLs) in which the latent viral genome is maintained. In this infection, known as Latency III (33), all the EBNA and LMP viral proteins are expressed together with various non-coding small RNAs (EBER and miRNAs). The EBV genes expressed in latency III cause the proliferation and survival of the LCL cells (49). In latency III, EBNA2 acts as a transcription factor to induce expression of the viral LMP genes and many cell genes (25). EBNA2 is known to be required for the continued proliferation of LCLs because an LCL (EREB2.5) engineered to

express an estrogen receptor EBNA2 fusion protein in the absence of wild type EBNA2 was dependent on estrogen for EBNA2 function and cell proliferation (24). In the tumour cells of the EBV associated cancer Burkitt's lymphoma (BL), where EBV infection is described as latency I (33), EBNA2 is not expressed, proliferation being caused primarily by the de-regulation of c-MYC caused by a chromosome translocation characteristic of BL (7). Some BL cells in culture adapt to acquire a latency III pattern similar to that found in LCLs but the Akata BL cell line retains latency I EBV gene expression in culture.

Natural sequence variation of EBV isolates has been described at many locations in the genome (3, 11, 12, 50) but the most prominent and biologically significant of these identified so far is the variation in EBNA2. EBV strains are classified as type 1 or 2 (also known as type A and B) according to the sequence of their EBNA2 gene (25). The protein sequences of type 1 and type 2 EBNA2 are only 55% identical, a remarkable difference in view of the very low degree of variation in most other genes in the EBV genome. Type 1 strains are found worldwide and efficiently immortalise human B cells to give LCLs but type 2 strains are much less effective at producing LCLs (32). Type 2 EBV strains are almost equally abundant as type 1 strains in Africa and some other regions of the world but are much less frequent than type 1 in Europe and the USA. A recombinant EBV type 2 strain in which the EBNA2 coding sequences were replaced with type 1 EBNA2 acquired the efficient immortalisation of B cells characteristic of type 1 EBV (9), showing that EBNA2 variation is the key difference, although additional variation in EBNA3 family genes and EBNA-LP linked to type 1 and type 2 has been described (29, 34).

The mechanism by which type 1 EBNA2 acts as a transcription factor has been studied in detail. EBNA2 contains a transcription activation domain but does not bind directly to DNA, instead binding to certain cell proteins that mediate the DNA binding in promoter regions (25). The most studied of these proteins is RBP-Jk (CBF1) but the cell transcription factors PU.1 and AP2 can also mediate its effects on some promoters. The LMP2A gene is a clear example of a promoter that is regulated by EBNA2 via RBP-Jk (52). In contrast, PU.1 and AP2 are also important for regulation of the promoter for LMP1 by EBNA2 (18, 19, 22, 27, 35, 36). In addition, EBNA2 can modify chromatin structure through recruitment of SWI/SNF (46-48). Although there has been extensive investigation of the mechanism of EBNA2 induction on transfected model promoters and reporter plasmids, the relative significance of the SWI/SNF complex activity and transcription activator/repressor recruitment on promoters at their natural locations in human chromosomes remains to be determined.

The sequences of type 1 and type 2 EBNA2 are quite similar in the parts of the proteins known to mediate binding to RBP-Jk and in the activation domain; the main sequence variation occurs elsewhere in the protein. Almost all published studies of EBNA2 mechanism have used type 1 EBNA2 but the ability of type1 and type 2 EBNA2 to induce gene expression has been compared on the promoters for LMP1 (43, 44) and CD23 (43).

In addition to the EBV LMP promoters and the Cp EBNA promoter, EBNA2 regulates many genes in the human genome. These EBNA2 target genes were initially studied individually but several recent microarray studies have identified large

numbers of genes regulated by EBNA2 type 1. Various approaches to identification of EBNA2 target genes have been described, using either LCLs with conditionally active EBNA2 (37, 51) or the effect of conditionally active EBNA2 in EBV negative BL cell lines (28). In our study (37) we sought to identify direct targets of EBNA2 regulation in the EREB2.5 LCL by inducing EBNA2 function in the presence of protein synthesis inhibitors. RNAs that were induced were considered to be directly regulated by EBNA2 in the sense that their induction did not depend on protein synthesis of intermediate genes. We have investigated the roles of some of these EBNA2 targets in detail, for example RUNX3 and PI3 kinase p55α (37, 38).

In contrast to LCLs where EBNA2 induces c-MYC, in latency I or EBV negative BL cells EBNA2 down regulates expression of the translocated c-MYC allele and causes cell cycle arrest (21). Although type 2 EBNA2 is relatively frequent in Africa and many BL cell lines contain type 2 EBV, only type 1 EBNA2 has so far been analysed in the context of c-MYC regulation in BL cells. In this paper we investigate functional differences between type 1 and type 2 EBNA2, studying the effects of physiologically normal levels of EBNA2 or ER-EBNA2 protein on regulation of cell genes and the EBV LMP1 gene. A previous report (9) showed approximately equal expression of LMP1 in comparable LCL cells expressing type 1 or type 2 EBNA2. We confirm this but reveal a different time response of LMP1 expression and identify several cell genes that are differentially regulated by type 1 and type 2 EBNA2, including a gene required for LCL proliferation. The results suggest mechanisms that might explain the different abilities of type 1 and type 2 EBV to convert B cells into LCLs.

Materials and Methods

EBNA2 plasmids

For clarity in this paper, the name pER-EBNA2 T1 will be used for p554-4 (24) and pEBNA2 T1 will be used for p554 (24); both plasmids were kindly provided by B. Kempkes. Analogous plasmids were constructed containing type 2 EBNA2. For construction of pER-EBNA2 T2 (also known as pERT2), EBNA2 type 2 was amplified by Pfu PCR from genomic DNA extracted from AG876 cells. The amplified product was cloned and sequenced to verify its identity to the published AG876 EBNA2 sequence (10, 12). Adaptors included in the PCR primers allowed substitution of the ER-EBNA2 sequence in pER-EBNA2 T1 with ER-EBNA2 T2. A similar strategy was used to clone pEBNA2 T2 (also known as pAG1) using the same AG876 EBNA2 DNA to substitute the EBNA2 sequence of pEBNA2 T1.

To make plasmids for in vitro translation of type 1 or type 2 EBNA2**,** EBNA2 sequences were Pfu PCR amplified from pER-EBNA2 T1 or T2 using primers with overhanging sequences carrying XbaI (5') and EcoRI (3') restriction sites. The amplified products were cloned into pSP64 (Promega). In vitro transcription and translation was performed using the TnT reticulocyte lysate system (Promega) with SP6 polymerase, using 1µg of template vector and 2µl of 10mCi/ml ^{35}S-methionine, in a 50 µl final reaction. Dilutions of the type 1 and type 2 in vitro translations were run on SDS-PAGE, blotted and the filter exposed on a phosphorimager. The same filter was then used for immunoblotting with PE2 antibody and the EBNA2 detected by ECL. Type 1 EBNA2 contains 10 Met residues and type 2 has 9: the images were compared using ImageQuant 5.0 software.

Cell culture

BL cell lines and the LCL EREB2.5 (24) were grown at 37^{o} in RPMI 1640 with 10% fetal calf serum and 50 units/ml penicillin/streptomycin. For EREB2.5 cells, RPMI 1640 medium lacking phenol red but containing 1μM β-estradiol was used. 293 cells were grown in DMEM with 10% fetal calf serum and penicillin/streptomycin.

For estrogen starvation and induction of EREB2.5 cells (Fig 4A), the cells were washed twice in unsupplemented RPMI and resuspended at 5x10^{5} cells/ml in medium without estrogen. After 4 days, cells were counted again and reincubated in fresh medium without estrogen. On the fifth day, protein synthesis inhibitors (50μg/ml cycloheximide and 25μg/ml anisomycin) were added if required (Fig 4A, middle panels) prior to addition of 1μM estrogen or ethanol as a solvent control. The induction with estrogen was continued for 4 hours in the 37°C incubator. Cells were harvested by washing twice in unsupplemented medium before protein extraction.

Transfection, ^{3}H-thymidine labelling and RNAi

For Amaxa transfection of EREB2.5 cells, 5x10^{6} cells growing in medium with estrogen were collected by centrifugation and resuspended in 100 μl of supplemented solution T (Amaxa Biosystems) at room temperature. 5 μg of plasmid DNA was added and the mixture was transferred to an Amaxa cuvette and nucleofected using Amaxa program A-23. 500μl of warmed medium was then added to the cuvette and cells were transferred to 6 ml of warmed medium. Estrogen was omitted from the culture medium after transfection for the experiments shown in Fig 1 and 4C.

For ^{3}H-thymidine labelling, cells suspended in 200 µl medium were aliquoted into wells of 96 well plates and incubated for 2 hours at 37ºC in the presence of 1µCi of ^{3}H-thymidine. Cells were harvested on to filters using a cell harvester (Skatron) and radioactivity was measured in a scintillation counter.

EBV negative Akata 31 BL (AK31) cells (20) expressing a conditional EBNA2 type 1 or type 2 were obtained by first electroporating $5x10^{6}$ cells with 5 µg of puro-oriP-EBNA1 vector (2) using a BioRad electroporator set at 960µF and 250V. 1 µg/ml puromycin was added 24 hours after transfection. The selected AK31 cell line expressing EBNA1 was grown out, characterized and then transfected with pER-EBNA2 T1 or pER-EBNA2 T2 vector using the same electroporation conditions. 1 mg/ml G418 was added 24 hours post transfection and selection was carried on in G418 and puromycin for about 4 weeks, by which time cells expressing EBNA1 and ER-EBNA2 had grown out. Daudi BL cells were transfected with pER-EBNA2 T1 or pER-EBNA2 T2 under the same electroporation conditions, and selected with 400 µg/ml G418.

293 cells were transfected using lipofectamine complexes formed by mixing 50 µl of serum free medium containing 8 µg of plasmid DNA with 50 µl of serum free medium containing 10 µl of lipofectamine and incubating for 20 minutes at room temperature. The complexes were added to $6x10^{6}$ cells at 90% confluence and incubated for 24 hours at 37ºC before replacing the medium and adding 1µg/ml puromycin. Cells were passaged as required to avoid confluence and counted after 8 days.

For shRNA RNAi of CXCR7, oligonucleotides GATCCCCAGCAAAGTAGCTTCGGGTCTTCAAGAGAGACCCGAAGCTACTT TGCTTTTTTGGAAA and AGCTTTTCCAAAAAAGCAAAGTAGCTTCGGGTCTCTCTTGAAGACCCGAA GCTACTTTGCTGGG were cloned into puro-oriP-SUPER, a puromycin resistant version of the pHEBo-SUPER plasmid described previously (38) and plasmid DNA was Amaxa electroporated into EREB2.5 cells. Transfected cells were grown in 1μg/ml puromycin and viable cell numbers were determined at the indicated time points later. Samples were taken on day 7 for RT-PCR analysis.

RNA extraction and RT-PCR

Total cell RNA was extracted using Trizol and cDNA was prepared using the Protoscript first strand cDNA synthesis kit (New England Biolabs). Before reverse transcription, RNA samples were treated with RQ1 RNasefree DNase (Promega). CXCR7 PCR used the following primers:

Splice 1 Fw: CAGCTTCAGATCTGGGTATTTATCC

Splice 2 Fw; GCAGCCAGCAGAGCTCACAGTTG

Splice 1/2 rev: TGGGCATGTTGGGACACATCACC

GAPDH was amplified with primers TGCCTCCTGCACCACCAACT and CGCCTGCTTCACCACCTTC.

ADAMDEC1 was amplified with primers CTCTCCCTACAAAAAACCAAGCAC and TGTGTGAAGTATCCTCTCAACCCG.

Microarray analysis

Microarray analysis was performed on Agilent G4112F 44K HD arrays (design ID 014850) exactly as recommended by the manufacturer (Two-Color Microarray-Based

Gene Expression Analysis V5.0.1, Agilent Technologies). Data extraction was performed with feature extraction software V9.1.3.1 by using the protocol file "GE2-v5_91_0806.xml". AK31:EBNA1/ER-EBNA2 type 1 or type 2 cells were pre-treated for 1hr with protein synthesis inhibitors, then with or without estrogen for 4 hrs in the presence of protein synthesis inhibitors. Total cell RNA was extracted and used for expression profiling. The experiment was performed in duplicate and the resulting RNAs were hybridised pairwise (estrogen-treated versus control) on four individual microarrays. The geometric mean of the four ratios/probe across all arrays was calculated. The output files from the Agilent Feature Extraction software for each microarray include the signal (MedianSignal), standard deviation of the signal (PixSDev), background (BGMedianSignal) and corrected signal (ProcessedSignal; dye-normalized signal after surrogate algorithm, used for computation of log ratio) for each channel (Cy3 and Cy5). From these, a signal to noise (S/N) ratio for each channel (= signal/background) and relative error (= SD signal/signal) were calculated. Analyses were done with the log ratio calculated from the corrected signals (Cy3 and Cy5). To describe the quality of the ratio value the smaller of the two S/N values was used while for the relative error the higher one was selected. The microarray data analysis was performed using the software Genedata Expressionist® Pro (Genedata AG, Basel, Switzerland).

Immunoblotting and ELISA

SDS gel electrophoresis and Western immunoblotting was performed as described previously (38). Antibodies and dilutions used were EBNA2, DAKO PE2 mouse monoclonal (1/500); LMP1, DAKO CS1-4 mouse monoclonal (1/500); IRF4, AB-cam ab27508 rabbit polyclonal (1/1000); IL-1β: ABcam ab10749 mouse monoclonal (1/200); Actin: Abcam AC-15 (1/20000); Cell Signalling Technologies anti-cleaved

human PARP Asp214: (1/1000); cMYC: Abcam 9E-10 mouse monoclonal (1/500). Secondary antibodies were HRP conjugated anti-mouse or anti-rabbit at 1/2000 and detection was by ECL (GE Healthcare).

ELISA for IL-1β used the Quantikine DLB50 kit (R&D systems). AK31:EBNA1/ER-EBNA2 T1 or T2 cells were treated with estrogen for 24 hours or kept untreated as controls and then protein was extracted in 200 µl CAT-ELISA buffer. The extract was added to the ELISA plate provided in the kit and incubated for 2 hours. The plate was washed 3 times then incubated for 1hr with secondary antibody, washed three times and developed, following the instructions with the kit.

Analysis of DNA content by Propidium Iodide staining

Cell were harvested and fixed in 70% ethanol in PBS overnight at 4 °C. Fixed cells were washed three times in 1ml cold PBS, then resuspended in 500 µl 50µg/ml propidium iodide (PI) in PBS containing RNaseA (0.5mg/ml) and incubated for 30 minutes at 37 °C prior to analysis. PI-stained cells were then analysed for DNA content by flow cytometry.

Apoptosis assays

For the JC-1 assay, cells were cultured at a density of 1 x 10^6 cells/ml in medium containing 2 µM JC-1 (Molecular Probes, Invitrogen) for 30 minutes at 37 °C, 5% CO_2. Positive control samples were also incubated with 50 µM membrane potential disrupter CCCP (Molecular Probes, Invitrogen) to uncouple mitochondria. After the incubation period, cells were washed in 2 ml warm PBS and resuspended in 500 µl PBS for analysis by flow cytometry.

To measure DNA laddering, cells were harvested after treatment and DNA was extracted as described (15). The extent of DNA fragmentation was visualised on a 1% agarose gel containing Ethidium Bromide.

Results

Assay for EBNA2 function distinguishes type 1 from type 2

Transfection of an oriP plasmid expressing wild-type type 1 EBNA2 into EREB2.5 cells was previously demonstrated to allow continued growth of the cells in the absence of estrogen. EBNA2 is known to be essential for the growth of LCLs and the wild type EBNA2 takes over from the ER-EBNA2, the episomal ER-EBNA2 plasmid eventually being lost from the cells (16, 24, 37). This procedure was adapted into an assay that functionally distinguishes type 1 from type 2 EBNA2. The type 1 EBNA2 sequences in the EBNA2 T1 expression plasmid (24) were replaced with type 2 EBNA2 coding sequence. The type 1 or type 2 EBNA2 plasmids were then transfected into EREB2.5 cells that had been growing normally with estrogen and estrogen was removed from the culture. Monitoring DNA synthesis by ^{3}H thymidine incorporation gave the expected continued DNA synthesis with type 1 EBNA2 but with type 2 EBNA2 there was only a brief period of DNA synthesis before incorporation of ^{3}H thymidine declined and cell proliferation ceased (Fig 1A). A negative control transfection in which a plasmid expressing no EBNA2 was transfected gave no significant ^{3}H thymidine incorporation. Such transfections eventually resulted in outgrowth in the absence of estrogen of typical LCLs with type 1 EBNA2 (Fig 1B). These cells expressed EBNA2 and after 3 months in culture had almost completely lost expression of the ER-EBNA2 fusion protein, as detected by

western blotting (Fig 1B), presumably because of passive loss of the ER-EBNA2 plasmid from the cells. The plasmids encoding type 1 and type 2 differed only in the EBNA2 coding region and expressed similar levels of EBNA2 protein at early times after transfection (3 days), as detected by Western blotting with the PE2 monoclonal antibody (Fig 1C). To check that the PE2 monoclonal antibody detects type 1 and type 2 EBNA2 with equal efficiency on a western blot, EBNA2 was prepared by in vitro translation incorporating ^{35}S methionine. Dilutions of the samples were analysed by western blotting with PE2 and a phosphorimager was used to measure ^{35}S Met incorporation on the same gel. The results (Fig 1D) showed that PE2 detected type 1 and type 2 EBNA2 with approximately equal efficiency in the western blotting assay.

In Figure 1A, the key point of difference in the time course of ^{3}H thymidine incorporation was about 3 to 4 days after transfection, when incorporation in the type 1 transfection was still increasing strongly as the cells proliferated but the culture with type 2 EBNA2 was stopping DNA synthesis. EBNA2 regulates expression of the viral LMP1 protein and LMP1 signal transduction is an important factor in proliferation and survival of EBV LCLs but it was noticeable that the level of LMP1 3 or 4 days after transfection and estrogen withdrawal was similar in the western blotting samples from the type 1 and type 2 transfections. In contrast, there were major differences in the expression level of some cell genes identified previously as EBNA2 targets, for example RUNX3 and IRF4 (Fig 1C). We showed previously that RUNX3 is required for proliferation of EREB2.5 LCLs (38) so this experiment indicates that differential expression of a cell gene that is directly controlled by EBNA2 correlates with the different proliferative capacity of type 1 and type 2 EBNA2.

Identification of cell genes differentially regulated by type 1 and type 2 EBNA2

Our previous strategy for identification of direct target genes of type 1 EBNA2 in the EREB2.5 LCL background (37) can not be applied to type 2 EBNA2 because the ER-EBNA2 (type 2) does not sustain cell proliferation. There were many similarities in the lists of EBNA2 target genes identified by our approach and that of EBNA2 expression in an EBV negative Burkitt's lymphoma cell line (28) so we therefore created EBV negative Akata (AK31) BL cell lines with constitutive expression of type 1 or type 2 ER-EBNA2. The ER-EBNA2 was expressed from episomal oriP vectors selected with G418 and the EBNA1 required for this plasmid maintenance was provided from another oriP plasmid in the cells under puromycin selection. There was characteristically some stabilisation of the EBNA2 protein when the cells were treated with estrogen (Fig 2), as is seen also in EREB2.5 cells. The AK31:EBNA1/ER-EBNA2 cell lines were treated with estrogen in the presence of protein synthesis inhibitors for 4 hours and total cell RNA was analysed by microarray expression profiling. Levels of cell RNAs were compared in response to type 1 or type 2 EBNA2 activation. Most cell genes are not regulated by EBNA2 activation but based on the filter criteria applied, approximately 150 genes were induced two fold or more, consistent with previous studies (28, 37, 51). Examples of these genes that were also classified as direct targets of EBNA2 regulation in EREB2.5 cells in our previous analysis (37) are shown in Fig 2. For most of these, there was no detectable difference in regulation by type 1 or type 2 EBNA2 but for a few genes there was significantly more induction by type 1 EBNA2. The genes regulated differentially in Fig 2 included MARCKS, IL1β, ADAMDEC1 and CXCR7 (also known as RDC1, CMKOR1). The induction of mRNA expression for these genes was about 3 fold higher with type 1 EBNA2.

Induction of IL1β was confirmed at the protein level in AK31:EBNA1/ER-EBNA2 (type 1) cells in response to estrogen activation of the EBNA2, both by ELISA assay (Fig 3A) and Western blotting (Fig 3B), but this protein was intracellular and could not be detected in the culture medium. RT-PCR for ADAMDEC1 confirmed its induction (Fig 3C) and MARCKS has been reported previously to be induced by EBV infection of BL cells (6). Interestingly, CXCR7 was the RNA most strongly induced by EBNA2 type 1 in our previous study on direct EBNA2 targets in EREB2.5 LCLs (37) and was also found to be strongly induced in the EBV negative BL cell background in other studies (28).

Induction of CXCR7 was confirmed by RT-PCR both in the AK31:EBNA1/ER-EBNA2 T1 cells and in EREB2.5 cells in response to estrogen activation of the EBNA2 (Fig 4A). Two known splice variants in the leader exons of CXCR7 and a novel splice of exon 2 directly to the coding exon were observed in the RT-PCR assays (Fig 4A). The differential regulation of CXCR7 and ADAMDEC1 by type 1 and type 2 EBNA2 observed in the BL cell lines (Fig 2) was also seen in EREB2.5 cells transfected with EBNA2 expression vectors followed by withdrawal of estrogen (Fig 4B), similar to the experiment shown in Fig 1. The time course of the experiment in Fig 4B was slightly slower than that shown in Fig 1 and the differential regulation of CXCR7 and ADAMDEC1 by type 1 and type 2 EBNA2 was most clearly seen 7 and 10 days after transfection, relative to a GAPDH control (Fig 4B). EREB2.5 cells were not unusual in expressing CXCR7; a similar pattern of expression was observed in a panel of LCLs (Fig 4C). These LCLs were derived from cord blood (C2-BL16, C2-Akata, IB4, EREB2.5) or peripheral blood (LCL3, Obaji LCL) B cells and

contained several different EBV strains (LCL3 contains B95-8 EBV). C2-BL16 contains a type 2 EBV but has a similar level of CXCR7 RNA to the other lines, suggesting that once an LCL has been established, cells with this level of CXCR7 may be selected, if CXCR7 is required for LCL proliferation or survival.

Using a similar shRNA plasmid transfection approach to that we applied previously to test the contribution of RUNX3 and p55α PI3kinase to LCLs (37, 38), we investigated the effect of depleting CXCR7. Knockdown of CXCR7, detected at the RNA level (Fig 5A, B), resulted in a severe impairment of accumulation of transfected EREB2.5 cells (Fig 5A) or LCL3 cells (Fig 5B) and cell death. In those experiments p53 RNAi and the empty vector were used as negative controls (37, 38) and affected neither the levels of CXCR7 RNA nor accumulation of the cells in culture. The shRNA plasmids were also transfected into 293 cells and puromycin selection was applied (Fig 5C). There was no difference between the CXCR7 and empty vector shRNA plasmids in the production of puromycin resistant 293 cells, indicating that the CXCR7 shRNA construct was not non-specifically toxic and did not prevent function of the puromycin resistance gene used in Fig 5A, B.

These results show that some cell genes (including CXCR7) can be differentially regulated by type 1 and type 2 EBNA2 and a CXCR7 gene product is required for LCL proliferation or survival.

Type 1 and type 2 EBNA2 both cause cell cycle arrest and apoptosis in EBV negative BL cell lines expressing EBNA1

In the experiment shown in Fig 2C, the effects of EBNA2 on cell gene expression were measured only 4 hours after activation of the ER-EBNA2 by estrogen. Earlier work has shown that there appears to be an incompatibility between EBNA2 expression and latency I BL cells that results in selection of tumour cells lacking EBNA2 expression (23). One potential mechanism for this is the ability of type 1 EBNA2 to down regulate the expression of the translocated c-Myc allele in BL cells and consequently cause cell cycle arrest (21). Many African BL tumours contain type 2 EBV so we therefore compared the effects of type 1 and type 2 ER-EBNA2 on AK31 BL cells over a longer time period. Both type 1 and type 2 ER-EBNA2 suppressed c-MYC protein levels (Fig 6A) and caused the previously described (21) accumulation of cells in the G1 phase of the cell cycle after about 24 hours (Fig 6B). In addition to the previously reported cell cycle arrest (21), these BL cells subsequently died by apoptosis in response to EBNA2 expression. The accumulation of cleaved PARP (Fig 6B), sub-diploid DNA content on FACS (Fig 6B), JC-1 assay (Fig 6C) and DNA laddering (Fig 6D) all confirmed the apoptotic mechanism of cell death. Similar effects of accumulation in G1 and subsequent apoptosis were also observed in DG75 EBV negative BL cells containing the same ER-EBNA2 plasmids (data not shown) Both type 1 and type 2 EBNA2 caused all these effects but in some experiments the PARP cleavage in response to type 2 EBNA2 was slightly slower (Fig 6A).

Differential regulation of LMP1 by type 1 and type 2 EBNA2

A previous report compared the levels of LMP1 in LCLs stably expressing type 1 or type 2 EBNA2 and found them to be similar (9). We transfected the ER-EBNA2 (type 1 and type 2) plasmids into Daudi BL cells, which contain an endogenous EBV genome with a deletion of the EBNA2 gene, and selected transfected cell lines with G418. Because LMP1 is normally induced by EBNA2, LMP1 protein is not usually present in Daudi cells at a significant level. When EBNA2 function was induced by adding estrogen, type 1 EBNA2 induced LMP1 much more rapidly than type 2 EBNA2 (Fig 7), even though the expression of type 2 EBNA2 was slightly stronger (Fig 7). This difference in LMP1 induction was large but transient; after 48 hours there was little difference in the levels of LMP1, consistent with the previously published result in cells stably expressing type 1 or type 2 EBNA2 (9). In contrast to the EREB2.5 cells (Fig 1), where the continued expression of LMP1 was measured following transfection of EBNA2, this Daudi cell system measures the effect of EBNA2 activation in cells that start with no LMP1 expression, more like the induction of LMP1 during infection of primary B cells by EBV.

Discussion

The important biological difference between type 1 and type 2 EBV strains is the much greater ability of type 1 strains to convert primary human B cells into continuously proliferating LCLs (9, 32). The systems we have devised allow study of gene regulation by EBNA2 at physiological levels on target genes in their normal regulatory context. The results suggest several possible reasons why type 1 EBV strains may produce LCLs more efficiently than type 2 strains. Timely induction of

LMP1 is important to allow survival of B cells driven into the cell cycle by EBNA2 and EBNA-LP and our results in Daudi cells suggest that type 1 EBNA2 induces LMP1 much more rapidly and efficiently than type 2 EBNA2.

Previous studies comparing the ability of type1 and type 2 EBNA2 to induce gene expression focussed on LMP1 (43, 44) and CD23 (43). Transient transfection assays of EBNA2 expression vectors into BL30/P3HR1 or Daudi cells assayed by immunoprecipitation of LMP1 gave a higher induction by type 2 EBNA2 in some experiments but equal induction in others (44). In contrast, transient transfection assays in BJAB cells gave about a 2.5 fold higher induction of an LMP1 promoter CAT reporter with type 1 EBNA2 and about a 1.5 fold higher induction of a CD23 promoter CAT construct (43). These results were interpreted to suggest that higher transactivation by type 1 EBNA2 might be the basis for the more efficient transforming properties of type 1 EBV. At that time the cooperation of EBNA2 with EBNA-LP was not taken into account.

All our experiments have been performed in the presence of the 2 repeat type 1 EBNA-LP that is expressed from p554 series plasmids (24). There is some evidence that type 1 and type 2 EBNA-LP can differ in their ability to cooperate with EBNA2 (29) in induction of the LMP1 promoter and that EBV sequence variation in the LMP1 promoter (studied in P3HR1 EBV) can cause it to respond to type 1 EBNA2 slightly differently from the prototypic B95-8 LMP1 promoter (18). In some respects it is surprising that we observed no outgrowth with type 2 EBNA2 in the EREB2.5 complementation assay since type 2 strains of EBV usually give rise to a low efficiency transformation of primary B cells. It is possible that the 2 repeat type 1

EBNA-LP expressed from both the type 1 and type 2 EBNA2 expression vectors used in this paper cooperates more effectively with the type 1 EBNA2 than the type 2 EBNA2 but there is no specific evidence for this. The EBNA-LP activity in the EREB2.5 cells is further complicated by the presence of the P3HR1 truncated EBNA-LP, which has recently been reported to be a gain of function mutant, acquiring the ability to inactivate protein phosphatase 2A (13). These additional levels of complexity have not yet been explored in our study but they do not affect our conclusion that there can be differential regulation of a relatively small number of cell genes by type 1 and type 2 EBNA2.

The Akata cell line used for the microarray analysis and apoptosis studies in this paper has an 8:14 translocation (41) similar to the BL cell lines studied previously (21) so the repression of c-MYC by EBNA2 shown in Fig 6 presumably occurs by the same mechanism involving the Ig Mu enhancer. In the Akata cells, type 1 and type 2 EBNA2 appeared to be equally effective in repressing c-MYC so there is no reason to think that the differential regulation of cell target genes we observed is mediated by c-MYC. The protein synthesis inhibitors present in the microarray study would also most likely prevent indirect effects that could occur via c-MYC. The mechanism by which apoptosis occurs in the Akata BL cells after cell cycle arrest (Fig 6) remains to be determined but the similar effects of type 1 and type 2 EBNA2 in BL cells are consistent with both EBV types being found in African BL tumours.

The experiments illustrated in Fig 1 in the EREB2.5 LCL involved Amaxa transient transfection and could only be performed on a small scale so it was only possible to test a few EBNA2 target genes by Western blotting (Fig 1C) or RT-PCR (Fig 4B). It

is noticeable that RUNX3, which is known to be required for LCL proliferation (38) and was differentially expressed in the LCL experiment (Fig 1C), was not in the list of differentially regulated target genes in the Akata BL system (Fig 2). Although RUNX3 was confirmed as an EBNA2 target gene in BL cells (28), it was only slightly induced in those experiments and it seems likely that differences in the transcription factor content in the different cell backgrounds will affect which genes are detected as EBNA2 targets, as will the different experimental procedures used. However, the biochemical functions of the four genes identified in Figure 2, namely MARCKS, IL1β□□ADAMDEC1 and CXCR7, are likely to be relevant to B cell proliferation and survival. ADAMDEC1 and CXCR7 were also shown to be differentially regulated by type 1 and type 2 EBNA2 in the LCL background (Fig 4B).

MARCKS (myristoylated alanine-rich protein kinase C substrate) is a PKC substrate and its phosphorylation has been used as a marker of PKC activation (1). It regulates membrane ruffling and cell spreading (30) and can reversibly inhibit phospholipase C (14) Targeted disruption of the gene encoding MARCKS in mice resulted in numerous developmental defects and perinatal death (40). MARCKS was previously shown to be induced by EBV infection (6) although in our experiments the induced level of MARCKS RNA was still very low so it is probably also regulated by another EBV gene product apart from EBNA2. IL1β is a cytokine involved in lymphocyte proliferation and has been reported previously to be expressed in EBV infected LCLs (26). Its function normally requires that it is secreted so that it can activate via its specific receptor but intracellular IL1β protein similar to that seen in our BL studies has been studied previously as an inhibitor of caspase activation (42) and intracellular IL-1α precursor form has been reported to function as a transcription regulator (8, 45)

so this intracellular IL-1β might be functional. ADAMDEC1 is a metalloprotease that is expressed in lymphocytes, dendritic cells and macrophages (5). Its precise function is not yet known but its expression has been used as a marker to distinguish splenic from peritoneal B cells of the B-1a class (39).

CXCR7 (RDC1, CMKOR1) was recently found to be the receptor for chemokine SDF-1/CXCL12 in T lymphocytes (4) although there is doubt about this being the ligand for CXCR7 in B cells (17). The function of CXCR7 in normal B cells has not yet been determined but we demonstrated (Fig 4B) that it is required for LCL proliferation or survival and a previous study identified CXCR7 as essential for transformation of endothelial cells by Kaposi's sarcoma-associated herpesvirus (31).

Further work will be required to characterize differential gene regulation by type 1 and type 2 EBNA2 but there does appear to be a small number of cell genes that are more differentially regulated than the great majority of EBNA2 target genes. We do not expect a single cell gene to complement the deficiency in type 2 EBV immortalization of B cells but it may be a relatively small number of genes that is required. The mechanism of the differential effects of type 1 and type 2 EBNA2 on these few genes remains to be determined but we conclude that differential induction of LMP1 and a relatively small number of cell genes correlates with the different transforming abilities of type 1 and type 2 EBV.

Acknowledgements

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Figure legends

Figure 1

A. EREB2.5 cells, which had been growing normally in estrogen, were washed to remove estrogen and transfected with EBNA2 T1 or T2 vector expressing EBNA2 type 1 or type 2 protein. Cells were cultured in the absence of estrogen and pulse labelled at the times indicated with ^{3}H-Thymidine.

B. Phase contrast images of cells from Fig 1A 7 days after transfection of EBNA2 plasmid showing proliferating cells only with type 1 EBNA2. Lower panel is a western blot for EBNA2 in 3 LCLs after 3 months of outgrowth.

C. Western blots of extracts from the same transfections assayed in part A 3 or 4 days after transfection as indicated for EBNA2, LMP1, IRF4, RUNX3 and actin as a loading control.

D. Comparison of detection of in vitro translated type 1 and type 2 EBNA2 by western blotting with PE2 antibody. Relative detection by Western blotting (WB) is similar to ^{35}S Met labelling during the in vitro translation.

Figure 2

Bar chart summary of microarray expression profiling comparing genes regulated by ER-EBNA2 type 1 and type 2 stably transfected in AK31 BL cells. Cells were pre-treated for 1hr with protein synthesis inhibitors, then with or without estrogen for 4 hrs in the presence of protein synthesis inhibitors. Total cell RNA was extracted and used for expression profiling on Agilent 44K microarrays (as described in the methods section). The experiment was performed in duplicate and the resulting RNAs were hybridised pairwise (estrogen-treated versus control) on individual microarrays. For each condition the values were averaged and a standard error was calculated.

Each column represents the ratio of the mean of values plus estrogen over the mean of the values minus estrogen (fold induction). The inset panel shows western blotting using PE2 antibody of ER-EBNA2 levels in the AK31:EBNA1/ER-EBNA2 cells with or without 4 hrs treatment with estrogen.

Figure 3

A. ELISA detection of IL1β. AK31:EBNA1/ER-EBNA2 type 1 or type 2 cells were cultured with or without estrogen for 24 hrs. The medium and cell extracts (IC) were assayed for IL1β by ELISA.

B. Cells were treated as in (A) and analysed by western blotting for IL1β

C. Cells were treated as in (A) and analysed by RT-PCR for ADAMDEC1 and GAPDH as a positive control.

Figure 4

A. Left panels: Estrogen starved EREB2.5 cells (E2.5) were treated with estrogen for 4 hours and RNA was extracted and analysed by RT-PCR for CXCR7 mRNA using indicated primer combinations (right panels) to detect alternately spliced forms. Middle panels: Estrogen starved EREB2.5 cells (E2.5) or AK31:EBNA1/ER-EBNA2 type 1 or type 2 cells (AK31) were pretreated with protein synthesis inhibitors for one hour (AK31) or 2 hours (E2.5) and the cells were then treated with or without estrogen for 4 hours in the presence of protein synthesis inhibitors (as in the microarray experiment in Fig 2). RNA was extracted and analysed by RT-PCR for CXCR7 mRNA using indicated primer combinations (right panels) Track M is size markers (100 bp ladder).

B. RT-PCR assay for CXCR7 or ADAMDEC1 mRNA in EREB2.5 cells 7 or 10 days after transfection with EBNA2 T1 or T2 vector and withdrawal of estrogen as in Figure 1. CXCR7 primers were those shown in upper panels of Fig 4A. GAPDH mRNA was also assayed to ensure equal input RNA in the samples.

C. RT-PCR assay for CXCR7 mRNA in a panel of LCLs with primers used in upper panels of Fig 4A. GAPDH mRNA was also assayed as a control.

Figure 5

A. Plasmids expressing shRNA for CXCR7 or p53 as a negative control or the control empty vector (V) were transfected into EREB2.5 cells and the transfected cells selected with puromycin. Live cell counts (trypan blue exclusion) are shown at the indicated times after transfection. CXCR7 RNA was assayed by RT-PCR 7 days after transfection using the exon 2 primer combination shown in Fig 4A upper panels.

B. As part A but in LCL3 cells (an LCL infected with B95-8 EBV). Here the CXCR7 RNA was assayed by RT-PCR 6 days after transfection.

C. To show that the CXCR7 shRNA plasmid was not non-specifically toxic to cells and that it did not prevent function of the puromycin resistance gene, the shRNA plasmids used in parts A and B were transfected into 293 cells and selected with puromycin. Ctrl sample received a plasmid lacking the puromycin resistance gene. Live cell counts 8 days after transfection are shown.

Figure 6

A. Type 1 and type 2 EBNA2 both repress MYC expression and drive apoptosis in AK31 BL cells. AK31:EBNA1/ER-EBNA2 T1 and T2 stable cells were induced with 5 μM β-estradiol for 0-72 hours. Extracts from cells were probed with indicated

antibodies.

B. Analysis of EBNA2 T1 and T2 driven apoptosis in AK31 cells. AK31:EBNA1/ER-EBNA2 T1 and T2 stable cells induced with 5 μM β-estradiol for 0-72 hours were ethanol fixed and stained with propidium iodide prior to analysis of DNA content by flow cytometry. Upper panels: DNA content analysis of AK31:EBNA1/ER-EBNA2 cells following ER-EBNA2 T1 activation demonstrating transient increase in G_0/G_1 populations followed by apoptosis at later time-points. Numbers inserted into histogram profiles denote % subdiploid, G_0/G_1 , S and G_2/M populations respectively. Lower panels: Bar chart comparing Sub-Diploid and G_0/G_1 DNA content for both T1 and T2 ER-EBNA2 stable populations.

C. Type 1 and type 2 EBNA2 drive mitochondrial/ intrinsic apoptosis in AK31 cells. AK31:EBNA1/ER-EBNA2 T1 and T2 stable cells were induced with 5 μM β-estradiol for 0-72 hours and analysed for apoptosis. The JC-1 assay was performed at each time point. Upper panel shows example profiles of CCCP-treated positive control, 0 hour and 72 -/+ activation of T1 ER-EBNA2 stable cells. Bar chart (lower panel) shows a comparison between EBNA2 T1/T2-driven intrinsic apoptosis or the percentage of normal (N) cells versus apoptotic (Ap) cells in both cell populations.

DNA

D. DNA laddering analysis was also performed on AK31:EBNA1/ER-EBNA2 T1 or T2 cells induced with 5 μM β-estradiol for 0-72 hours.

Figure 7

Daudi BL cells stably transfected with ER-EBNA2 type 1 or type 2 were cultured with or without estrogen for the indicated times and protein extracts of the cells were

analysed by western blotting for EBNA2 (PE2 antibody), LMP1 (CS1-4 antibody) or actin as a loading control.

Figure 1

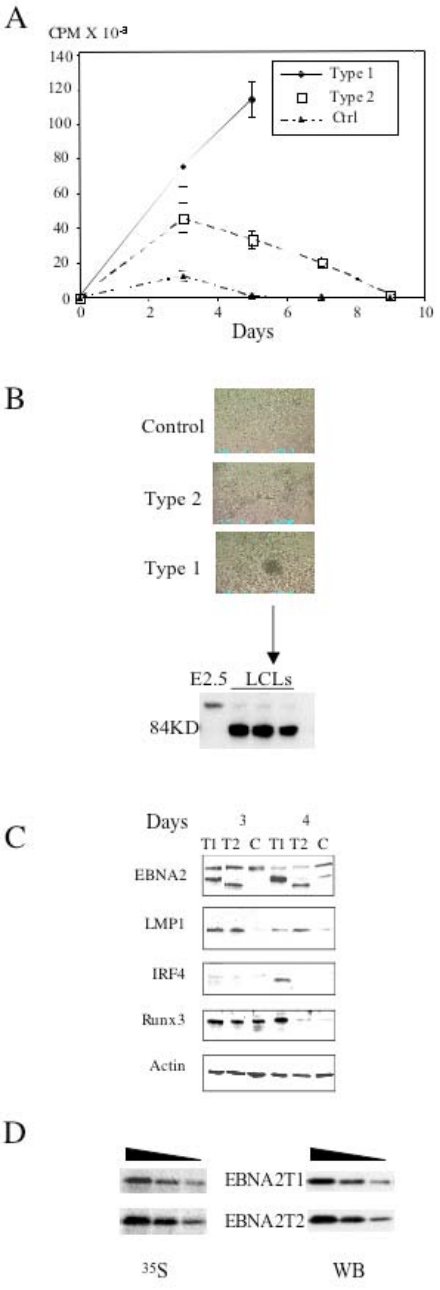
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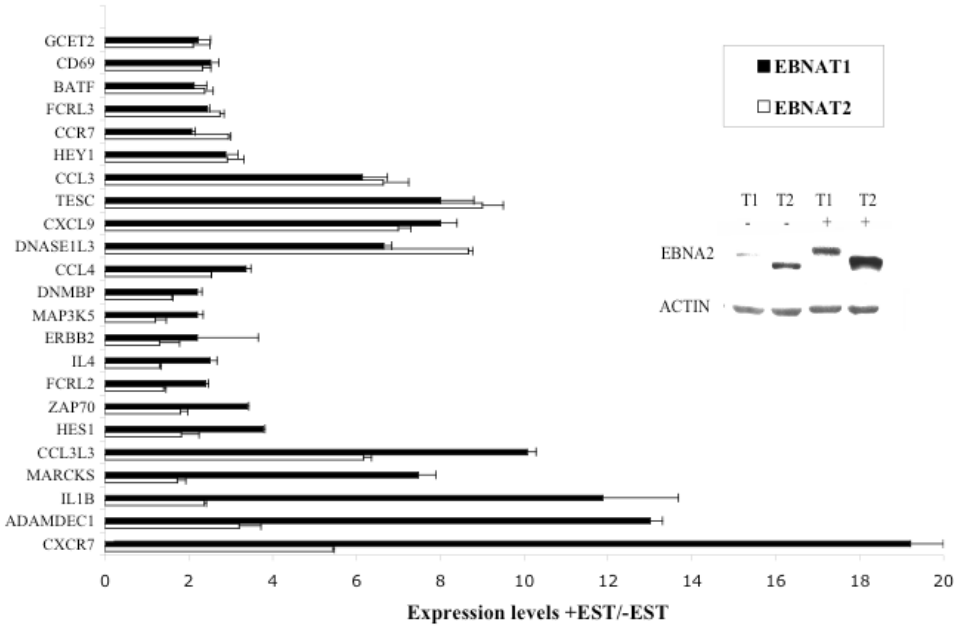
Figure 2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IL4
FCRL2
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Figure 3

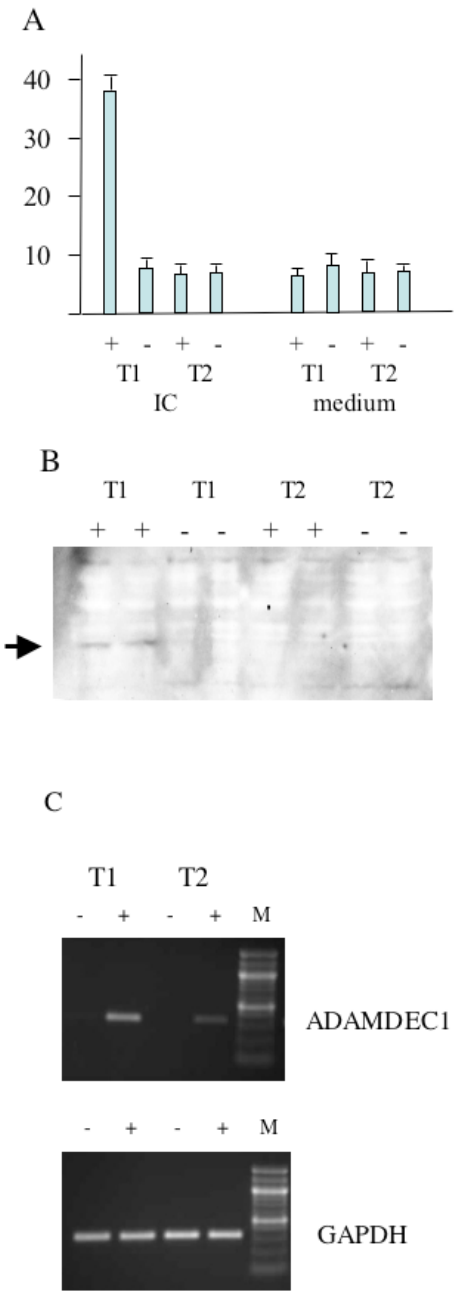
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Figure 4

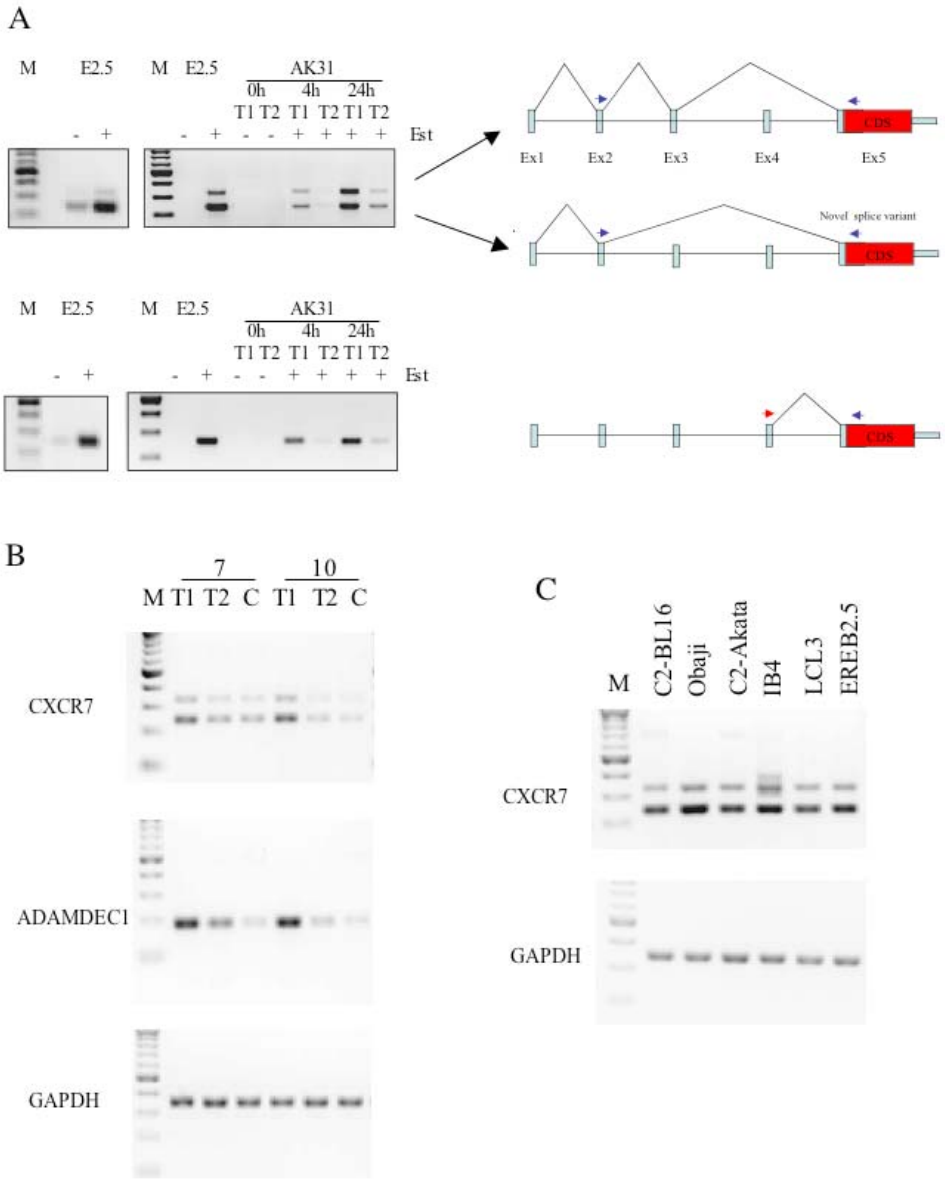
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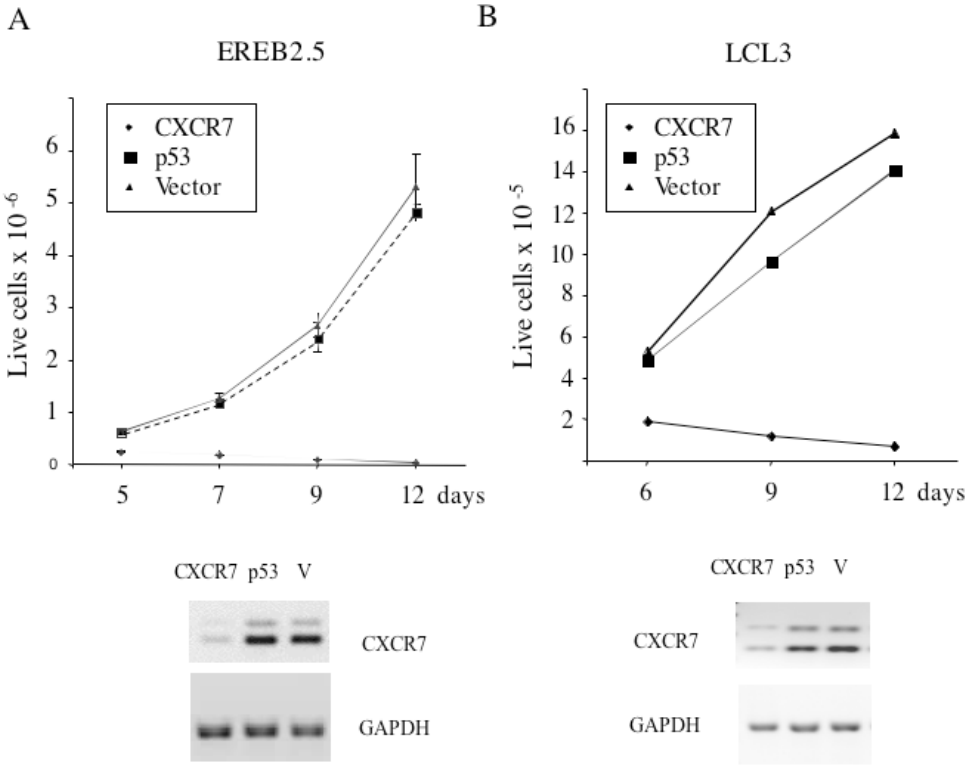
Figure 5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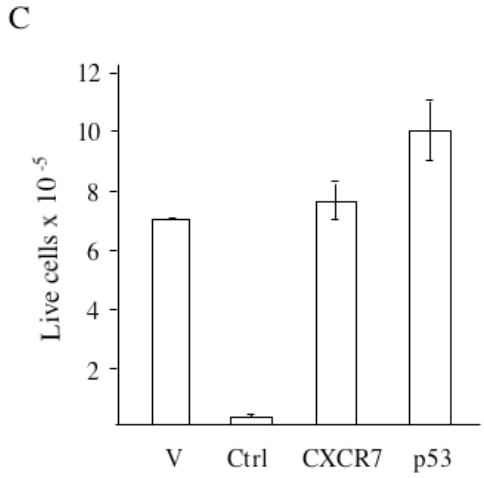
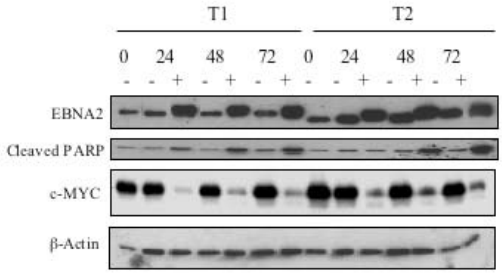
12 days
CXCR7 p53 V
CXCR7
GAPDH
CXCR7 p53 V
CXCR7
GAPDH


C
Live cells x 10 -5
2
4
6
8
10
12
V
Ctrl
CXCR7
p53

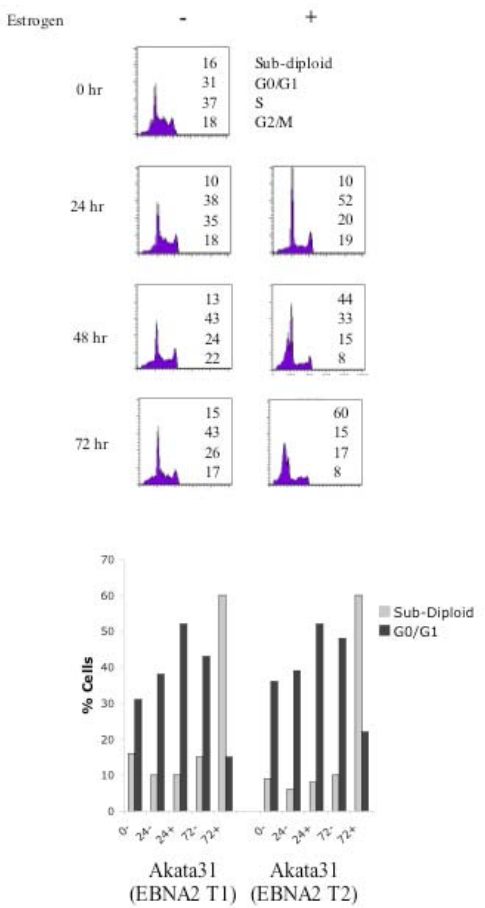
1
2

Figure 6 A, B

A



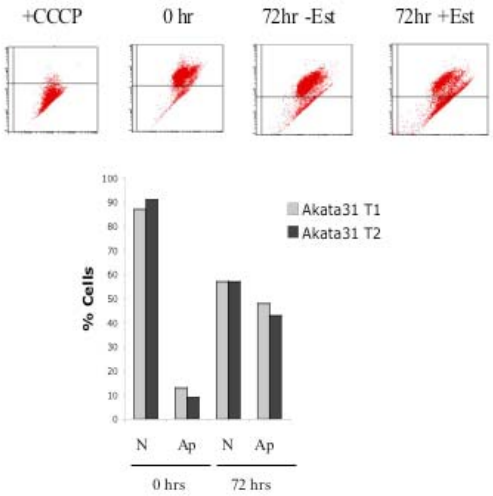
B



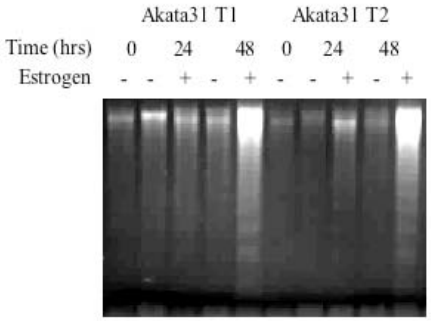
1
2

Figure 6 contd

C

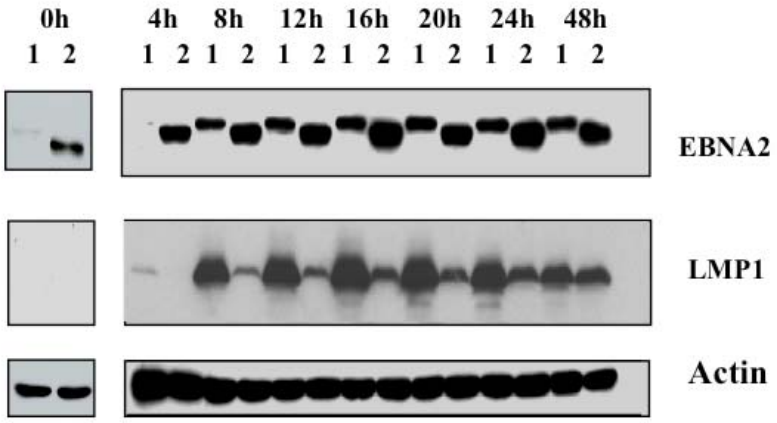


D



1
2

Figure 7


0h
4h
8h
12h
16h
20h
24h
48h
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
EBNA2
LMP1
Actin

1