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Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: Maintenance of tight latency with efficient reactivation upon induction

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Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (KS) and at least two B cell lymphoproliferative diseases: primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). B cells derived from PEL are latently infected, and can be induced to lytic replication by treatment with chemical agents like TPA or butyrate, which have pleiotropic effects on host cell signaling and chromatin structure. Most of these lines also display moderate levels of spontaneous lytic induction, which complicates analysis of latency. Here we describe the creation of latently infected cell lines derived from SLK endothelial cells that (i) display tight control of KSHV latency, with little spontaneous reactivation and (ii) are efficiently inducible by doxycycline, avoiding the need for pleiotropic inducing agents. These cells produce substantial quantities of infectious KSHV, and should be useful for studies of the latent-lytic switch and the impact of lytic replication on host cell biology.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), belongs to the gamma-herpesvirus subfamily and shows homology to herpesvirus saimiri and Epstein-Barr virus (EBV). KSHV was first identified based on its association with an endothelial neoplasm called Kaposi's sarcoma, but was soon discovered to cause at least two lymphoproliferative diseases: multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL) (reviewed in (Ganem, 2010)).

PEL cells grow readily in culture, and have been the most widely used lines for the in vitro study of KSHV. In the ground state, they are latently infected: the viral genome is present as a nuclear plasmid, and expresses only a handful of viral genes. However following treatment with certain chemical agents (see below), they can be induced to enter the lytic transcriptional program. In this program, virtually the entire viral genome is transcribed, in a temporally regulated cascade of gene expression – early genes are regulatory and catalytic functions that prepare the cell for viral DNA replication; following genomic replication, the late genes encoding structural components are expressed. Lytic replication is highly cytopathic, and results in the generation of large quantities of infectious virus in the culture supernatant. Subsequently, several labs have shown that many adherent lines in culture can be latently infected following exposure to KSHV virions (Bechtel et al., 2003; Vieira et al., 2001).

Most existing KSHV-infected lines, however, have experimental limitations. For example, most PEL cells have substantial levels of spontaneous lytic reactivation, complicating the analysis of latent gene expression (Renne et al., 1996). Conversely, many adherent cell lines are poorly inducible with chemical agents (Bechtel et al., 2003). In addition, the chemicals that are used to induce lytic replication – HDAC inhibitors (e.g., sodium butyrate or valproate) or phorbol esters (e.g., TPA) – are pleiotropic, with broad effects on chromatin structure and signal transduction (respectively). As such, it is difficult to rigorously examine the biology of the host cell during the latent-lytic switch, since host cell homeostasis is itself so profoundly affected by the exogenous inducing stimuli.

The principal viral actor in the latent-lytic switch is the protein RTA (replication and transcription activator), encoded by KSHV ORF 50 (Lukac et al., 1998, 1999; Sun et al., 1998; Xu et al., 2005). RTA is a sequence-specific transcriptional activator that can also interact with host transcription factors to induce genes whose promoters lack canonical RTA response elements (RREs) (Chen et al., 2000; Deng et al., 2002; Liang et al., 2002; Liang and Ganem, 2004; Lukac et al., 2001; Sadler et al., 1999; Song et al., 2001). Ectopic expression of RTA induces cells from latency, and mutant viruses lacking functional RTA cannot reactivate in response to any inducing stimuli (Xu et al., 2005). Thus, RTA expression is both necessary and sufficient to trigger lytic reactivation (Lukac et al., 1998, 1999; Sun et al., 1998). PEL cells latently infected with KSHV have been transduced with vectors allowing inducible expression of RTA, generating cells that

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are inducible with doxycycline (Nakamura et al., 2003), thereby obviating the need for pleiotropic inducers. But these cells retain the high rate of spontaneous lytic reactivation of their parental line, and also lack isogenic uninfected counterparts that serve as essential controls for many types of biochemical experiment. Also, since they are of B cell origin they do not allow examination of viral replication in the other important lineage infected by KSHV, the endothelial cell.

In search of endothelial cell lines, which (i) allow efficient KSHV infection, (ii) maintain tight and stable latency when once established and (iii) support robust viral reactivation upon induction, a panel of cell lines were screened. Here we report that SLK cells, which are uninfected endothelial cells derived from a gingival KS lesion of an HIV-negative renal transplant recipient (Siegal et al., 1990), fulfill many of these criteria. To construct SLK derivatives that were inducible without pleiotropic chemicals, we first engineered a derivative of SLK (termed iSLK) that expresses a doxycycline-inducible RTA transgene. To generate iSLK.219 cells, iSLK cells were then latently infected with a recombinant KSHV.219 virus, which constitutively expresses puromycin N-acetyl-transferase and GFP while RFP is expressed during lytic replication (Vieira and O'Hearn, 2004). The mass culture was inducible by doxycyline, but further induced by addition of HDAc inhibitors. A subclone was derived that displays highly efficient induction by doxycycline alone. These cells provide a reliable source for generating large quantities of rKSHV.219 viruses from endothelial cells without induction by agents like TPA or HDAC inhibitors that disturb host cell physiology.

2. Materials and methods

2.1. Cells and reagents

SLK, Phoenix and Vero76 cells were maintained in DMEM supplemented with 10% FBS and 1% glutamate and penicillin/streptomycin. BJAB, Ramos, Jurkat, SUP-T1 cells were cultured in RPMI 1640 media containing 10% FBS and 1% glutamate and antibiotics. TIME, LEC, BEC, HUVEC, cells were cultured in EGM-2 MV media, supplemented with 2% FBS and various growth factors (Lonza, Allendale, NJ). Hygromycin was purchased from Invitrogen (Carlsbad, CA), Puromycin from Invivogen (San Diego, CA), and G418 from Sigma (St. Louis, MO). QBI293 cells were purchased from QBiogene (Carlsbad, CA).

2.2. Generation of retroviruses

A retrovirus-based inducible expression system (pRetro-X Tet-ON Advanced Inducible Expression System) was purchased from Clontech (Mountainview, CA). Retroviruses constitutively expressing rtTA (a Tet-On transactivator) were obtained by transfecting pRetroX-Tet-On Advanced into Phoenix cells. Culture supernatants containing retroviral particles were harvested at days 2–4 post-transfection and filtered through 0.22 μm membrane. RTA, encoded by ORF50, was amplified by PCR (forward primer: GATCGCGGCCGCATGGCGCAAGATGACAAGGG, reverse primer: GGCCGAATTCTCAGTCTCGGAAGTAATTACGCC) and cloned into pRetroX-Tight-Hyg using NotI and EcoRI site. This vector contains 7 copies of the tet operator sequences linked to a minimal CMV promoter, which drives expression of the RTA gene. Sequence was verified (Elim Biopharmaceuticals). Retroviruses, expressing RTA, were generated as described above.

2.3. Generation of iSLK.219

Doxycycline-inducible SLK cells were generated by transducing cells first with retroviruses expressing rtTA, in the presence of polybrene (8 μg/ml) for 3 h at 2500 RPM. rtTA-expressing SLK cells were selected by G418 at 800 μg/ml for 2 weeks. rtTA-expressing SLK cells were transduced again with retroviruses expressing RTA under tet operator control and transductants were selected by hygromycin at 1200 μg/ml for 2 weeks. Resulting doxycycline-inducible SLK (iSLK) cells were maintained in culture medium containing both G418 and hygromycin. iSLK cells were infected with rKSHV.219 (Vieira and O'Hearn, 2004) and virus-harboring iSLK cells were selected by puromycin at 1 μg/ml for 2 weeks. To increase the virus genome copy number in the resulting cells, iSLK.219 cells were cultured in increasing concentration of puromycin (up to 10 μg/ml) by 1 μg/ml increment. At each puromycin concentration, iSLK.219 cells were passed 3 times before puromycin concentration was increased to the next level. Resulting iSLK.219 cells (iSLK.219p10), selected at 10 μg/ml puromycin, were subcloned at 1000 cells in a 150-mm culture dish and 12 subclones were picked and cultured for subsequent experiments. Doxycycline-inducible stable Vero76 cells, harboring rKSHV.219, were prepared similarly as described above. Inducible cells were maintained in the presence of antibiotics at all times.

2.4. KSHV infection with free virus and viral stock titration

KSHV was delivered to various primary and established cells by spinoculation (2500 RPM for 90 min) (Vieira and O'Hearn, 2004; Yoo et al., 2008) at varying multiplicity of infection (MOI). Unbound viruses were washed off and infected cells were cultured for 48 h before FACS analysis or selection with puromycin. Virus stocks were prepared from induced Vero cells harboring rKSHV.219 (Vieira and O'Hearn, 2004). Procedure for virus concentration from induced culture supernatant is described elsewhere (Bechtel et al., 2003; Myoung and Ganem, 2011b). Titers of KSHV stocks were determined by infecting serially diluted stocks on QBI293A cells with subsequent enumeration of GFP-expressing cells by FACS analysis (Vieira and O'Hearn, 2004; Myoung and Ganem, 2011a).

Flow cytometry: iSLK.219 cells or QBI293A cells were first trypsinized and washed once with incomplete PBS and fixed by 0.5% paraformaldehyde in PBS for 5 min at RT. Cells were washed in cold incomplete PBS and resuspended in FACS staining buffer, containing 4% FBS and 0.09% sodium azide. GFP and/or RFP expression in cells was examined on LSR II (BD Bioscience, San Jose, CA). Data analysis was performed by FlowJo software.

Statistical analysis: Data represented in this study are either a representative one or shown as the means ± standard deviations (SD) of 2–3 independent experiments. Statistical significance of differences in the mean values was evaluated by paired Student's *t* test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Generation of doxycycline-inducible SLK cells, harboring rKSHV.219

The following cell lines were first screened for their infectibility by KSHV and their ability to support lytic reactivation at MOI 10 by spinoculation in the absence of polybrene: TIME, FO-1, 293, and SLK cells. TIME cells supported efficient viral entry as assessed by GFP expression (~50%), but the growth of infected TIME cells was very slow, and infected cells eventually died, largely due to spontaneous lytic KSHV reactivation (data not shown). FO-1 and 293 cells demonstrated high levels of rKSHV.219 infection (analyzed by GFP expression), roughly ~30% and 90%, respectively. However, FO-1 cells displayed poor inducibility while 293 cells died of extensive spontaneous lytic replication after infection. In addition, surviving 293 cells were refractory to reactivation by HDAc inhibitors