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Increased tumorigenicity of cells carrying recombinant human herpesvirus 8

N. Cloutier¹, O. van Eyll¹, M.-E. Janelle¹, S. Lefort¹, S.-J. Gao², and L. Flamand¹

¹ Laboratory of Virology, Rheumatology and Immunology Research Center, Centre Hospitalier Universitaire de Québec, pavillon CHUL and Faculty of Medicine, Laval University, Sainte-Foy, Québec, Canada

² The University of Texas Health Science Center at San Antonio, San Antonio, TX, U.S.A

Summary

Human herpesvirus 8 (HHV-8) infection is associated with the development of Kaposi's sarcoma and primary effusion lymphoma. The cloning of the HHV-8 genome into a bacterial artificial chromosome (BAC) allows researchers to mutate and identify the relative importance of HHV-8 genes essential for growth and replication in tissue culture systems. However, *in vivo* models to study the impact of such mutations are very limited. Consequently, the objective of this study was to determine whether cells carrying the HHV-8 BAC would form tumors when injected into mice, enabling the use of this model to assess the influence of viral gene mutation on tumorigenesis. To do so, 293T and 293T-E1 cells carrying recombinant HHV-8 were injected into SCID mice and tumor growth was analyzed. Our results clearly show that mice injected with 293T-E1 cells had a significantly higher tumor incidence level as well as increased tumor volumes and weights compared to mice injected with 293T control cells. Cells carrying the HHV-8 genome grew faster and more aggressively in SCID mice than control 293T cells, highlighting the oncogenic properties of HHV-8. The model presented could therefore be used for the identification of HHV-8 genes contributing to tumorigenesis in the context of the entire viral genome.

Introduction

Human herpesvirus 8 (HHV-8) is an oncogenic virus associated with the development of at least two malignancies: Kaposi's sarcoma (KS) [8] and primary effusion lymphoma (PEL) [6]. HHV-8 is also linked to some cases of multicentric Castleman's disease (MCD), a B-lymphocyte lymphoproliferative disorder [24]. Epidemiological studies are in strong support of a direct association between HHV-8 infection and the development of these pathologies. Furthermore, *in vitro* infection of primary endothelial cells results in long-term cellular survival with the development of a distinctive spindle shape morphology, reminiscent of KS lesions [9,11]. Several HHV-8 oncogenes, including *ORFK1*, *ORF74*, *ORF71*, and *ORFK9*, when ectopically expressed, cause endothelial and/or fibroblast cell transformation *in vitro* as well as tumor development *in vivo* [2,4,10,12,16,21]. Some models using HHV-8-infected cells to induce tumor development *in vivo* have been described. For example, PEL cells injected into SCID mice induce tumor development only under certain conditions (in the presence of matrigel, etc.) [25]. More recently, a first model using telomerase-immortalized endothelial cells (TIVE cells) demonstrated the oncogenic potential of HHV-8, although several difficulties were encountered for the generation of persistently infected endothelial cells [1].

In vivo immortalization and transformation of primary B lymphocytes by HHV-8 has not yet been achieved, although several cell lines carrying infectious HHV-8 have been isolated from PEL patients, suggesting that lymphoid B cells are targets for infection by this virus [3,5,6, 23].

Studies on kinetic of infection of endothelial cells with HHV-8 have indicated that during the initial phase (<12 h), both lytic and latent proteins are expressed, followed by gene expression restricted to latent genes (*ORF73-71*) in more than 90% of the cells [13,29]. The small percentage of cells infected produce infectious virions and numerous factors that influence cell growth and survival.

Most of our current understanding on the role of HHV-8 proteins has been obtained through ectopic overexpression of individual viral genes. The lack of animal models susceptible to infection as well as the difficulty of genetically manipulating the viral genome have limited pathogenesis studies. Recently, a recombinant bacterial artificial chromosome (BAC) containing the entire HHV-8 genome (HHV-8 BAC) was generated [30]. Using such technology, essential *in vitro* roles for RTA (*ORF50*) in viral reactivation, LANA (*ORF73*) in viral episome maintenance, and glycoprotein B (*ORF8*) for viral egress were clearly demonstrated, highlighting the usefulness of the HHV-8 BAC [14,27,28]. The lack of suitable animal models allowing the study of the tumorigenic potential of cells carrying WT HHV-8 prompted us to tackle this issue. Consequently, the purpose of the present work was to develop a mouse model that would compare the tumor formation of 293T and 293T cells carrying HHV-8. The model presented here allows the possibility to study the relative contribution of HHV-8 genes/proteins in tumor development in the context of the entire viral genome.

Materials and methods

Cells

HEK293T (ATCC) were cultured in DMEM (Megacell) containing 3% HI-FBS. 293T-E1 cells were generated essentially as described for the generation of 293-E1 and cultured in DMEM containing 3% HI-FBS and 150 µg/mL hygromycin [30]. With the exception of a slightly greater transfection efficiency, 293T-E1 behaved exactly as 293-E1 cells (S.-J. Gao personal communication).

In vitro proliferation

The *in vitro* proliferation rate of 293T and 293T-E1 was determined by plating 3.5×10^4 cells (in triplicate) in the wells of a 12-well plate. On days 1, 2, 3 and 4, cells were trypsinized and counted using a hemacytometer.

Immunofluorescence assay

293T and 293T-E1 cells were fixed in cold acetone for 10 min and air-dried. Cells were first reacted with a rat anti-LANA, mouse anti-K1 (2H5) (generously provided by Dr Jung) [15], and rabbit anti-vIL-6 for 1 h at room temperature. Slides were washed three times for 5 min in PBS and then incubated with Alexa 568-labeled anti-rat IgG, Alexa 568-labeled anti-mouse IgG or Alexa 568-labeled anti-rabbit IgG (Invitrogen) for 1 h at room temperature. Slides were washed three times and the nuclei were stained by incubating with 0.1 µg/mL of 4'-6-diamino-2-phenylindole (DAPI) (Invitrogen) for 5 min at room temperature. After three additional 5-min washes in PBS, the slides were mounted with 80% glycerol in PBS and observed using fluorescence microscopy (BX51 Olympus, Canada).

Injection of cells into SCID mice

Three independent experiments were carried out. For each experimental group, a total of 11 CB17 female SCID mice (28–35 days old) were injected subcutaneously on both hind legs with 0.1 mL PBS containing 1×10^7 293T or 293T-E1 cells. Mice were observed regularly for the appearance of tumors (tumor incidence) and behavior. Upon growth, tumor size was determined 3 times per week using a caliper. At sacrifice, tumors were excised, measured, weighed and chopped into small blocks before being processed for real-time PCR, immunohistochemistry (IHC), and flow cytometry analysis following Hoechst 33342 staining.

Real-time PCR

Total RNA was extracted from cells and from pieces of tumors using the TRIZOL reagent (Invitrogen, Ontario, Canada). All RNA samples were treated with DNase to eliminate residual genomic DNA. HHV-8 *ORF26*, *ORF50*, *ORF73*, *ORFK2* and human *GAPDH* gene expression were determined using the following forward (F) and reverse (R) oligonucleotide primers (Sigma-Aldrich) and probes (P) (IDTDNA, Coralville, IA, USA): *ORF26* F 5'-GCT CGA ATC CAA CGG ATT TG-3', R 5'-AAT AGC GTG CCC CAG TTG C-3', P 5'-FAM-TTC CCC ATG GTC GTG CCG C-BHQ1-3', *ORF50* F 5'-CGC AAT GCG TTA CGT TGT TG-3', R 5'-GCC CGG ACT GTT GAATCG-3', P 5'-FAM-ACC TGT GCC CCC TCT TCG ACA CC-BHQ1-3', *ORF73* F 5'-CGC GAA TAC CGC TAT GTA CTCA-3', R 5'-GGA ACG CGC GCC TCA TAC GA-3', P 5'-FAM-ACA TCA CCA CCC CAC AGA CCT GGA G-BHQ1-3', *ORFK2* F: 5'-TCT GAC GAA GAC CTT AGG ATG GGA C-3', *ORFK2* R: 5'-AGT CCC TGA AGC CTC CCT AAT AGA C-3', *ORFK2* P: 5'-FAM-AAG ACG CAC TAC AGT CCA CCC AAA-BHQ1-3', *hGAPDH* F 5'-CGA GAT CCC TCC AAA ATC AA-3', R 5'-TTC ACA CCC ATG ACG AAC AT-3', P 5'-HEX-TGG AGA AGG CTG GGG CTCAT-BHQ1-3'.

Hoechst 33342 analysis

Upon excision of tumors, one section was fixed with 4% paraformaldehyde. One million cells were extracted, washed twice with $1 \times$ PBS and incubated with 1.8 μ g/mL Hoechst 33342 (Invitrogen) overnight at room temperature. The percentage of human and mouse cells in tumors was determined using differences between murine and 293T cell DNA content (G1, G2 and S phase of the cell cycle) of the sample by FACS analysis (EPICS ELITE ESP, Beckman) using Multicycle AV software (Phoenix Flow System Inc., San Diego, CA).

Immunohistochemistry

Upon excision, tumors were immediately cut into pieces, and one section was fixed with 4% formalin. Paraffin-embedded blocks of tumors were cut into 3- μ m sections and processed for IHC using standard technique. Briefly, the sections were fixed on slides by an overnight incubation at 60 °C, dewaxed in toluene and gradually rehydrated. Treating the tissue sections with 3% hydrogen peroxide solution for 20 min eliminated endogenous peroxidase activity. Antigen retrieval was done by microwaving tissue in 10 mmol/L citric acid buffer for 5 min, then cooling at room temperature for 45 min. The sections were blocked with 3% fetal bovine serum for 30 min at room temperature. Tissues were analyzed using rat anti-LANA mAb (Advanced Biotechnologies Inc., Maryland, USA), rabbit anti-K8 [20] (generously provided by Professor Henri Gruffat), rabbit anti-GFP (Clontech, USA) and rabbit anti-vIL-6 (Advanced Biotechnologies Inc.) specific antibodies for 1 h at room temperature, followed by 2 five-min washes with Tris-buffered saline containing 0.1% Tween-20 (TBS-T). The primary antibodies were detected using a combination of biotinylated donkey F(ab')₂ anti-mouse IgG/peroxidase-conjugated streptavidin (Jackson Immuno Research) or a goat anti-rabbit-HRP (NEB, Ontario, Canada) as the secondary antibodies for 45 min at room temperature, followed by 2 five-min washes with TBS-T. Addition of diaminobenzidine (DAB), a chromogen that precipitates and forms brown deposits upon oxidation, was used to visualize antibody-labeled cells. Tissue

section nuclei were counterstained with hematoxylin, dehydrated with ethanol and toluene and mounted for examination by light microscopy (BX51, Olympus, Canada) using Permount (Fisher Scientific, Canada). Positive and negative immunohistochemistry controls were used.

Statistical analyses

Differences in tumor incidence were determined using the Fisher's exact test. Differences in tumor volume and cellular composition were estimated using the non-parametric Mann-Whitney test and Student's *t*-test, respectively, while differences in tumor weight were determined using ANOVA with Bonferroni's multiple comparison test. A *p* value < 0.05 was considered significant for all statistical tests.

Results

293T-E1 cells carrying wild-type recombinant HHV-8

We were first interested in characterizing some properties of 293T-E1 cells. As previously described for 293-E1 cells [30], 100% of 293T-E1 cells express GFP, although slight variations in GFP intensity were observed, as determined using fluorescence microscopy (Fig. 1A) and FACS analysis (data not shown). Next, we determined the *in vitro* proliferation rate of 293T-E1 cells in comparison to the parental cell line 293T. The results obtained, up to 4 days (Fig. 1B), indicate that these cell lines had similar *in vitro* proliferation rates. We also performed an immunofluorescence assay (IFA) to detect latent and lytic viral genes expressed in resting 293T-E1 cells (Fig. 1C). For a better appreciation of the cellular localization of immunoreactive proteins, nuclei were stained blue using DAPI. As can be observed in Fig. 1C, 293T-E1 expressed the LANA antigen with a typical punctate nuclear pattern, and other than residual background fluorescence, no immunoreactivity of the anti-LANA antibody was detected in 293T control cells. LANA protein was detected in more than 90% of 293T-E1 cells (data not shown). Analysis of the K1 early lytic protein expression indicates that approximately 5% of 293T-E1 cells were positive for K1 (Fig. 1D). K1 staining was cytoplasmic, in agreement with previous work reporting K1 as a membrane-bound lytic protein [15]. Once again, no staining was observed in control 293T cells. Lastly, we performed IFA to detect the K2 gene product, vIL-6. No staining was detected in control 293T cells, while approximately 5% of 293T-E1 cells showed cytoplasmic immunoreactivity with the anti-vIL-6 antibodies used (Fig. 1E). *In vitro*-cultured 293T-E1 cells released approximately 10^5 DNase-protected HHV-8 virions/mL, as determined by quantitative PCR using ORF26 primers (data not shown).

In vivo growth of 293T cells carrying WT HHV-8

Our experiments indicated that the *in vitro* growth of 293T cells carrying WT HHV-8 BAC is identical to that of control 293T cells. Knowing that HHV-8 encodes several oncogenes and/or growth factors reported to affect cell growth, we next determined whether similar conclusions could be drawn under *in vivo* conditions. To do so, we used an animal model that allows 293T to form tumors. Female SCID mice (*n* = 11/group) were injected subcutaneously on both hind leg with 10 million exponentially growing 293T or 293T-E1 cells/site. Tumor incidence and tumor volume were recorded 3 times/week until sacrifice, at which time tumor weight and precise tumor volume were determined. Incidence of tumor development, as determined by the number of tumors that have grown out of 22 injected sites (2 sites/mouse), is presented in Fig. 2A. All results are presented as mean $\pm$ standard deviation from 3 independent experiments. The first animal group to show detectable tumor growth was the one injected with 293T-E1. On day 13 after cell injection, 17% (293T-E1) of the injection sites on mice developed tumors. Tumor incidence continued to increase, and by day 15, 60% of the sites injected with 293T-E1 developed tumors. At that time, the incidence of tumor development for 293T control cells was 4.5%. This difference (293T versus 293T-E1) was considered extremely significant (*p* < 0.0004). By day 17, the difference in incidence was still

considered very significant ($p = 0.0037$), with 15% of sites injected with 293T cells developing tumors as compared to 82% for 293T-E1. By day 19, tumor incidence for the 293T group reached 60% and was no longer considered significantly different from the 293T-E1 group. These results indicate that 293T cells harboring WT BAC HHV-8 cause tumor formation more rapidly and more frequently than cells not carrying HHV-8 during the first 17 days after cell injection.

When tumor growth could be detected, an estimation of the tumor volume (mm^3) was made every 2–3 days. The mean $\pm 95\%$ confidence interval (CI) of tumor volume in function of time is presented in Fig. 2B. Statistical analyses could not be performed before day 17 as no (day 13) or a single tumor in 3 different experiments (day 15) developed for 293T cells. On day 17, 293T-E1 tumors were, on average, 3–4 times larger than those of 293T cells ($**p < 0.001$). Similar findings were observed for days 19 and 21 after cell injection. By day 21, all mice were euthanized and the tumors were excised, photographed, weighed and precisely measured. Representative figures of tumors excised from animals injected with 293T and 293T-E1 cells are presented in Fig. 2C.

Quantitative expression of viral transcripts in 293T-E1 cells and tumors

To have a better and more quantitative evaluation of the viral transcripts expressed under *in vitro* and *in vivo* conditions, we isolated total RNA from resting 293T-E1 cells and 293T-E1 tumors and performed RT-Q-PCR for *ORFK2*, *ORF73*, *ORF26* and *ORF50* (Fig. 3). Total RNA was extracted from tumors from three different tumor-bearing mice by three independent protocols. All samples were normalized using human GAPDH housekeeping gene expression. We analyzed the expression of latent (LANA [*ORF73*]) and lytic (viral IL-6 [*ORFK2*], minor capsid protein [*ORF26*] and RTA [*ORF50*]) genes to determine whether HHV-8 gene expression patterns differ between *in vitro*-cultured cells and in tumors. As seen in Fig. 3, expression of *ORFK2*, *ORF73*, *ORF26* and *ORF50* transcripts was similar under *in vitro* and *in vivo* conditions. K2, LANA, ORF26 or RTA transcripts were not detected in 293T control cells (data not shown). Considering that the relative amplification efficiency between primer sets has not been determined, quantitative comparison between transcripts (e.g. ORF50 versus ORF73) was not attempted.

Characterization of 293T and 293T-E1 tumor cells

After sacrifice, tumors were excised and analyzed. Tumors from 293T-E1 weighed, on average, three times more than tumors from 293T cells, a difference considered significant ($p < 0.02$) (Fig. 4A). Similarly, the mean volume of tumors excised from mice grafted with 293T-E1 cells was, on average, 3 times larger than that of tumors from mice injected with 293T cells ($p < 0.02$).

We also analyzed the cellular composition of 293T and 293T-E1 tumors. After excision of tumors, one part was fixed with 4% paraformaldehyde. The percentage of mouse and human cells in tumors was determined by Hoechst 33342 labeling and FACS analysis using the difference of DNA composition between these 2 species. We extracted cells from 5 different regions of the same tumor to determine whether tumors were homogenous. Our results indicate that tumors were 95% uniform (data not shown). We next compared the cellular composition of five 293T and five 293T-E1 tumors. As shown in Table 1, 293T tumors were composed of $45 \pm 5\%$ and $55 \pm 5\%$ mouse and human cells, respectively. 293T-E1 tumors were mainly composed of human cells ($73 \pm 2\%$), a result that differed significantly ($p < 0.02$) from that obtained for 293T tumors. Of the human cells present in the 293T-E1 tumors, most ($76 \pm 11\%$) were GFP⁺, indicating HHV-8-BAC retention under *in vivo* conditions. No GFP was detected in 293T tumors (data not shown).

These results were confirmed by immunohistochemistry using anti-GFP antibodies. As shown in Fig. 4B (last row), many cells from 293T-E1 were positive for GFP, suggesting that HHV-8 BAC was maintained. Background staining was observed for 293T cells. Tumor sections were also examined for expression of the LANA antigen. As expected, 293T control cells did not show any reactivity with the anti-LANA antibody (Fig. 4B, first row). LANA-positive cells were detected in tumors originating from 293T-E1 cells. Staining was nuclear, as expected, and a small number of cells expressed high levels of LANA (arrow). Tumors were also examined for lytic protein expression using anti-K8 and anti-vIL-6 antibodies. A few K8- and vIL-6 positive cells were detected in 293T-E1 tumors (Fig. 4B, second and third rows). For K8, the staining was nuclear, while anti-vIL-6 staining showed cytoplasmic immunoreactivity. No K8- or vIL-6-expressing cells were detected in 293T control cells.

Discussion

Studies of HHV-8 pathogenesis and oncogenic potential are hampered by the lack of animal models susceptible to HHV-8 infection. Cell-free transmission of HHV-8 infection into animals has had very limited success. In one study, the results suggest very limited replication in rhesus macaques without any symptoms related to HHV-8 infection [22]. Very recently, cell-free infection and replication of HHV-8 in NOD/SCID mice has been reported [18]. Such a model will certainly be useful for HHV-8-related immunological studies. However, the lack of clinical symptoms and/or development of HHV-8-related tumors in the macaque and the SCID mouse model limit the usefulness of these models for the study of HHV-8 pathogenesis. Other animal models that have been used to study HHV-8-related tumor development consisted of injecting PEL cells into immunodeficient animals. Subcutaneous or intraperitoneal injection of PEL cells into such mice leads to the development of solid tumors or ascites, respectively [5,19,25]. Treatment of PEL-cell-bearing mice with a combination of interferon- α and azidothymidine exemplify the usefulness of such a model for the testing of compounds against virally-induced lymphoproliferative diseases [26]. Recently, a mouse model for studying the HHV-8 latency was developed using long-term-infected telomerase-immortalized endothelial (TIVE) cells [1]. Two long-term HHV-8-infected TIVE cell lines were successfully obtained, which formed tumors when injected into nude mice.

The main goal of our study was to develop a small animal model that could be used to assess the relative contribution of HHV-8 genes within the context of the entire viral genome. We used 293T-E1 cells carrying the entire HHV-8 genome cloned within a bacterial artificial chromosome and compared their tumorigenic potential to that of parental 293T cells in order to determine whether, in this system, HHV-8 contributed to tumor cell formation and growth. Our results clearly show that mice injected subcutaneously with 293T cells carrying the HHV-8 genome developed tumors more frequently than those injected with 293T cells. In addition, the rate of growth of tumors was significantly higher for 293T-E1 than for control cells. In fact, by day 22, the mean tumor weight and volume of tumors from 293T-E1 cells was three times greater than that of 293T cells. The percentage of human cells in 293T-E1 tumors was significantly greater than that from 293T tumors. These results suggest that although a similar proliferation rate was observed *in vitro*, under *in vivo* conditions, HHV-8-infected cells can grow faster and form tumors more rapidly than 293T cells. In this model, the sex of the animals did not influence the rate of tumor growth (data not shown). These results clearly show that HHV-8 contributes to an increase in the tumorigenicity of 293T cells. This might have been anticipated considering the vast body of literature reporting on HHV-8-encoded oncogenes/growth factors and angiogenic factors. However, expression of genes driven by strong heterologous promoters might differ than what is observed in the context of the entire viral genome. Our data add to these existing models by demonstrating that expression of HHV-8 genes in the context of the entire genome leads to increased cell tumorigenicity. Experiments are currently underway to obtain a human endothelial cell line persistently infected with the

HHV-8 recombinant BAC. But as many investigators have experienced, this is a difficult task, and this explains, at least in part, why our initial efforts have focused on the use of the 293T cell line. Very recently, however, a published report has indicated the formation of KS-like tumors in nude mice following the transfection of the HHV-8 BAC into murine bone marrow endothelial-lineage (mECK36) cells [17]. The model presented by Mutlu et al. elegantly demonstrates the oncogenic properties of the HHV-8 BAC and an essential role for v-GPCR in angiogenesis and sarcomagenesis [17]. Unlike our 293T-E1 cells, which have comparable latent and lytic HHV-8 transcript levels under in vitro and in vivo conditions, mECK36 tumors express lytic genes much more than in vitro-cultured mECK36 cells. Whether this is related to the inability of mECK36 cells to generate infectious virions remains undetermined.

In conclusion, we have developed a small animal model that could prove useful for assessing the relative contribution of HHV-8 genes/proteins in tumor development. The impact of a deletion or mutation of HHV-8 genes on tumorigenesis can be monitored by injecting 293T cells carrying the mutated HHV-8 into SCID mice and analyzing tumor growth compared to control cells carrying WT HHV-8. Considering that many virally encoded proteins have redundant functions, the relative importance of individual proteins in pathogenesis should be addressed in the context of a mutant virus. The present animal model could also be adapted for the testing of potential anti-HHV-8 drugs, in a manner analogous to the model described using PEL cells [26]. The identification of viral determinants associated with pathogenesis represents the first step toward the development of appropriate therapeutics, and although not perfect, this animal model offers the possibility of addressing the relative contribution of selected HHV-8 proteins to pathogenesis, in relation to other virally-encoded HHV-8 proteins.

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Fig. 1.

293T-E1 cells carrying wild-type recombinant HHV-8. **(A)** 293T and 293T-E1 cells observed under bright field and fluorescent microscopy (magnification 40×). **(B)** In vitro proliferation rates of 293T and 293T-E1 cells. Cells were plated at 3.5×10^4 cells (in triplicate) in the wells of a 12-well plate. On days 1, 2, 3 and 4, cells were trypsinized and counted using a hemacytometer. Results are expressed as mean cell number $\pm 95\%$ CI as a function of time from two independent experiments. **(C–E)** Immunofluorescence assay on 293T and 293T-E1 cells. Acetone-fixed cells were incubated with rat-anti-LANA **(C)**, mouse anti-K1 **(D)** or rabbit anti-K2 **(E)** followed by incubation with Alexa 568-conjugated secondary antibody (*red*). Nuclei were stained with DAPI (*blue*). The localization of the proteins, relative to the nucleus, is presented in the merged pictures (magnification 60×)

Fig. 2.

293T and 293T-E1 tumor incidence, weight and volume following injection into SCID mice. CB17 female SCID mice ($n = 11/\text{group}$) were injected subcutaneously on both hind legs with 10^7 293T or 293T-E1 cells. **(A)** Mice were observed every 2 days for the appearance of tumors. Results are expressed as mean tumor incidence $\pm$ SD as a function of time. * $p < 0.005$. **(B)** Upon growth, tumor size was determined using a caliper 3 times per week until sacrifice. Tumor size $\geq 125 \text{ mm}^3$ was considered positive and included for statistics. Results are expressed as mean tumor size 395% CI. * $p < 0.0006$, ** $p < 0.0001$ as determined by Mann-Whitney test. **(C)** Representative tumors from mice injected with 293T and 293T-E1 upon sacrifice

Fig. 3.

RT-QPCR analysis of 293T-E1 in cells and tumors obtained from mice. Relative levels of ORFK2, ORF26, ORF73 and ORF50 transcripts in 293T-E1 cells were determined by extracting total RNA followed by RT-QPCR using specific primers. All samples were normalized using GAPDH mRNA levels. The results are expressed as mean gene expression $\pm$ SD from three independent samples. 293T-E1 tumors were analyzed at day of sacrifice. On day 22 after tumor cell injection, tumors were excised and total RNA extracted. Viral gene expression was determined by RT-QPCR. Human GAPDH mRNA levels were used for normalization. Representative results from one protocol (out of 3) are presented. Results are expressed as mean relative gene expression $\pm$ SD derived from at least three different tumors. * $p < 0.05$, ** $p < 0.02$ as determined by unpaired t -test with Welch correction

Fig. 4.

Characterization of 293T and 293T-E1 tumors. (A) 293T and 293T-E1 tumor weight and volume. Mean tumor weight $\pm 95\%$ CI and mean tumor volume $\pm 95\%$ CI upon sacrifice. $*p < 0.05$, $**p < 0.0005$. (B) Immunohistochemistry on tumors grown in SCID mice. Paraffin-embedded blocks of tissues (293T and 293T-E1 tumors) were cut into 3- μm sections and processed for IHC. Tissues were analyzed using rat anti-LANA, rabbit anti-K8, rabbit anti-vIL-6, or rabbit anti-GFP specific antibodies. Goat anti-rabbit-HRP or biotinylated donkey F (ab')₂ anti-mouse IgG/streptavidin peroxidase were used as secondary reagents. The proteins were made visible by a brown deposit. Tissues were counterstained with hematoxylin. Arrows indicate the strongly positive cells (magnification 60 $\times$)

Table 1

Percentage of mouse and human cells in 293T and 293T-E1 tumors by Hoechst analysis

	<i>n</i>	% Mouse cells	% Human cells
		Mean $\pm$ SEM	Mean $\pm$ SEM
293T tumors	5	45 $\pm$ 5 *	55 $\pm$ 5 *
293T-E1 tumors	5	27 $\pm$ 2	73 $\pm$ 2

* $p < 0.02$ relative to 293T-E1 cells as determined using Student's *t*-test.