The human fetal glial cell line SVG p12 contains infectious BK polyomavirus (BKPyV)

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Abstract

The human fetal glial cell line SVG was generated in 1985 by transfecting primary fetal brain cells with a plasmid containing an origin-defective mutant of simian virus 40 (SV40). The cells, which express SV40 large T-antigen, support the replication of human JC polyomavirus (JCPyV) and have been used for JCPyV studies but also for other studies where cells of neural origin were desirable. We intended to use the SVG p12 cells from ATCC for antiviral drug studies with JCPyV. However, during initial experiments, immunofluorescence microscopy controls unexpectedly revealed cells expressing the late viral proteins VP1, VP2/VP3 and agno. This was confirmed by western blotting. Since the agnoprotein antiserum is specific for BKPyV-agnoprotein, infection with BKPyV was suspected. Indeed specific BKPyV PCR of SVG p12 supernatants revealed a viral load of >1x10^{10} genomic equivalents/ml. Negative staining electron microscopy showed characteristic polyomavirus virions and infectious BKPyV was transmitted from SVG p12 supernatant to other cells. Long-range PCR covering the viral genome followed by DNA sequencing, identified BKPyV strain UT as well as deletion derivatives. This was confirmed by next generation sequencing. JCPyV (MAD-4) was found to infect apparently uninfected and BKPyV-infected SVG p12 cells. In total, 4 vials from 2 different ATCC lots of SVG p12 cells dating back to 2006 contained BKPyV, whereas the subclone SVG-A was negative. In conclusion, SVG p12 cells from ATCC contain infectious BKPyV. This may have affected results and interpretation of previous studies and caution should be taken in future experiments.
Importance

This work reveals that one of the most frequently used cell lines for JC polyomavirus (JCPyV) research, the SV40-immortalized human fetal glial cell line SVG obtained directly from ATCC, contains infectious BK polyomavirus (BKPyV) of UT strain and a spectrum of defective mutants. The UT strain has been previously found in urine and in tumours of different patients but is also frequently used for research. It is therefore not clear if BKPyV was present in the brain tissue used to generate the cell line or if this is a contamination. Although a productive JCPyV of SVG cells was not dependent on prior BKPyV infection, the unknown BKPyV infection may have influenced the results of studies performed in these cells. However, the frequently used subclone SVG-A did not contain BKPyV. The interpretation of past results should therefore be reconsidered and cells tested for BKPyV before new studies are initiated.
Introduction

The family of human polyomaviruses now includes 12 viruses which seem to at least partly coexist in the human host (1). The first identified and best studied human polyomaviruses are JC (JCPyV) and BK (BKPyV) (2, 3). These viruses independently infect most humans early in life and thereafter establish lifelong latent infections in the epithelial cells of the renalurinary tract, with occasional reactivation and shedding in urine (4, 5). Although BKPyV and JCPyV infections are usually benign, severe opportunistic diseases may occur in immunocompromised host.

JCPyV is the causative agent of progressive multifocal leucoencephalopathy (PML), mainly affecting HIV-positive/AIDS patients, individuals receiving immunomodulatory treatment against autoimmune diseases, such as multiple sclerosis and patients receiving immunosuppressive therapy after organ transplantation (6). BKPyV is the causative agent of polyomavirus-associated nephropathy (PyVAN) in kidney transplant patients and polyomavirus-associated haemorrhagic cystitis (PyVHC) in bone marrow transplant patients (7). Unfortunately, there are currently no effective antiviral drugs against polyomaviruses and survival is mainly dependent on recovery of polyomavirus specific immune function.

The viral structure, genome organization and replication of both JCPyV and BKPyV are closely related to the better studied monkey polyomavirus, simian virus 40 (SV 40). The circular double stranded DNA genome consists of about 5200 bp and is arranged in early viral gene region (EVGR) and late viral gene region (LVGR), separated by a non-coding control region (NCCR) containing the origin of replication, promoters and enhancer sequences. The EVGR encodes the regulatory proteins small tumor antigen (sTag) and large tumor antigen (LTag) (8). In addition JCPyV encodes the derivatives T'135, T'136 and T'165 (9) while BKPyV encodes TruncTag
(10). LTag plays a pivotal role in viral genome replication, transcription and virion assembly (11). Presumably LTag also optimizes the conditions for viral replication by interacting with p53 and pRb-family proteins, thus preventing growth arrest and apoptosis and facilitating expression of E2F-dependent growth-inducing genes, driving resting host cells into the cell cycle (11, 12). The LVGR encodes the non-structural agnoprotein and the viral capsid proteins 1, 2 and 3 (VP1-3) forming the icosahedral capsid.

Animal models to study JCPyV and BKPyV replication and disease have been missing. Only recently, mice with human thymus and lymphocytes were generated to study the JCPyV specific immune responses (13). During PyVAN and PyVHC, BKPyV replicates extensively in renal tubular epithelial cells and bladder epithelial cells, respectively (14). Primary cultures of these cells provide good model systems for in vitro studies of BKPyV replication (15-17). In addition, BKPyV can successfully replicate in a large variety of cells or cell lines. By contrast, JCPyV is more difficult to propagate and an authentic cellular model system is lacking (6). The main cause of PML pathology is JCPyV infection of oligodendrocytes, however, oligodendrocytes are difficult to culture unless they are immortalized. Astrocytes may possibly be infected via direct contact with internalized JCPyV-infected oligodendrocytes (18). Recently 293TT cells which constitutively express SV40 LTag were found to support replication of archetype JCPyV (19). These cells were originally derived from a human embryonic kidney but are probably of neuronal linage (20).

The SV40-immortalized glial cell line SVG, was originally developed as a model system facilitating research on JCPyV replication. The cell line was derived from human fetal glial cells immortalized with a plasmid conferring constitutive SV40 LTag expression (21, 22). The SV40-strain used contained a deletion in the origin of
replication which affected two of the three LTag binding sites thus preventing replication of the SV40 genome.

Based on the expression of glial fibrillary acidic protein (GFAP) and lack of the myelin marker galactocerebroside (GC), the cell line was originally characterized as an astrocyte cell line (21). However, the cells ability to increase expression of GFAP and to synthesize GC when the appropriate growth signals were present caused speculations that the cells were neural progenitor cells with the potential to differentiate (23). This speculation was supported by the apparent lack of estrogen receptor alpha (ERα) expression which is usually found in astrocytes (24, 25) and also by their undifferentiated epithelial phenotype when grown in minimal essential medium supplemented with 10% FBS (23).

The cell line denoted SVG p12, although the passage number is said to be unknown, has been provided by the ATCC since 1987. SVG p12, SVG and subclones of SVG have been used in several JCPyV studies (26-41) and in more than 30 other studies where cells of neural origin appeared desirable. Unfortunately, the source of the SVG cells was not clearly specified for some of these studies.

Here we report our unexpected finding that a subpopulation SVG p12 cells, obtained directly from ATCC, is productively infected with BKPyV. This stowaway-virus may have influenced research performed in these cells and should be considered in interpretation of past results and in future experiments.
Material and methods

Cells and virus

Human fetal glial (SVG p12) cells (ATCC CRL-8621, www.atcc.org) was cultured in Minimum Essential Medium Eagle (Sigma Aldrich M4655) containing 10% fetal bovine serum (FBS). African green monkey kidney epithelial (Vero) cells (ATCC CCL-81) was cultured Dulbecco’s Modified Eagle’s Medium (Sigma Aldrich D5671) with 10% FBS and 1x GlutaMAX-I (Invitrogen Cat# 35050). SV40 transformed African green monkey kidney fibroblast (COS-7) cells (ATCC CRL-1651) were cultured in Dulbecco’s Modified Eagle’s Medium with 5% FBS and 1x GlutaMAX-I. A subclone of SVG, SVG-A (42), was kindly provided by Dr. Walter Atwood, Brown University and cultured in Minimum Essential Medium Eagle (Sigma Aldrich M4655) containing 10% FBS.

Human Renal Proximal Tubular Epithelial cells (RPTECs) were acquired from ScienCell™, (www.sciencellonline.com) and cultured in Renal Epithelial Growth Medium (REGM) (Lonza, www.lonzabioscience.com) containing 0.5% FBS. All experiments were performed with RPTECs at passage 4.

Infectious JCPyV (MAD-4) (ATCC VR-1583) supernatants obtained from COS-7 cells and infectious BKPyV (Dunlop) (ATCC 45025) supernatants obtained from Vero cells were used for infection and as PCR controls.

Viral infection

Supernatants from SVG p12 cells were used to infect RPTECs and Vero cells. As a positive control BKPyV (Dunlop) supernatant was used. SVG p12 cells were infected with JCPyV (MAD-4) and SVG-A cells were infected with BKPyV (Dunlop). All infections were carried out for 2 h before surplus infectious units were removed and
cells were washed once with phosphate buffered saline (PBS) and complete medium was added.

**Immunofluorescence staining, microscopy and digital image processing**

Immunofluorescence staining was performed as previously described (43). The following primary antibodies were used: polyclonal rabbit antisera directed against SV40 VP1 (ab53977, Abcam), SV40 VP2 + VP3 (ab53983, Abcam), and BKPyV agnoprotein (44, 45), two mouse monoclonal antibodies directed against SV40 LTag (Pab 416, Abcam and Pab 419, Santa Cruz Biotechnology) and a mouse monoclonal antibody directed against JCPyV VP1 (ab34756, Abcam). While ab53977, ab53983, and Pab 416 all cross-react with BKPyV proteins, the agnoprotein antisera only recognizes BKPyV agnoprotein, ab34756 only recognizes JCPyV VP1 and Pab 419 only recognizes SV40 LTag. As secondary antibodies a combination of anti-mouse conjugated with Alexa Fluor 568 (1:500; Molecular Probes, www.lifetechnologies.com) and anti-rabbit conjugated with Alexa Fluor 488 (1:500; Molecular Probes) were used. Nuclei were labeled with DRAQ5 (Biostatus, www.biostatus.com). Images were captured using a Zeiss Axiovert 200 confocal microscope equipped with an LSM510-META confocal module using the LSM5 software version 3.2 (Carl Zeiss).

**Western blotting**

SVG p12 cells cultured for 4 days in a 25 cm² flask were lysed in 1.5 ml of radioimmunoprecipitation assay buffer (150 mM sodium chloride, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0), collected and stored at -70°C. Next, total protein was measured using the EZQ
Protein Quantitation Kit (Invitrogen) according to the manufacturer’s instructions using a microplate reader (Infinite F200 Pro, Tecan). Cell lysates containing 7.2 µg of total protein were separated by 4-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Invitrogen) and blotted onto a PVDF (polyvinylidene fluoride) membrane (LI-COR Biosciences, www.licor.com). The membrane was blocked with Odyssey™ blocking buffer (LI-COR Biosciences) and incubated with the following primary antibodies: polyclonal rabbit antisera directed against BKPyV VP1 (46) and BKPyV agnoprotein (44, 45) and two different monoclonal antibodies directed against SV40 LTag (Pab 416, Abcam, and Pab 419, Santa Cruz). In addition, a monoclonal mouse antibody directed against the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245; Abcam, www.abcam.com) was used. As secondary antibody, a combination of IRDye 800CW Goat anti-Rabbit (LI-COR Biosciences) and IRDye 680RD Goat anti-Mouse (LI-COR Biosciences) was used before detection with LI-COR Odyssey Infrared detection system.

Quantification of extracellular and intracellular BKPyV load

Extracellular BKPyV DNA load was determined by quantitative PCR (qPCR) of supernatants using primers and a probe targeting the BKPyV LTag gene (47). Supernatants were diluted in dH₂O (1:100) and boiled for 5 minutes. Five µl was used for a 25 µl PCR reaction. Intracellular BKPyV DNA load was determined by performing the same qPCR on DNA extracted from cells using the GenoM-48 platform (GenoVision) with MagAttract DNA mini M48 kit (Qiagen cat# 953336) or the QIAsymphony (Qiagen, www.qiagen.com).

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Negative staining electron microscopy

Supernatants collected from SVG p12 cells 4 days after seeding were ultracentrifuged in Beckmann ultra-clear tubes (40 ml) in a SW28 rotor at 28000 rpm for 90 minutes at 4°C. The supernatant was removed and the pellet dissolved in 1 ml dH₂O. As a positive control supernatants from Vero cells 3 weeks post infection with BKPyV Dunlop were processed in parallel. The pellet suspensions were fixed with 2% paraformaldehyde (PFA) and negative staining was performed. In short, 10 µl of virus suspension was deposited on carbon coated copper grids and air dried for 5 minutes before 10 µl of 3% uranyl acetate was added and a 15 minutes finale air drying was performed. The grids were examined in a JEOL JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV.

Long-range PCR

Supernatants from SVG p12 cells or from RPTECs inoculated with SVG p12 supernatant were harvested and prepared for long-range PCR as previously described for qPCR. Long-range PCR was performed with partly overlapping primers targeting the VP1-gene (adapted from (48) and a high fidelity Phusion polymerase (New England Biolabs M0530S) according to the manufacturer’s instructions. Briefly, a reaction volume of 50 µl was used, consisting of 10 µl 5x Phusion HF buffer, 1 µl dNTP mix (10 mM), 1 µl of each primer Eco-F and Eco-R (10 µM) (Table 1), 0.5 µl Phusion HF polymerase, 5 µl of the diluted supernatants and the remainder dH₂O. The cycling program started with initial denaturing at 98°C for 30 sec followed by 35 cycles of 98°C for 10 sec, 70°C for 30 sec and 72°C for 2.5 min and finishing with elongation at 72°C for 10 min. The annealing temperature of 70°C was set based on melting temperature calculations using the New England Biolabs Tm calculator.
Five µl of the PCR product was run on a 0.8% agarose gel at 110 V for 400 min and visualized with GelRed (Biotium) using the GelDoc XR gel imager (BioRad). The remaining 45 µl of the PCR product was purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel Gmbh) according to the manufacturer’s instructions, and stored at 4°C until sequencing.

Cloning of long-range PCR products

The long-range PCR products were prepared for TA cloning using the TOPO® XL PCR Cloning kit (Invitrogen K7030-20). A 3’ adenine overhang was created by adding 0.2 µl deoxyadenosine triphosphate and 0.5 µl Taq polymerase (both Sigma Aldrich) to 20 µl of the PCR product and incubating in a thermocycler at 72°C for 10 min. The PCR product was purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel Gmbh) and 4 µl of the purified PCR product was used for TA cloning according to the manufacturer’s instructions. Plasmids from 10 colonies were purified using PureLink® Quick Plasmid Miniprep Kit (Invitrogen) and the presence of inserts was confirmed by restriction enzyme digestion with EcoRI (New England Biolabs) followed by agarose gel electrophoresis.

Conventional DNA sequencing

Sanger sequencing of the whole genome of BKPyV was accomplished using 20 primers with binding sites dispersed along both strands of the genome of BKPyV (Table 1) while shorter inserts were sequenced using M13R and T7 primers (Table 1). The BigDye® v3.1 sequencing kit (Applied Biosystems) was used according to the manufacturer’s instructions. The products of the sequencing reactions were
analyzed by capillary electrophoresis at the DNA sequencing core facility at the University Hospital of Northern Norway. Sequences were analyzed using the Geneious software version 6.1.3 (Biomatters, www.geneious.com).

DNA preparation and rolling circle amplification

Supernatant from SVG p12 culture was centrifuged twice at 1000 rcf for 15 min. DNA was then extracted from 200 µl supernatant using the QiaSymphony SP and DNA mini kit (Qiagen, Hilden, Germany), generating 100 µl DNA extract with a BKPyV DNA load of 4.7x10^9 genomic equivalents (GEq)/ml. Rolling circle amplification was performed using 0.5 µl of the DNA and the TempliPhi 100 Amplification Kit (GE Healthcare Lifescience, Little Chalfont, UK), according to the instructions of the manufacturer. A non-template control was processed in parallel to ensure the specificity of the sequencing. The concentration of the amplified DNA was determined using the PicoGreen system (Invitrogen). An amount of 500 ng was processed further for the library preparation.

Next generation sequencing (NGS)

The TemphiPhi amplified DNA from the supernatant and the non-template control were fragmented by nebulization, using the Roche GS Titanium Rapid Library Preparation Kit (454 Life Science/Roche, Branford CT, USA) according to the manufacturer's instructions. The libraries were labelled with different multiplex identifiers (MID) and processed together. Briefly, the combined libraries were amplified by emulsion PCR and the clonally amplified DNA molecules were enriched and purified using the Roche GS Junior Titanium emPCR kit, following the
manufacturer's instructions. Finally, the enriched fragments were sequenced on the Roche GS Junior Instrument, using the Roche GS Junior Titanium sequencing kit.

NGS data analysis: mapping and *de novo* assembly

The reads of the SVG p12 library were analysed in groups of maximum 4000 reads using the Roche GS Reference mapper software (version 2.7) and the CLC Genomics Workbench for *de novo* assembly (version 6.0.3). The obtained contigs were compared to the NCBI sequence database using the BLAST algorithm.
Results

SVG p12 cells from ATCC are infected with BKPyV

The SVG p12 cells are transformed with a plasmid containing an origin deficient SV40 genome and all cells are therefore supposed to express SV40 LTag (21). As an initial characterization of the cells before JCPyV infections and antiviral studies, immunofluorescence staining was performed with two different monoclonal antibodies directed against SV40 LTag (Pab 416 and Pab 419) and two polyclonal antisera directed against the N- and C-terminal part of BKPyV LTag (81048 and 81178) (44) also known to cross-react with SV40 LTag. As expected, immunofluorescence microscopy revealed nuclear LTag expression in all cells and this was independent of the different LTag antibodies used (Figure 1A Pab 419 and Figure 1B, Pab 416, for 81048 and 81178 data not shown). The staining was very strong in some cells but weaker in others.

Considering the possibility that the origin-deficient plasmid originally used to transform the SVG p12 cell line could give rise to SV40 late proteins and to exclude nonspecific staining with other potentially useful antibodies, we included antibodies and antisera directed against SV40, JCPyV and BKPyV late proteins in our initial characterization. Indeed a small subpopulation of cells stained positive for VP1 (Figure 1A) as well as VP2/VP3 (results not shown). Even more unexpectedly, immunofluorescence with an anti-BKPyV agnoserum stained a subpopulation of the cells (Figure 1B). While the SV40 VP1 and VP2/VP3 antiserum are known to cross-react with BKPyV and JCPyV proteins and therefore did not identify the virus involved, the rabbit polyclonal agnoprotein antiserum is specific for BKPyV agnoprotein and, in our experience, does not cross-react with either SV40- or JCPyV agnoprotein (S. Sørensen, B. N. Sharma and C.H. Rinaldo, unpublished data). The
positive agnoprotein staining therefore suggested the presence of BKPyV in the SVG p12 cells.

In order to confirm the expression of BKPyV agnoprotein and VP1, and at the same time confirm the expression of SV40 LTag, western blotting was performed on SVG p12 cell extracts. As a positive control for LTag expression, cell extracts from COS-7 were included while cell extracts from Vero cells were used as a negative control for LTag expression and BKPyV infection. The results confirmed that SVG p12 expressed BKPyV agnoprotein (~7kDa) as well as VP1 (40 kDa) and as anticipated LTag (80 kDa) (Figure 1C). As expected COS-7 cells only expressed LTag whereas uninfected Vero cells did not express any of the targeted PyV proteins (Figure 1C).

To independently address the presence of BKPyV DNA, supernatants from SVG p12 cells were analysed 4 days post-seeding by a BKPyV specific quantitative real-time PCR (qPCR). A viral DNA load of >1x10^{10} Geq/ml was found, demonstrating the presence BKPyV DNA in the supernatant.

To exclude the possibility that the cells or assays had been contaminated in the lab in Tromsø, a new vial of SVG p12 cells with a different lot number was ordered from ATCC and tested directly in Basel. Again BKPyV proteins were detected by immunofluorescence staining and extracellular BKPyV DNA load was found by qPCR (results not shown). According to the data sheet, the first lot of SVG p12 cells had been cryopreserved at ATCC in 2006 while the new lot was cryopreserved in 2010. Therefore, a third order of the second lot was processed directly for DNA extraction and BKPyV qPCR in the accredited diagnostic laboratory in Basel and a forth order of the same lot was processed directly for DNA extraction and BKPyV qPCR in the accredited diagnostic laboratory in Tromsø. The results showed that both vials contained more than 1 x 10^{10} Geq/ml BKPyV, further confirming that the SVG p12
cells stored at ATCC were infected with BKPyV. Apparently BKPyV has been present in these cells since 2006 or earlier.

**SVG p12 cultures produce infectious BKPyV**

Given the high BKPyV genome viral load in supernatants from SVG p12 cells, we next investigated whether or not viral particles were released. Supernatants from SVG p12 cells were harvested at 4 days post seeding, enriched by ultracentrifugation and used for negative staining electron microscopy. As a positive control, a supernatant from BKPyV (Dunlop) infected Vero cells was used. The negative staining revealed numerous viral particles with a diameter of about 40-50 nm and small surface projections characteristic of polyomavirus capsomers (Figure 2A). Similar viral particles were found in supernatants from BKPyV infected Vero cells (result not shown).

To investigate whether the virus released from the SVG p12 cell line consisted of infectious BKPyV, SVG p12 cell supernatants were used to infect RPTECs and Vero cells. Vero cells are known to be semi-permissive while RPTECs are highly susceptible for BKPyV (15, 49). Cells were fixed at 3 days post infection (dpi) and immunofluorescence staining performed with primary antibodies directed against BKPyV agnoprotein and SV40 LTag. Microscopy clearly demonstrated infection of RPTECs (Figure 2B) expressing only LTag or LTag and agnoprotein. Similar results were obtained for Vero cells (data not shown). The results demonstrate that SVG p12 cells were infected by BKPyV and produced infectious BKPyV capable of initiating infection in RPTECs and Vero cells.
SVG p12 cultures contain a mixture of complete and defective BKPyV UT genomes

To determine genome characteristics of the BKPyV strain infecting the SVG p12 cell line, the NCCR was amplified by PCR and sequenced. The cells were found to contain BKPyV of the UT strain. The NCCR of the UT strain was first time detected by PCR of urines of patients in North Norway in 1990 but then denoted TU (50) (GenBank M34049). In 2005 the complete genome of a virus with an identical NCCR, apparently an isolate from the urine of a patient in the United States with a solid tumor, was submitted to the GenBank and named UT strain (GenBank DQ305492) (51). The UT NCCR lacks a full R-block and contains a partly duplicated P-block, Q-block and R-block. Compared to the GenBank sequences only one nucleotide was missing in the O-block (nt 127).

To characterize the full-length genome, a long-range PCR using a high fidelity polymerase was performed on SVG p12 cell supernatants. As a positive control a supernatant from BKPyV Dunlop infected RPTECs was used. Agarose gel electrophoresis revealed several PCR products with sizes around 1200 bp, considerably lower than the expected ~5000 bp large BKPyV genome (Figure 3A, 4 days).

We reasoned that the full genome was not well amplified due to low copy numbers. In order to increase the likelihood of detecting full length genomes, the SVG p12 cells were seeded and maintained in culture for 58 days and the long-range PCR was repeated. This time a weak band corresponding to the whole genome was obtained, again together with several other lower sized bands making the PCR product unsuitable for whole genome cloning (Figure 3A, 58 days).
We therefore hypothesized that infection of more permissive cells would favor the production of virus and thereby enrich for the complete BKPyV genome. A supernatant from SVG p12 cells was therefore passaged twice on RPTECs, each time for 6 days, before supernatant was harvested and long-range PCR performed. A supernatant from BKPyV (Dunlop) infected RPTECs was used as a positive control. This time, agarose gel electrophoresis revealed one strong band with the expected size of the BKPyV genome (Figure 3B). The complete PCR product was DNA sequenced by the Sanger method using 20 primers producing overlapping sequences. The sequencing revealed that the 5189 bp genome amplified from SVG p12 supernatants was almost identical with the BKPyV UT genome (GenBank DQ305492) with the exception of the previously mentioned nucleotide missing in the O-block (nt 127) and a point mutation in the intergenic region between agnoprotein and VP2 (nt 629 A>G transition).

We also investigated the identity of the lower sized bands detected by long-range PCR directly on supernatants from SVG p12 cells. To reduce the chance that this was nonspecific amplification of cellular DNA or partly degraded free viral DNA released from dead cells, DNase-treatment of the cell supernatant was performed prior to long-range PCR. However, the approach failed to remove the lower sized bands thereby suggesting that the DNA was encapsidated or otherwise protected (data not shown). A TA-cloning of the heterogeneous PCR product was therefore performed. PCR of 10 colonies using primers in the TA vector revealed inserts ranging from approximately 300 bp to 2000 bp. The inserts were sequenced with the Sanger method and all were found to contain BKPyV DNA in fragments ranging from 100 bp to about 900 bp (Figure 3C). Three of the 10 clones also contained part of or the complete previously identified UT NCCR. The subgenomic fragments covered
less than 50% of the BKPyV UT genome and included different parts of the genome. This suggested a population of highly fragmented genomes.

To further investigate the presence of BKPyV genome fragments in the SVG p12 cell supernatant we also performed two separate PCRs targeting the EVGR and a large part of the LVGR (**Figure 3D**) (primers and PCR conditions in Table 1). Agarose gel electrophoresis of the PCR products revealed several bands migrating faster than the strong and distinct bands in the positive control (**Figure 3D**). Since these results were obtained with different primers than used for long-range PCR they supported our finding of highly fragmented BKPyV genomes in SVG p12 cell supernatants.

In order to validate these results in an independent manner, next generation sequencing (NGS) was performed after rolling-circle amplification of DNA extracted from SVG p12 supernatants. A total of 70 818 reads was obtained, including 22 610 for the SVG p12 library and 43 855 for the non-template control library. The median read length was 496 nucleotides (range 40 to 733) with an average quality score of 37.05 (standard deviation 7.79). None of the reads of the non-template control library could be matched to the BKPyV reference, excluding contaminating DNA. The de novo assembly of the SVG p12 reads yielded a contig of 5182 bp (1753 reads, average depth 113.38), with 8 mismatches to the BKPyV UT strain (GenBank DQ305492) (**Figure 4A**). All mismatches were located in homopolymer stretches, a common artifact of pyrosequencing. Interestingly the de novo assembly also resulted in several contigs containing deletions in the VP2-VP3-VP1 region, ranging from nucleotide 720 to 1990 (**Figure 4A and 4B**). A total of 10 350 reads (45.3%) of the SVG p12 library could be mapped to the BKPyV UT genome. Of those, 85 reads contained both ends of the deletion confirming that this extended from residue 726 to residue 1990. Moreover, the depth of the NGS reads was strongly reduced in the
agno-VP2-VP3-VP1 region compared to the rest of the genome suggesting that variants with deletion in this area were dominant (Figure 4A).

Thus, SVG p12 cells seemed to be productively infected by BKPyV UT but also produced circular subgenomic DNA fragments that seemed to be encapsidated. Passage of infectious units to RPTECs appeared to select for full-length genomes suggesting that virions with fragmented genomes are not infectious or are less fit than BKPyV UT, at least in RPTECs.

SVG p12 cells with and without BKPyV late protein expression can be infected by JCPyV

Since SVG p12 cells have been used in at least two published JCPyV studies (32, 39) it cannot be excluded that these studies were unknowingly performed with BKPyV present. In order to find out if JCPyV infection in SVG p12 requires co-infection with BKPyV, SVG p12 cells were infected with JCPyV (MAD-4). The cells were fixed 4 dpi and JCPyV and BKPyV infected cells were identified by immunofluorescence staining using a combination of a monoclonal antibody specific for JCPyV VP1 and the polyclonal BKPyV agnoprotein antiserum specific for BKPyV infected cells. Microscopy revealed that, overall, a low number of SVG p12 cells were infected with either of viruses (Figure 5A). However, some cells were clearly co-infected with JCPyV and BKPyV (Figure 5B).

Thus, SVG p12 cells seem to be able to support the JCPyV lifecycle in parallel to and independently of detectable BKPyV infection. However, co-infections of cells with JCPyV and BKPyV do also occur.
The SVG-A cell line, a frequently used subclone of SVG cells, does not contain BKPyV.

Based on these results, the question arose whether or not the SVG-A cell line established by limiting dilution assay of SVG cells (42) and commonly used for JCPyV studies (29-31, 33, 37, 41, 52-55) was also harboring BKPyV. A supernatant harvested from SVG-A cells at 4 days post seeding was subjected to BKPyV qPCR. Intracellular DNA was also extracted and subjected to BKPyV qPCR. In addition, immunofluorescence staining with several late protein antibodies was performed. The supernatant and intracellular DNA were both negative for BKPyV DNA and the cells only expressed SV40 LTag (Figure 6A). Since SVG-A cells were found to be negative for BKPyV infection, we investigated whether the SVG-A cells represented a subpopulation of SVG with resistance to BKPyV infection. Therefore SVG-A cells were seeded and infected by BKPyV (Dunlop). At 3 dpi cells were fixed and immunofluorescence staining was performed with polyclonal BKPyV agnoprotein antiserum. Microscopy revealed numerous BKPyV infected cells (Figure 6B). Thus, SVG-A cells do not contain BKPyV but are permissive for BKPyV infection.
Discussion

The SVG p12 cell line is one of few human cell lines available for propagation of JCPyV (6, 34). As such, a number of previous JCPyV studies including drug efficacy studies (27, 36) as well as production of antigen for antibody detection assays (26) have utilized SVG or subclones of these cells. Moreover, SVG cells have been used in other settings where human glial cells are relevant, such as investigation of cerebral HIV replication (56).

We report here that the SVG p12 cell line as obtained directly from ATCC is productively infected with BKPyV. This infection, which has been undetected until now, may have influenced critical results of previous studies and must be taken into consideration in future studies utilizing these cells.

The lines of evidence supporting the presence of infectious BKPyV in the SVG p12 cell line are as follows: Firstly, immunofluorescent staining of SVG p12 cells with antiserum directed against BKPyV agnoprotein, previously shown not to cross-react with SV40 or JCPyV agnoprotein, showed the characteristic cytoplasmic staining pattern seen in BKPyV infected cells (45, 57). This result was confirmed in a separate laboratory and by western blot. Secondly, a high viral load was measured by qPCR targeting BKPyV specific sequences within LTag in SVG p12 supernatants and directly in a vial from ATCC upon arrival in the laboratory. These results were also confirmed in a second laboratory. In total, 4 vials from two different lots tested positive. Thirdly, the cells produced viral particles that were indistinguishable from the positive control by electron microscopy. Fourthly, the virus was infectious as demonstrated by immunofluorescent staining of RPTECs, the natural host cells for BKPyV, and Vero cells following exposure to SVG p12 supernatants. Finally, full genome sequencing identified BKPyV in two separate laboratories using different
methods for both initial amplification and sequencing reactions. Both laboratories converged on the same sequence, that of the UT strain of BKPyV. Sequencing also showed a spectrum of defective mutants, some of which were DNAse protected ie likely encapsidated, characteristics both consistent with a cell culture origin of the viral DNA rather than typical PCR contamination. Based on these findings, we feel confident that a subpopulation of SVG p12 from ATCC is indeed productively infected with BKPyV UT strain.

While both NGS and conventional sequencing of the cloned long-range PCR product indicated that several variants with deletions in the coding region coexisted in the SVG p12 cell supernatant, only NGS detected the apparent dominant variants. These variants had an approximately 1260 bp deletion in the VP2-VP3-VP1 region which encompassed the target sequences used for long-range PCR prior to conventional sequencing (Figure 3C and Figure 4B). This result illustrates the advantages of using an unbiased amplification protocol. In addition, 4 distinct deletion mutants were identified by long-range PCR and conventional sequencing (Figure 3C). Moreover, PCR of the EVGR and LVGR followed by gel electrophoresis showed a spectrum of different product sizes (Figure 3D) confirming that SVG p12 cells produced a wide range of fragmented BKPyV genomes. Cloning and sequencing of more than 10 colonies from the long-range PCR would probably have confirmed this.

We cannot fully exclude that some of the fragmented genomes found are due to amplification artifacts caused for instance by generation of secondary structures during the amplification prior to pyrosequencing (58) or from the rolling circle amplification (59). However, the observation of deletion mutants by different methods supports the notion of fragmented genomes.
It is unlikely that BKPyV carrying deletions removing more than 25% of the genome (Figure 3C and Figure 4B) would be independently infectious. Indeed, some of the fragmented genomes are also expected to be replication incompetent due to deletion of the origin of replication. This notion was indirectly substantiated in our work, as only full length BKPyV UT replicated to high levels when the virus was passaged twice in RPTECs. In the context of the chronically infected LTag producing SVG p12 cells, the defective viruses were probably supplied with the missing viral proteins in trans by coexisting and replicating complete virus. This phenomenon has previously been demonstrated for BKPyV (60) as well as for JCPyV (61, 62). It is also supported by our DNase treatment data which suggest that the defective genomes were encapsidated. Perhaps generation of defective genomes was driven by constitutive SV40 LTag expression as this protein is known to cause replicative stress and mitotic dysfunction leading to both structural and numerical chromosome instability (63).

Whatever the mechanism, generation of defective mutants in polyomavirus cell culture has previously been observed and seems to depend on both host and viral factors (64, 65). Although the spectrum of defective mutants suggests that the infection is longstanding, it is no definitive proof.

When did BKPyV first enter the SVG p12 cell line? Was BKPyV already present in the fetal brain tissue when the SVG cell line was established in 1985 or did it enter later as a contamination of the cell line? There have been some reports of BKPyV in the CNS of adults (66-70) and one group has reported finding BKPyV DNA in fetal brains (71). It is more likely, however, that BKPyV entered as a contamination of the cell line. Unfortunately NCCR and full genome sequencing shed little light on the origin of the contaminating virus, as BKPyV UT appears to be a relatively common variant, having been found in patient samples both in North Norway in 1990 (50) and
in the USA in 2005 (51). Of note, this strain has also been used in several research labs (10, 46, 72-74). Although we cannot pinpoint the infection temporally, we can be certain that BKPyV has been present in the cells at least since 2006 based on ATCC records.

A comprehensive account of the possible implications for studies conducted in SVG cells is beyond the scope of this article. The studies running the greatest risk of erroneous results are those involving the closely related JCPyV. As shown here overt BKPyV infection is not needed for JCPyV to infect and replicate in SVG p12 cells. For JCPyV replication the most important characteristic of these cells is probably the high level of SV40 LTag expression. SV40 LTag has been previously shown to support JCPyV DNA replication (75). However, we did also find JCPyV and BKPyV coinfected cells and BKPyV may have influenced the JCPyV replication in these cells. Also infection of neighboring cells with BKPyV may have indirectly influenced JCPyV replication. Moreover, the high homology of the JCPyV and BKPyV genomes and their antigenic similarity which is between 63 to 83% for the different proteins (76), may have led to false interpretation of viral protein and DNA levels. More unsettling is the use of viral antigens purified from SVG cells for diagnostic purposes (26). If the SVG cells used in this study were contaminated with BKPyV it is highly likely that the purified viral particles contained a mixture of BKPyV and JCPyV antigens. Diagnostic tests based on such antigens would exhibit cross-reactivity to JCPyV- and BKPyV-reactive sera. Clearly this would affect the antibody seroprevalence which significantly differs between BKPyV and JCPyV.

That only a subpopulation of the SVG p12 cells expresses BKPyV proteins may suggests that the SVG p12 cell line is heterogeneous. This is supported by a recent study showing that SVG cells give rise to clonal cell lines with different phenotypes.
We therefore speculate that subcloning of SVG p12 cells might give rise to uncontaminated cell lines. Importantly we found that the SVG-A subclone was not infected by BKPyV and the research performed in these cells therefore seems to be cleared from suspicion. It is not completely clear to us when this subclone was generated and the details on how this was performed but given their permissivity for JCPyV, the SVG-A cells may be a useful substitute for SVG p12 cells.

In conclusion, a subpo population of SVG p12 cells from ATCC, has been productively infected with BKPyV UT and virions with fragmented genomes at least since 2006. This may have affected previous studies in unknown ways. It is therefore crucial that investigators that have used these cells examine their cells for BKPyV and if needed re-examine their results carefully. Naturally this must also be considered in all future experiments with these cells.

Acknowledgement

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Figure legends

Figure 1. The SVG p12 cell line expresses BKPyV late proteins.
SVG p12 cells were fixed 4 days post seeding and indirect immunofluorescence staining was performed using different combinations of primary antibodies; A) SV40 VP1 rabbit polyclonal antiserum (green, Alexa 488) with the SV40 specific LTag mouse monoclonal antibody, Pab419 (red, Alexa 568), and B) BKPyV agnoprotein rabbit polyclonal antiserum (green, Alexa 488) with SV40 LTag mouse monoclonal antibody, Pab416 (red, Alexa 568). The DNA (nucleus) was stained with DRAQ5 (blue) and both images were acquired by confocal microscopy with a 40 x objective.
C) Western blot of cell lysate (7.2 μg protein/lane) from SVG p12, COS-7 and Vero cells 4 days post seeding. The membrane was labelled with SV40 LTag mouse monoclonal antibody, Pab 416, BKPyV VP1 rabbit polyclonal antiserum, BKPyV agnoprotein rabbit polyclonal antiserum and anti-GAPDH mouse monoclonal antibody. M= molecular weight marker; MagicMark™ XP western standard (Invitrogen).

Figure 2. The SVG p12 cell line produces infectious BKPyV.
A) Electron microscopy of negatively stained viral particles from supernatant harvested from SVG p12 cells 4 days post seeding.
B) Immunofluorescence staining of RPTECs 3 days post exposure to supernatant harvested from SVG p12 cells 4 days post seeding. Indirect immunofluorescence staining was performed using a combination of BKPyV agnoprotein rabbit polyclonal antiserum (green, Alexa 488) and SV40 LTag mouse monoclonal antibody, Pab 416.
The DNA (nucleus) was stained with DRAQ5 (blue). Images were acquired by confocal microscopy with a 40 x objective.

**Figure 3. The BKPyV DNA isolated from the SVG p12 cell line consisted of a mixture of complete and defective genomes.**

A) PCR products generated from long-range PCR of SVG p12 supernatants 4 days post seeding and 58 days post seeding were separated on a 0.8% agarose gel. A supernatant harvested from BKPyV-infected RPTECs 3 days post infection was included as a positive control.

B) PCR products generated from long-range PCR of RPTEC supernatant after infection with a SVG p12 supernatant (the virus was passaged twice in RPTECs). A supernatant harvested from BKPyV-infected RPTECs 3 days post infection was included as a positive control.

C) Defective genomes detected by long-range PCR followed by TA cloning and Sanger sequencing. The color code to the different regions of the genome: NCCR = Blue bar, LVGR = Green bar, Intergenic region = Gray bar, EVGR= Yellow bar, Deletions = Thin line. The star indicates the binding sites of the partly overlapping primers used for long-range PCR.

D) Schematic display of the BKPyV UT genome with the different reading frames annotated and with the PCR amplified regions marked in red and subsequent PCR products from EVGR and LVGR respectively, separated on a 1% agarose gel. PCR product from the BKPyV Dunlop plasmid served as a positive control. M= 1 Kb Plus DNA Ladder (Invitrogen).
Figure 4. The BKPyV DNA isolated from the SVG p12 cell line consisted of a mixture of complete and defective genomes as detected by NGS.

A) Coverage per nucleotide of the 454 reads when aligned to the BKV UT genome. The positions of the dominant deletion, as well as the BKPyV open reading frames are indicated.

B) Defective genomes detected by NGS. Number of reads are indicated next to the defective genomes and the average coverage is given in parentheses. The color code to the different regions of the genome: NCCR = Blue bar, LVGR = Green bar, Intergenic region = Gray bar, EVGR = Yellow bar, Deletions = Thin line. The star indicates the binding sites of the partly overlapping primers used for long-range PCR.

Figure 5. JCPyV can infect SVG p12 cells both with and without BKPyV late protein expression.

SVG p12 cells were infected with JCPyV and indirect immunofluorescence staining performed 4 days post infection using a combination of the BKPyV specific agnoprotein rabbit polyclonal antiserum (green, Alexa 488) and the JCPyV specific JCPyV VP1 mouse monoclonal antibody (red, Alexa 568). The DNA (nucleus) was stained with DRAQ5 (blue). Images were acquired by confocal microscopy with a 40x objective.

A) Cells infected with either BKPyV or JCPyV

B) Cell co-infected with BKPyV and JCPyV
Figure 6. SVG-A cells do not express BKPyV late proteins inherently but are permissive for BKPyV infection.

Indirect immunofluorescence staining of SVG-A cells was performed using a combination of BKPyV agnoprotein rabbit polyclonal antiserum (green, Alexa 488) and SV40 LTag mouse monoclonal antibody, Pab416 (red, Alexa 568). The DNA (nucleus) was stained with DRAQ5 (blue). Images were acquired by confocal microscopy with a 20x objective.

A) 3 days post seeding

B) 3 days post infection with BKPyV Dunlop.
References


Schweighardt B, Atwood WJ. 2001. HIV type 1 infection of human astrocytes is restricted by inefficient viral entry. AIDS research and human retroviruses 17:1133-1142.


Table 1: Primers and PCR conditions

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