Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 237 (2016) 166–173

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Generation and Characterization of *Eptesicus fuscus* (Big brown bat) kidney cell lines immortalized using the *Myotis polyomavirus* large T-antigen

Arinjay Banerjee[a], Noreen Rapin[a], Megan Miller[b], Philip Griebel[c], Yan Zhou[c], Vincent Munster[b], Vikram Misra[a,*]

[a] *Department of Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada*
[b] *Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT, USA*
[c] *Vaccine and Infectious Disease Organization – International Vaccine Center (VIDO-InterVac), University of Saskatchewan, Saskatoon, Saskatchewan, Canada*

Article history:
Received 11 May 2016
Received in revised form 6 September 2016
Accepted 7 September 2016
Available online 14 September 2016

Keywords:
Big brown bat
Kidney
Cell-line
MERS-CoV
PED-CoV
VSV
HSV

A B S T R A C T

It is speculated that bats are important reservoir hosts for numerous viruses, with 27 viral families reportedly detected in bats. Majority of these viruses have not been isolated and there is little information regarding their biology in bats. Establishing a well-characterized bat cell line supporting the replication of bat-borne viruses would facilitate the analysis of virus-host interactions in an *in vitro* model. Currently, few bat cell lines have been developed and only Tb1-Lu, derived from *Tadarida brasiliensis* is commercially available. Here we describe a method to establish and immortalize big brown bat (*Eptesicus fuscus*) kidney (Efk3) cells using the *Myotis polyomavirus* T-antigen. Subclones of this cell line expressed both epithelial and fibroblast markers to varying extents. Cell clones expressed interferon beta in response to poly(I:C) stimulation and supported the replication of four different viruses, namely, vesicular stomatitis virus (VSV), porcine epidemic diarrhea coronavirus (PED-CoV), Middle-East respiratory syndrome coronavirus (MERS-CoV) and herpes simplex virus (HSV). To our knowledge, this is the first bat cell line from a northern latitude insectivorous bat developed using a novel technology. The cell line has the potential to be used for isolation of bat viruses and for studying virus-bat interactions in culture.

1. Introduction

It is speculated that bats are an important reservoir host for several viruses, such as Ebola virus (family *Filoviridae*, genus *Ebolavirus*), Marburg virus (family *Filoviridae*, genus *Marburgvirus*), severe acute respiratory syndrome coronavirus (SARS-CoV; family *Coronaviridae*, subfamily *Coronavirinae,* genus *Betacoronavirus*), Middle-East respiratory syndrome coronavirus (MERS-CoV; family *Coronaviridae*, subfamily *Coronavirinae,* genus *Betacoronavirus*), porcine epidemic diarrhea coronavirus (PED-CoV; family *Coronaviridae*, subfamily *Coronavirinae,* genus *Alphacoronavirus*) and Hendra and Nipah viruses (family *Paramyxoviridae*, genus *Henipavirus*). There is evidence that many of these viruses have been transmitted from bats to other hosts where they caused serious disease (Drexler et al., 2012; Changula et al., 2014; Wacharapluesadee et al., 2015). Over 200 different viruses from 27 families have been detected in bats [reviewed in (Moratelli and Calisher, 2015)] but most of these viruses have yet to be isolated and there is scant information regarding the biology of these viruses in bats.

Bats are genetically diverse and are found dispersed across much of the planet. With over 1200 species, bats display major differences in their behavior, feeding habits and the viruses they harbor [reviewed in (Moratelli and Calisher, 2015)]. Very little is known, however, about bat immune responses and if these differ across genera and species. Currently a single bat cell line (Tb1-Lu, ATCC number CCL-88, derived from the lung of *Tadarida brasiliensis*) is available through the American Type Culture Collection. Research groups have established other bat cell lines, from fruit and insectivorous bats using established techniques such as using the *SV40* T-antigen and expressing human telomerase reverse transcriptase (hTERT), but these are not commercially available yet (Crameri et al., 2009; Jordan et al., 2012; Maruyama et al., 2014).

* Corresponding author.
E-mail address: vikram.misra@usask.ca (V. Misra).

http://dx.doi.org/10.1016/j.jviromet.2016.09.008
0166-0934/

Bats are the only mammals capable of true flight and as such they may have unique physiological adaptations. For example, they display unique strategies for neutralizing the DNA-damaging by-products of oxidative metabolism produced as a result of increased metabolic activity (Shen et al., 2010). Zhang et al. hypothesize that bats have evolved and accumulated genetic changes as a result of their adaptation to flight. This is to limit collateral damage caused by by-products of an elevated metabolic rate (Zhang et al., 2013). These genetic changes may be important in the expansion and contraction of important gene families, including genes involved in the innate response pathway (Zhang et al., 2013).

North American bat species are at risk of drastic population depletion due to white-nose syndrome (Knudsen et al., 2013; Alves et al., 2014) and conducting terminal *in vivo* experiments might not be entirely possible in future. Establishing stable bat cell lines would provide an alternative for conducting *in vitro* host-pathogen studies. Experiments using cultured bat cells could provide useful preliminary information on bat innate immune defense responses, virus-cell interactions and cellular physiology.

There are several established methods for immortalizing primary cells. The first involves the introduction and stable expression of genes coding for the Simian virus 40 (*SV40*) large T antigen (SV40Tag). The large T antigen binds to and attenuates the tumor suppressor protein p53 and proteins of the retinoblastoma tumor suppressor family (pRb, p130 and p107). This promotes DNA replication and cell division. This method has been used to immortalize cells from a number of species including human (Mayne et al., 1986), rabbit (Scott et al., 1986) and rat (Lechardeur et al., 1995).

The second method involves the introduction and stable expression of the catalytic subunit of the human telomerase reverse transcriptase (hTERT). Ectopic expression of hTERT has been successfully used to immortalize primary cells in a range of mammalian species such as goat mammary epithelial cells (He et al., 2009) and canine Schwann cells (Techangamsuwan et al., 2009). This enzyme subunit prevents the shortening of telomeres with repeated cell divisions and thus prevents cellular senescence.

Here we describe a method for establishing and characterizing a kidney cell line (Efk3) from *Eptesicus fuscus* (the N. American Big brown bat) using the *Myotis polyomavirus* T antigen (MyPVTag). We characterized the capacity of MyPVTag to enhance DNA replication in Vero cells and found that it significantly increased their DNA content. We then transfected MyPVTag into primary bat kidney cells and sub-cloned several cell lines. We characterized the lineage of these clones and tested their expression of the interferon beta (IFN beta) gene in response to polyinosinic-polycytidylic acid (poly(I:C)) stimulation. We further tested three cloned kidney cell lines for their ability to support the replication of viruses from the families *Coronaviridae*, *Herpesviridae* and *Rhabdoviridae*. The parental cell line and clones were capable of expressing IFN beta and supported the replication of viruses such as vesicular stomatitis virus (VSV; family *Rhabdoviridae*, genus *Vesiculovirus*), herpes simplex virus (HSV; family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Herpesvirus*), PED-CoV and MERS-CoV. PED-CoV and MERS-CoV are viruses for which transmission from bats, either directly or via an intermediate reservoir, has resulted in high mortality in pigs (Lee, 2015) and humans (Coleman and Frieman, 2014), respectively. VSV and HSV are members of viral families that have previously been detected in bat species [reviewed in (Moratelli and Calisher, 2015)]. Although *E. fuscus* primary embryonic cells have been described before (Qian et al., 2013), to our knowledge, this is the first cell line established from a northern latitude insectivorous bat that was transformed by using a viral element (MyPVTag) selected from a known bat virus. Furthermore, the established kidney cell lines were able to support the replication of selected viruses from three different virus families.

2. Materials and methods

2.1. Ethics statement

All procedures related to the handling and euthanasia of bats were submitted to and approved by the Committee on Animal Care and Supply of the University of Saskatchewan Animal Research Ethics Board (protocol #20090036) and were in accordance with regulations approved by the Canadian Council on Animal Care.

2.2. Cell culture

A moribund male *E. fuscus* bat submitted to the laboratory was humanely euthanized. Brain, liver, lungs, spleen and kidney were harvested. Each organ was finely minced, and incubated at room temperature in 0.5% trypsin-EDTA (Gibco, USA) with agitation. Periodically cells were recovered after neutralizing trypsin with fetal bovine serum (FBS; Seradigm, USA) added to 5%. Cells were resuspended in Dulbecco's Minimal Essential Medium (DMEM; Corning, USA) containing penicillin (Gibco, USA), streptomycin (Gibco, USA) and amphotericin B (Sigma, USA), placed in 75 cm^2 flasks (Cellstar, Germany) and incubated at 37 °C in an atmosphere of 5% CO_2. Only kidney cells grew to form a monolayer. These cells were recovered by trypsinization, diluted 1/3 and re-plated. Cell samples at various passages were cryopreserved in DMEM containing 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (EMD Chemicals, USA).

Bat kidney cells were immortalized by using ViaFect (Promega, USA) to transfect cells with either 2.5 μg of pcDNA3 (Invitrogen, USA) empty vector or plasmids expressing either *SV40* large T-antigen (SV40Tag) or *Myotis polyomavirus* large T-antigen (MyPVTag). Transfected cells were cultivated in DMEM containing 10% FBS and Geneticin reagent (InvivoGen, USA). Only cells transfected with MyPVTag continued to replicate. Cells were confirmed to be *E. fuscus* cells by amplifying and sequencing a segment of mitochondrial cytochrome b transcripts (Parson et al., 2000). MRC5 cells (ATCC CCL-171) were cultured in MEM medium (Corning, USA) supplemented with 10% FBS (Seradigm USA), 1/100 non-essential amino acids (NEAA; Gibco), 1/100 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco) and 1/1000 gentamycin (Gibco, USA). Vero cells (Elaine Van Moorlehem, Vaccine and Infectious Disease Organization – International Vaccine Center (VIDO-InterVac)) were cultured in DMEM with Glutagro (Corning, USA) supplemented with 10% FBS (Seradigm, USA) and penicillin/streptomycin. All cell lines were checked and controlled for mycoplasma by a semi-nested PCR (described below).

2.3. Chromosome spread

Efk–3B cells were seeded at a concentration of 5×10^5 in a T-75 flask. The cells were grown up to 80% confluency and treated with 0.1 μg/ml Colcemid (Roche, USA) as mentioned previously (Howe et al., 2014). The cells were processed, spread on slides and chromosomes were stained with Giemsa staining solution as mentioned previously (Howe et al., 2014).

2.4. Plasmid cloning

The MyPVTag was amplified from the *Myotis polyomavirus* whole genome (National Centre for Biotechnology Information (NCBI), Accession number NC_011310.1) cloned in a TOPO vector (Invitrogen, USA) and sub-cloned into a pcDNA3 (Invitrogen, USA) backbone. SV40Tag (a generous gift from Ivan Sadowski, University of British Columbia) was also sub-cloned in pcDNA3. The pcDNA3 plasmids encoding the T-antigens were used for transfection studies.

2.5. Flow cytometry

To quantify DNA in T-antigen (T-ag) transfected Vero cells, the cells were seeded at a concentration of 5×10^5 cells/well in 6-well plates. The cells were transfected with 2.5 μg of either SV40Tag (SV40Tag in pcDNA3), MyPVTag (MyPVTag in pcDNA3) or pcDNA3 using Lipofectamine 2000 (ThermoFisher Scientific, USA). The cells were harvested and prepared for flow cytometry twenty-four hours after transfection. Briefly, cells were harvested and re-suspended in Dulbecco's Phosphate-Buffered Saline (DPBS) (ThermoFisher Scientific, USA). Cells were fixed in 70% ice-cold ethanol for 30 mins and stained for the respective T-antigens using 0.8 μg/ml mouse anti-SV40Tag (cross-reactive for SV40Tag and MyPVTag) (Molecular Probes, USA). The secondary antibody cocktail contained 0.01 mg/ml propidium iodide (Molecular Probes), 0.2 mg/ml RNase A (Sigma, USA), 4.0 μg/ml goat anti-mouse immunoglobulin-Alexa488 conjugate (Molecular Probes) and 0.1% Triton X-100 (Sigma, USA) in DPBS. The cells were filtered through 64 μm nylon mesh prior to analyses. For analyzing the cell lineage of the clones, intracellular staining using the commercial BD fixative (BD Biosciences, USA) was carried out following the manufacturer's recommendation. Murine monoclonal antibodies against vimentin (1/200 dilution of Monoclonal anti-vimentin, clone VIM 13.2, mouse ascites fluid IgM) (Sigma-Aldrich, USA) and cytokeratin were used (1/200 dilution of monoclonal anti-cytokeratin 8.13, clone K8.13, mouse ascites fluid IgG2a isotype) (Sigma, USA). Secondary antibodies used were 0.625 μg/ml goat anti-mouse IgM (μ)-FITC conjugate (Caltag/Invitrogen, USA) and 0.25 μg/ml goat anti-mouse IgG2a-FITC conjugate (Caltag/Invitrogen, USA). Cells were analysed using FacsCalibur (BD Biosciences, USA) with forward scatter detection using a photodiode with 488/10 nm bandpass filter and side scatter detection PMT with Brewster-angle beam splitter. FITC was detected with a 488 nm laser and 530/30 nm band pass filter. For each sample 50,000 events were accumulated and analyzed with CellQuest Pro (BD Biosciences, USA).

2.6. Nucleic acid extraction, PCR and qRT-PCR

All RNA extractions were performed using the RNeasy Plus Mini kit (QIAGEN, Germany) as per manufacturer's instructions. cDNA was prepared using the QuantiTect Reverse Transcription kit (QIAGEN) as per manufacturer's instructions. One μg of RNA was used for cDNA preparation. cDNA was used as a template for the quantification of target genes. Conventional PCR (polymerase chain reaction) to determine the cell lineage of the clones was performed using primers specific for *E. fuscus* vimentin (BBB–Vimentin–F–TCAAGAATACCCGCACCAACG and BBB-Vimentin–R– ACTGCTGACGGACGTCGCGC) and cytokeratin (BBB-Cytoker–F– GAAGACCTACAAGGTGTCCAC and BBB-Cytoker–R–CCATCTCGGGTCTCAATCTTC). Primers were designed using the annotated *E. fuscus* genome on NCBI (Accession No. vimentin – XM_008148829.1; cytokeratin – XM_008140727.1). After initial denaturation for 3 min at 94 °C, the remaining 35 PCR cycles were at 94 °C/30s, 60 °C/30 s and 72 °C/1 min. The final extension was at 72 °C for 10 min.

Conventional PCR for the detection and identification of *E. fuscus* cytochrome B was performed using primers CytB US – CCCCHCCHCAYATYAARCCMGARTGATA and CytB DS – TCRACDGGNTGYCCTCCDATTCATGTTA. After initial denaturation for 3 min at 94 °C, the remaining 35 PCR cycles were at 94 °C/30s, 55 °C/30 s and 72 °C/1 min. The final extension was at 72 °C for 10 min.

Semi-nested PCR using primers specific to the 16 s rRNA gene of mollicutes was performed for the detection of mycoplasma in cell lines. Primers were designed as mentioned previously (Kong et al., 2001; Yoshida et al., 2003). Briefly, primers My-1-ACGGCCCADACTYCTACGGRAGGCAGCAGTA and My-2-CCRTGCACCAYTTGTCWHHHBGWWAACCTC were used for the first PCR. After initial denaturation for 3 min at 94 °C, the remaining 40 PCR cycles were at 94 °C/30s, 64 °C/30 s and 72 °C/1 min. The final extension was at 72 °C for 10 min. Primers My-2 and My-3-GTAATACATAGCTCGCAAGCGTTATC were used for the second PCR. After initial denaturation for 3 min at 94 °C, the remaining 35 PCR cycles were at 94 °C/30s, 60 °C/30 s and 72 °C/1 min. The final extension was at 72 °C for 10 min.

For IFN beta quantification, qPCR assays targeting the IFN beta transcripts and the normalizer (GAPDH, Glyceraldehyde-3-phosphate) were performed for the clones. Stratagene's MX3005P PCR (Stratagene, USA) cycler was used in conjunction with Quantifast SYBR Green PCR kit (QIAGEN). Primers used were Interferon beta (BBB_IFNbeta-F-GCTCCGATTCCGACAGAGAAGCA and BBB_IFNbeta-R-ATGCATGACCACCATGGCTTC) and GAPDH (BBB_GAPDH-F-GGAGCGAGATCCCGCCAACAT and BBB_GAPDH-R-GGGAGTTGTCATACTTGTCATGG). Primers were designed using the annotated *E. fuscus* genome (NCBI, Accession No. Interferon beta: XM_008145044.1 and GAPDH: XM_008144826.1). Samples were prepared as previously mentioned (Rapin et al., 2014). The products were quantified based on the amount of relative IFN beta expression. Briefly, cells were either transfected with 750 ng/ml poly(I:C) (InvivoGen, USA) using Lipofectamine 2000 (ThermoFisher Scientific, USA) or mock transfected. For quantifying PED-CoV transcripts, primers were designed to amplify the PED-CoV nucleocapsid (N) gene (GenBank accession number KF272920), (PEDV-s GCAACAACAGGTCCAGATCTC) and (PEDV-r CTCCACGACCCTGGTTATTTC). For qPCR, after the initial denaturation step of 95 °C for 10 min, the remaining 40 cycles were at 95 °C/30 s, 55 °C/1 min and 72 °C/1 min. The absorbance reading was taken after the 55 °C step. Relative fold change in gene expression between the two groups of cells was calculated and plotted after normalizing the Ct values for IFN beta using GAPDH. Three housekeeping genes were tested (GAPDH, beta-actin and beta-2-microglobulin) and none showed variation between treated and mock treated samples. Thus GAPDH was used for normalizing the data. Difference of one Ct indicates a two-fold difference in gene expression.

PCR and qRT-PCR products were confirmed on a gel and sequenced (Macrogen, South Korea). Reaction efficiencies for qRT-PCR primers were calculated to be between 95 and 105%.

2.7. Cell division

Total number of viable cells was determined by using a hemocytometer to count viable cells by trypan blue exclusion method. Cells were cultivated in 6-well plates, trypsinized and re-suspended in media at every time point.

2.8. Virus replication

Efk3 parental cell line and three subclones were inoculated with VSV-Indiana strain (Dr. Ellis' lab at the University of Saskatchewan), HSV, PED-CoV (Dr. Zhou's lab at Vaccine and Infectious Disease Organization – International Vaccine Center (VIDO-InterVac)), and MERS-CoV (strain EMC/2012, Dr. Fouchier at Erasmus Medical Center in the Netherlands). For VSV inoculations, WST_{50} concentration of the virus was used to infect the cells, which were seeded in 12 well plates at a concentration of 2×10^5 cells/well. WST_{50} was determined as that dilution of virus that produced 50% cell death as measured using the WST-1 assay. WST-1 assay is similar to the MTT assay (Heldt et al., 2006). Briefly, cells were seeded in 96 well plates at a density of 1×10^4/well and analyzed with the WST assay at the indicated time points. 10 μl WST-1 reagent (Roche, USA) was added to each well and incubated at 37 °C for 1 h. Colour developed was measured at 450 nm with a reference wavelength of

650 nm using Molecular Devices Vmax spectrophotometer. Intensity of the colour developed is directly proportional to the number of viable cells in the wells. Cells cultured in 12-well plates were inoculated with 100 μl virus (VSV) for 1hr, rinsed with sterile PBS and medium replaced with DMEM containing 10% FBS. Cells and the supernatant were harvested at 4 and 48 h post infection and frozen at −80 °C. After freeze-thawing the supernatant and cells three times, virus from Efk3 parental cell line and the subclones was titrated in Vero cells using the WST_{50} assay. Virus was quantified using the WST_{50} assay and formula as described by Heldt et al. (Heldt et al., 2006). For HSV titration, MRC5, Efk1B, Efk2F and Efk3 B subclones were seeded in triplicates at a concentration of 2.5×10^5 cells/well in 6 well plates. The cells were inoculated with HSV at a multiplicity of infection (MOI) of 1. Viral inoculum (0.5 ml) was replaced with complete medium and plates frozen at 0 and 24 h post inoculation. Plates were freeze-thawed 3x, transferred to 15 ml tubes and centrifuged at 2000 rpm for 5 min. Supernatant was collected and serially diluted 1:10 down to 10^{-6} and titrated in Vero cells (1×10^5 cells/well in 24-well plates). For quantifying the virus, 100ul of diluted virus was added to the wells and incubated for an hour. The inoculum was replaced with 0.5 ml of DMEM + 1% pooled human serum (MP Biomedical Cellect). Plaques were counted under the microscope 5 days later.

Multistep replication kinetics were determined by inoculating wells of cells in triplicate with MERS-CoV (strain EMC/2012) with a MOI of 0.01, 50% tissue culture infectious dose ($TCID_{50}$) per cell. One hour after inoculation, cells were washed once with DMEM and culture medium replaced. Culture supernatants were sampled at 0, 24, 48, 72, 96 and 120 h after inoculation. MERS-CoV was titrated by end-point titration performed in quadruplicate using Vero E6 cells cultured in DMEM supplemented with 2% fetal calf serum, 1 mM L-glutamine (Lonza, USA), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were inoculated with ten-fold serial dilutions of virus, and scored for cytopathic effect 5 days later. The $TCID_{50}$ was calculated by the method of Spearman-Karber (Hamilton et al., 1977).

For PED-CoV infection, cells were seeded in 24-well plate at a density of 1.2×10^5/well and cultured overnight. Cells were inoculated with PED-CoV at an MOI of 1. Cells were harvested and total RNA extracted at 0, 24, 48, 72 and 96 h. Virus replication was quantified by qRT-PCR.

2.9. Statistics

Significance between T-ag data was calculated by Mann Whitney *U* test for two independent samples using IBM SPSS (Version 21).

3. Results

3.1. Characterizing the MyPVTag (Myotis polyomavirus large-T antigen)

The *SV40* large T-antigen is well characterized and is known to enhance DNA replication in cells (An et al., 2012) and immortalize primary cells (Crameri et al., 2009). Our laboratory has previously detected a novel polyomavirus in *M. lucifugus* (Misra et al., 2009). To determine if the *Myotis polyomavirus* T-ag shared the ability of its *SV40* homologue to induce DNA replication, we transfected Vero cells with plasmids encoding genes for the two T-antigens. We then confirmed that cells expressed T antigen by immunostaining and compared the DNA content of T antigen expressing cells with cells transfected with the pcDNA null plasmid. Fig. 1 shows cells expressing *Myotis polyomavirus* and *SV40*T antigen contained more DNA than cells transfected with pcDNA plasmid. SV40Tag expressing Vero cells showed the highest increase in DNA content.

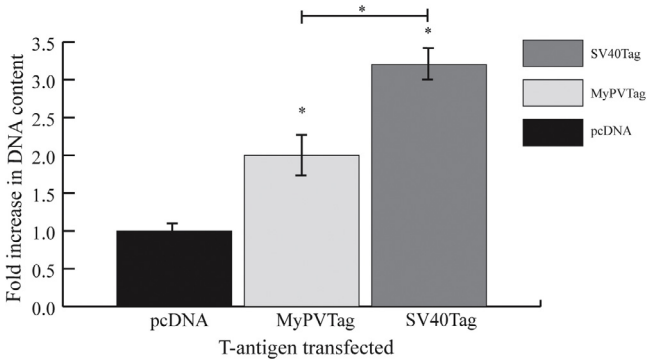

Fig. 1. Transfection with *SV40* and MyPV T-antigens increases DNA content in cells. Vero cells were transfected with plasmids expressing either SV40Tag, MyPVTag or empty vector (pcDNA). Twenty-four hr after transfection, cells were immunestained for cytoplasmic T-antigen and with propidium iodide to quantify DNA. The DNA content of T-antigen and pcDNA transfected cells was determined by flow cytometry and expressed as the fold increase in DNA content relative to pcDNA transfected cells. The ratio for cells transfected with pcDNA was taken as '1'. Experiments were done in triplicate and mean values plotted. Error bars represent standard deviation. Statistical difference was calculated using Mann-Whitney *U* test for two independent samples. * < 0.05.

3.2. Establishing and immortalizing E. fuscus kidney cells

We attempted to immortalize primary cells derived from the kidney of *E. fuscus* by transfection with plasmids expressing SV40Tag, MyPVTag or empty vector (pcDNA). We observed clusters of cells in cultures transfected with either T-ag but after several passages, only the MyPVTag transfected primary cells (Efk) continued to replicate. The immortalized Efk cell line was cloned by limiting dilution to generate three clones (Efk1, 2 and 3). 8 clones were further isolated by end point dilution of Efk1, Efk2 and Efk3 cells. We established the clones as separate cell lines and characterized representative clones from each of the three clones i.e. Efk1, Efk2 and Efk3, along with the parental Efk3 cells for their cell type markers, interferon beta response, virus susceptibility and cell division rates. We determined the number of chromosomes in Efk–3 B to rule out the possibility of chromosome number abnormality in immortalized cells (Supplementary Fig. 1). Efk–3 B had 2n = 50 chromosomes, which is normal for genus *Eptesicus* (Fedyk and Ruprecht, 1983).

3.3. Lineage of the Efk clones

We screened the clones for cytokeratin, a lineage-specific marker for epithelial cells (Xie et al., 2015) and vimentin, a lineage marker for fibroblasts (Zschemisch et al., 2014). Since specific antibodies are not available for bat cytokeratin and vimentin, we used antibodies specific to the human proteins. No positive staining of the bat clones was observed with either anti-vimentin or anti-cytokeratin antibodies when cells were analyzed with flow cytometry (data not shown). We then screened the cell lines for expression of vimentin and cytokeratin transcript by conventional PCR. Five of the eight clones analyzed contained detectable transcripts for both cytokeratin and vimentin. In contrast, only vimentin transcripts were detected in primary Efk cells at passage 8 (Table 1).

3.4. Interferon beta production

We characterized the Efk clones for their capacity to respond to polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of double-stranded RNA (Mian et al., 2013), through analysis of

Table 1
Transcripts for cell lineage markers vimentin (fibroblast) and cytokeratin (epithelial) are expressed by the Efk clones. The ability of the cells to respond to poly(I:C) treatment with increased IFN beta gene expression was detected by qRT-PCR. Along with the clones, a parental cell line (Efk3) and primary kidney cells (Efk; not transfected with either T-ag) were compared. + = PCR product detected, – = no PCR product detected.

Clone	Marker type – Cytokeratin	Marker type – Vimentin	IFN beta fold increase
1H	–	+	6,517.03
2A	–	+	33,225.42
2B	+	+	18,432.95
1A	+	+	39,786.73
1B	–	+	61,572.56
1E	+	+	6,746.85
2F	+	+	42,938.97
3B	+	+	7,912.95
Efk3 (uncloned)	–	+	10,155.68
Efk (primary cells)	–	+	60,100

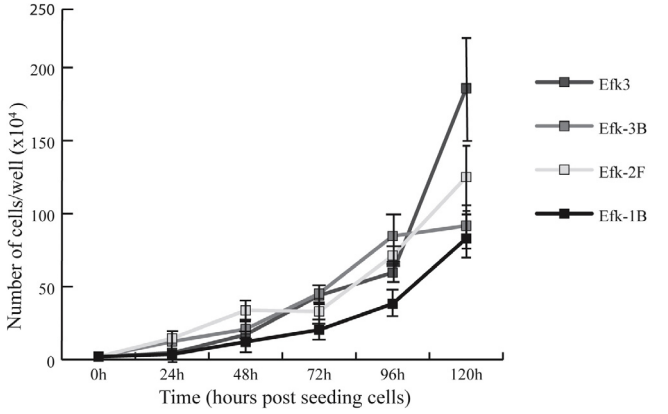

Fig. 2. Efk3 and Efk clones multiplication curve. The cell division curve for the three clones and Efk3 parental cell line was determined by counting viable cells at the indicated time-points.

interferon beta transcription. Poly(I:C)-treated primary Efk cells displayed an average of 60,000-fold increase in interferon beta transcripts when compared to mock transfected cells. All clones displayed increased interferon beta transcription following poly(I:C) treatment, with the level of increase ranging from six thousand to over sixty thousand. (Table 1).

3.5. Cell multiplication rates

All clones displayed similar multiplication rates when assayed with the WST-1 reagent (data not shown). We determined the cell division rates of three subclones (1B, 2F and 3B) and the parental Efk3 cell line by counting viable cells using a hemocytometer at various time points after seeding (Fig. 2). The three clones and parental cell line (Efk3) did not differ from one another in their multiplication rates.

3.6. Efk cells competence for virus infection

We evaluated the Efk3 parental cell line and three clones (1B, 2F and 3B) for their competence in supporting VSV, HSV, PED-CoV and MERS-CoV replication. As a positive control, Vero cells were infected with MERS-CoV and PED-CoV and MRC5 cells were infected with HSV and VSV. VSV caused rounding, sloughing and detachment of MRC5 cells in culture, PED-CoV caused syncytia in Vero cells and MERS-CoV caused rounding and sloughing off of Vero cells. Cytopathic effects (CPE) such as plaques or rounding and sloughing off of cells following infection with VSV and MERS-CoV was observed with all Efk clones. Two of the three clones responded to PED-CoV infection with cytopathology. In contrast, Efk-2F did not exhibit noticeable CPE 24 h.p.i. with PED-CoV (Fig. 3). The positive control cell lines exhibited CPE 24 h.p.i. following infection with the respective viruses.

4. Discussion

The evolutionary pressures of flight are thought to have conferred upon bats unique physiological adaptations. Most viruses that have been transmitted from bats to other species have been studied in animal models of human disease or in cell lines of non-bat origin. In addition, most *in vitro* studies of mammalian innate immune and anti-viral responses have been performed in human and rodent cell lines. The results from these studies may not accurately represent pathogen-host interactions that occur in bats. Establishing bat cell lines enable researchers to study relevant virus-host interactions in a system that more closely resembles the reservoir host. Cell lines have been established from fruit bats (Crameri et al., 2009; Virtue et al., 2011), *Myotis myotis* (He et al., 2014), *Tadarida brasiliensis* and other insectivorous bats (Maruyama et al., 2014). Primary embryonic cells have been developed from *E. fuscus* (Qian et al., 2013) but an immortalized *E. fuscus* cell line capable of supporting the replication of viruses from three diverse viral families is not commercially available yet.

Historically, cells have been immortalized by either using the large T-antigen from *SV40*, which is a monkey virus or by the ectopic expression of hTERT. We characterized the MyPVTag and SV40Tag's ability to enhance DNA replication in cells. Both MyPVTag and the SV40Tag significantly increased DNA content in Vero cells. *SV40* T-ag is known to enhance DNA content in cells (Ohkubo et al., 1994; Ahuja et al., 2005) and interestingly the bat polyomavirus T-antigen shared similar properties. *Myotis polyomavirus* belongs to the same family as *SV40*. Large T-ags from both these viruses were transfected to immortalize the *E. fuscus* kidney cells, but only cells expressing MyPVTag gave rise to stable cell lines. We do not know why SV40Tag failed to immortalize bat cells. However, it might be possible that the expression of MyPVTag, derived from a virus found in this bat, could have been favored by the cellular machinery over the SV40Tag derived from *SV40*, which has not been detected in big brown bats yet.

Clones 2B, 1A, 1E, 2F, 3B expressed transcripts for lineage-specific proteins of both epithelial and fibroblast cells and Clones 1H, 2A and 1B expressed mRNA for vimentin. Since primary bats cells at passage 8 had detectable transcript for vimentin alone, it is possible that the immortalization procedure may have altered transcription in some of the clones. There is evidence, however, that tumor cells can co-express both epithelial and fibroblast markers (Viale et al., 1988). It is also possible that MyPVTag immortalized a mixture of both epithelial and fibroblast cells and the epithelial cells had a replication advantage during the process of limiting dilution cloning. When cells were being passaged, some cells were more strongly adhered to the plastic and were not removed by trypsiniza-

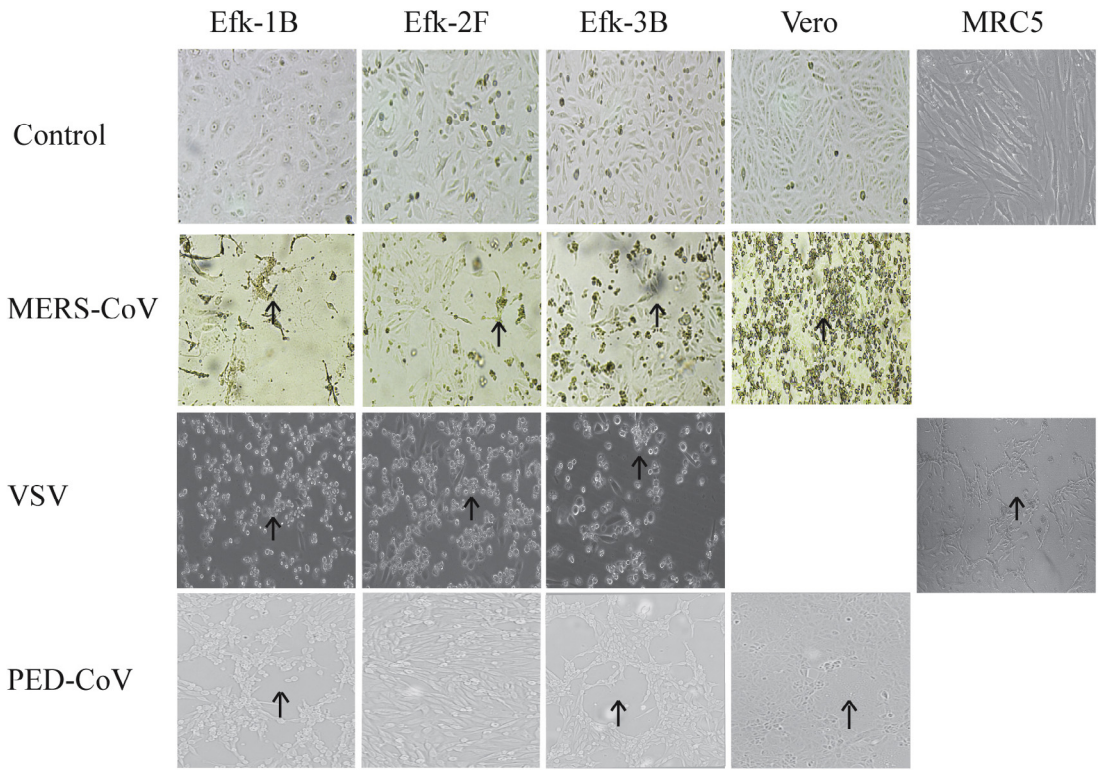

Fig. 3. CPE observed in Efk cells. CPE observed following infection with MERS-CoV, VSV and PED-CoV is indicated by arrows.

tion. This process could have selected for particular cell types based on their adherent properties.

Relatively little is known about the innate immune response of insectivorous bats to viral infection. We were able to generate an insectivorous bat cell line capable of upregulating IFN beta gene expression in response to a known innate immune stimulus. An early interferon response is known to inhibit replication of some viruses (Katze et al., 2002). Interferon response in terms of interferon beta transcript upregulation by bat cell lines, mostly cell lines from fruit bats, has been demonstrated before (Hagmaier et al., 2007; Crameri et al., 2009; Biesold et al., 2011; Virtue et al., 2011). We analyzed the capacity of the Efk clones to respond to poly(I:C) stimulation by monitoring interferon beta gene expression. This synthetic analogue of dsRNA is usually used as a pathogen-associated molecular pattern (PAMP) to stimulate interferon responses (Crameri et al., 2009). The clones responded to poly(I:C), as we observed a several thousand-fold increase in interferon beta transcript when compared to mock treated cells. Immortalization of cells is sometimes known to compromise the ability of the cells to transcribe the interferon beta gene (Biesold et al., 2011). The Efk clones, however, retained a remarkable but variable capacity to respond to poly(I:C).

West Nile virus, Eptesipox virus, novel group I coronavirus and American bat vesiculovirus are examples of viruses that have been detected in *Eptesicus fuscus* (Donaldson et al., 2010; Moratelli and Calisher 2015). The ability of this cell line to support the replication of viruses from three different viral families demonstrates the potential application of this cell line to isolate and study viruses in bats that have previously only been detected using PCR and sequencing. Further work can be done to identify receptors specific to these viruses. VSV, HSV, PED-CoV and MERS-CoV grew to varying levels in the Efk clones. Clone 2F did not exhibit any visual cytopathology on infection with PED-CoV although virus replication was detected by qRT-PCR. This could be due to a lower level of virus replication in 2F (Fig. 4D) as the images (Fig. 3) were taken 48 h post-infection. At this point we do not know if 2F mounts a more robust interferon response or if it lacks other factors required for PED-CoV replication during the initial 48 h post-infection.

In conclusion, we established a stable kidney cell line from a northern latitude bat, which has the capacity to respond to a known innate immune stimulus with transcription of IFN beta. Furthermore, this cell line supported the replication of viruses from three virus families known to be harbored by bats. Not much is known about innate immune responses in bats and how they are activated during viral infections. Establishing well-characterized cell lines from relevant bat reservoir species is the first step in addressing the many questions that researchers have about innate immunity in bats (Baker et al., 2013; Moratelli and Calisher 2015). This cell line will help us better understand the bat innate responses and how they may contribute to the absence of overt disease symptoms when bats are infected with these viruses.

Authors' note

The *E. fuscus* kidney cell line developed here is now available for research through Kerafast, USA. The goal of this study was to generate a cell line that would allow researchers to potentially study viruses derived from bats in a reservoir *in vitro* model.

Acknowledgements

We thank Nathalie Berube and Shirley Hauta from VIDO-InterVac for their technical support. Funding for the research was obtained from a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant awarded to Vikram Misra, Integrated Training Program in Infectious Disease, Food Safety and Public Policy funded by NSERC/CREATE and the Department of Veterinary Microbiology, University of Saskatchewan. Vincent Munster and Megan Miller were supported by the Intramural

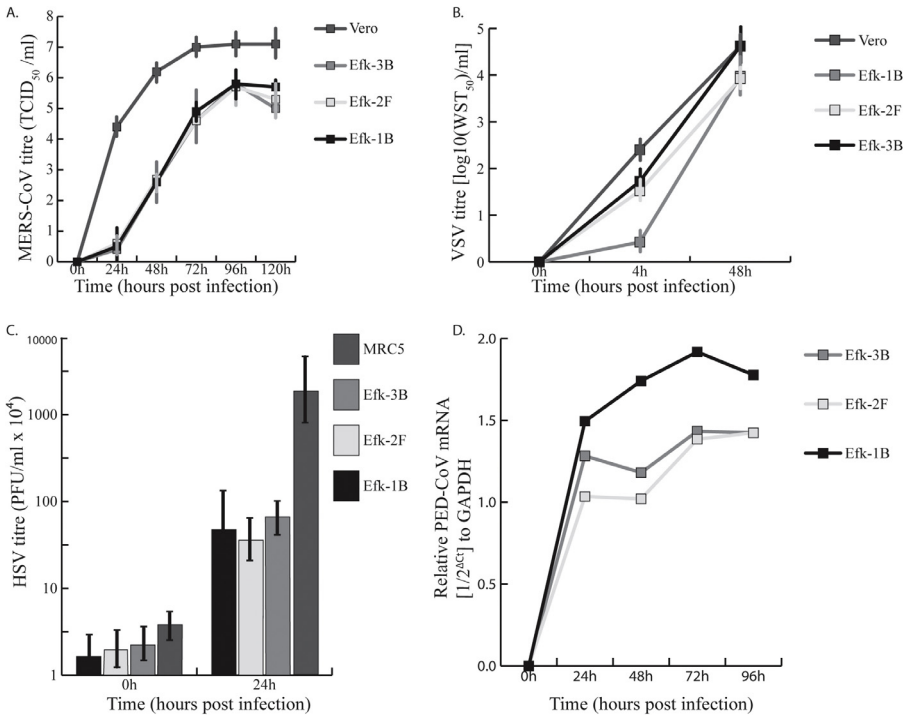

Fig. 4. Efk clones support replication of viruses. The amount of virus (or viral nucleic acid) produced by Efk cell clones was measured by determining $\log_{10}$ $TCID_{50}$ (MERS-CoV Fig. 4A), WST_{50} (VSV, Fig. 4B), plaque assay (HSV, Fig. 4C) and qRT-PCR (PED-CoV, Fig. 4D). The data confirm that the viruses replicated in the Efk cell lines as well as the relevant positive control cell lines (Vero, MRC5).

Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2016.09.008.

References

Ahuja, D., Saenz-Robles, M.T., Pipas, J.M., 2005. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. Oncogene 24 (52), 7729–7745.

Alves, D.M., Terribile, L.C., Brito, D., 2014. The potential impact of white-nose syndrome on the conservation status of north american bats. PLoS One 9 (9), e107395.

An, P., Saenz Robles, M.T., Pipas, J.M., 2012. Large T antigens of polyomaviruses: amazing molecular machines. Annu. Rev. Microbiol. 66, 213–236.

Baker, M.L., Schountz, T., Wang, L.F., 2013. Antiviral immune responses of bats: a review. Zoonoses Public Health 60 (1), 104–116.

Biesold, S.E., Ritz, D., Gloza-Rausch, F., Wollny, R., Drexler, J.F., Corman, V.M., Kalko, E.K., Oppong, S., Drosten, C., Muller, M.A., 2011. Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat Eidolon helvum. PLoS One 6 (11), e28131.

Changula, K., Kajihara, M., Mweene, A.S., Takada, A., 2014. Ebola and Marburg virus diseases in Africa: increased risk of outbreaks in previously unaffected areas? Microbiol. Immunol. 58 (9), 483–491.

Coleman, C.M., Frieman, M.B., 2014. Coronaviruses: important emerging human pathogens. J. Virol. 88 (10), 5209–5212.

Crameri, G., Todd, S., Grimley, S., McEachern, J.A., Marsh, G.A., Smith, C., Tachedjian, M., De Jong, C., Virtue, E.R., Yu, M., Bulach, D., Liu, J.P., Michalski, W.P., Middleton, D., Field, H.E., Wang, L.F., 2009. Establishment, immortalisation and characterisation of pteropid bat cell lines. PLoS One 4 (12), e8266.

Donaldson, E.F., Haskew, A.N., Gates, J.E., Huynh, J., Moore, C.J., Frieman, M.B., 2010. Metagenomic analysis of the viromes of three North American bat species: viral diversity among different bat species that share a common habitat. J. Virol. 84 (24), 13004–13018.

Drexler, J.F., Corman, V.M., Muller, M.A., Maganga, G.D., Vallo, P., Binger, T., Gloza-Rausch, F., Cottontail, V.M., Rasche, A., Yordanov, S., Seebens, A., Knornschild, M., Oppong, S., Adu Sarkodie, Y., Pongombo, C., Lukashev, A.N., Schmidt-Chanasit, J., Stocker, A., Carneiro, A.J., Erbar, S., Maisner, A., Fronhoffs, F., Buettner, R., Kalko, E.K., Kruppa, T., Franke, C.R., Kallies, R., Yandoko, E.R., Herrler, G., Reusken, C., Hassanin, A., Kruger, D.H., Matthee, S., Ulrich, R.G., Leroy, E.M., Drosten, C., 2012. Bats host major mammalian paramyxoviruses. Nat. Commun. 3, 796.

Fedyk, S., Ruprecht, L.A., 1983. Chromosome of some species of Vespertilionid Bats.I. Banding patterns of *Eptesicus serotinus* chromosomes. Acta Theriol. (Warsz.) 28 (9), 159–170.

Hagmaier, K., Stock, N., Precious, B., Childs, K., Wang, L.F., Goodbourn, S., Randall, R.E., 2007. Mapuera virus, a rubulavirus that inhibits interferon signalling in a wide variety of mammalian cells without degrading STATs. J. Gen. Virol. 88 (Pt (3)), 956–966.

Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11 (7), 714–719.

He, Y.L., Wu, Y.H., He, X.N., Liu, F.J., He, X.Y., Zhang, Y., 2009. An immortalized goat mammary epithelial cell line induced with human telomerase reverse transcriptase (hTERT) gene transfer. Theriogenology 71 (9), 1417–1424.

He, X., Korytar, T., Zhu, Y., Pikula, J., Bandouchova, H., Zukal, J., Kollner, B., 2014. Establishment of myotis myotis cell lines – model for investigation of host-pathogen interaction in a natural host for emerging viruses. PLoS One 9 (10), e109795.

Heldt, C.L., Hernandez, R., Mudiganti, U., Gurgel, P.V., Brown, D.T., Carbonell, R.G., 2006. A colorimetric assay for viral agents that produce cytopathic effects. J. Virol. Methods 135 (1), 56–65.

Howe, B., Umrigar, A., Tsien, F., 2014. Chromosome preparation from cultured cells. J. Vis. Exp. (83), e50203.

Jordan, I., Munster, V.J., Sandig, V., 2012. Authentication of the R06E fruit bat cell line. Viruses 4 (5), 889–900.

Katze, M.G., He, Y., Gale Jr., M., 2002. Viruses and interferon: a fight for supremacy. Nat. Rev. Immunol. 2 (9), 675–687.

Knudsen, G.R., Dixon, R.D., Amelon, S.K., 2013. Potential spread of white-nose syndrome of bats to the northwest: epidemiological considerations. Northwest Sci. 87 (4), 292–306.

Kong, F., James, G., Gordon, S., Zelynski, A., Gilbert, G.L., 2001. Species-specific PCR for identification of common contaminant mollicutes in cell culture. Appl. Environ. Microbiol. 67 (7), 3195–3200.

Lechardeur, D., Schwartz, B., Paulin, D., Scherman, D., 1995. Induction of blood-brain barrier differentiation in a rat brain-derived endothelial cell line. Exp. Cell Res. 220 (1), 161–170.

Lee, C., 2015. Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. Virol. J. 12 (1), 193.

Maruyama, J., Miyamoto, H., Kajihara, M., Ogawa, H., Maeda, K., Sakoda, Y., Yoshida, R., Takada, A., 2014. Characterization of the envelope glycoprotein of a novel filovirus, lloviu virus. J. Virol. 88 (1), 99–109.

Mayne, L.V., Priestley, A., James, M.R., Burke, J.F., 1986. Efficient immortalization and morphological transformation of human fibroblasts by transfection with SV40 DNA linked to a dominant marker. Exp. Cell Res. 162 (2), 530–538.

Mian, M.F., Ahmed, A.N., Rad, M., Babaian, A., Bowdish, D., Ashkar, A.A., 2013. Length of dsRNA (poly I:C) drives distinct innate immune responses, depending on the cell type. J. Leukoc. Biol. 94 (5), 1025–1036.
Misra, V., Dumonceaux, T., Dubois, J., Willis, C., Nadin-Davis, S., Severini, A., Wandeler, A., Lindsay, R., Artsob, H., 2009. Detection of polyoma and corona viruses in bats of Canada. J. Gen. Virol. 90 (Pt (8)), 2015–2022.
Moratelli, R., Calisher, C.H., 2015. Bats and zoonotic viruses: can we confidently link bats with emerging deadly viruses? Mem. Inst. Oswaldo Cruz 110 (1), 1–22.
Ohkubo, Y., Kishimoto, T., Nakata, T., Yasuda, H., Endo, T., 1994. SV40 large T antigen reinduces the cell cycle in terminally differentiated myotubes through inducing Cdk2, Cdc2, and their partner cyclins. Exp. Cell Res. 214 (1), 270–278.
Parson, W., Pegoraro, K., Niederstatter, H., Foger, M., Steinlechner, M., 2000. Species identification by means of the cytochrome b gene. Int. J. Legal Med. 114 (1–2), 23–28.
Qian, Z., Dominguez, S.R., Holmes, K.V., 2013. Role of the spike glycoprotein of human Middle East respiratory syndrome coronavirus (MERS-CoV) in virus entry and syncytia formation. PLoS One 8 (10), e76469.
Rapin, N., Johns, K., Martin, L., Warnecke, L., Turner, J.M., Bollinger, T.K., Willis, C.K., Voyles, J., Misra, V., 2014. Activation of innate immune-response genes in little brown bats (Myotis lucifugus) infected with the fungus pseudogymnoascus destructans. PLoS One 9 (11), e112285.
Scott, D.M., MacDonald, C., Brzeski, H., Kinne, R., 1986. Maintenance of expression of differentiated function of kidney cells following transformation by SV40 early region DNA. Exp. Cell Res. 166 (2), 391–398.
Shen, Y.Y., Liang, L., Zhu, Z.H., Zhou, W.P., Irwin, D.M., Zhang, Y.P., 2010. Adaptive evolution of energy metabolism genes and the origin of flight in bats. Proc. Natl. Acad. Sci. U. S. A. 107 (19), 8666–8671.
Techangamsuwan, S., Kreutzer, R., Kreutzer, M., Imbschweiler, I., Rohn, K., Wewetzer, K., Baumgartner, W., 2009. Transfection of adult canine Schwann cells and olfactory ensheathing cells at early and late passage with human TERT differentially affects growth factor responsiveness and in vitro growth. J. Neurosci. Methods 176 (2), 112–120.
Viale, G., Gambacorta, M., Dell'Orto, P., Coggi, G., 1988. Coexpression of cytokeratins and vimentin in common epithelial tumours of the ovary: an immunocytochemical study of eighty-three cases. Virchows Arch. A 413 (2), 91–101.
Virtue, E.R., Marsh, G.A., Baker, M.L., Wang, L.F., 2011. Interferon production and signaling pathways are antagonized during henipavirus infection of fruit bat cell lines. PLoS One 6 (7), e22488.
Wacharapluesadee, S., Duengkae, P., Rodpan, A., Kaewpom, T., Maneeorn, P., Kanchanasaka, B., Yingsakmongkon, S., Sittidetboripat, N., Chareesaen, C., Khlangsap, N., Pidthong, A., Leadprathom, K., Ghai, S., Epstein, J.H., Daszak, P., Olival, K.J., Blair, P.J., Callahan, M.V., Hemachudha, T., 2015. Diversity of coronavirus in bats from Eastern Thailand. Virol. J. 12 (57).
Xie, X., Pang, M., Liang, S., Yu, L., Zhao, Y., Ma, K., Kalhoro, D.H., Lu, C., Liu, Y., 2015. Establishment and characterization of a telomerase-immortalized canine bronchiolar epithelial cell line. Appl. Microbiol. Biotechnol. 99 (21), 9135–9146.
Yoshida, T., Maeda, S.I., Deguchi, T., Miyazawa, T., Ishiko, H., 2003. Rapid detection of Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma parvum, and Ureaplasma urealyticum organisms in genitourinary samples by PCR-microtiter plate hybridization assay. J. Clin. Microbiol. 41 (5), 1850–1855.
Zhang, G., Cowled, C., Shi, Z., Huang, Z., Bishop-Lilly, K.A., Fang, X., Wynne, J.W., Xiong, Z., Baker, M.L., Zhao, W., Tachedjian, M., Zhu, Y., Zhou, P., Jiang, X., Ng, J., Yang, L., Wu, L., Xiao, J., Feng, Y., Chen, Y., Sun, X., Zhang, Y., Marsh, G.A., Crameri, G., Broder, C.C., Frey, K.G., Wang, L.F., Wang, J., 2013. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. Science 339 (6118), 456–460.
Zschemisch, N.H., Eisenblatter, R., Rudolph, C., Glage, S., Dorsch, M., 2014. Immortalized tumor derived rat fibroblasts as feeder cells facilitate the cultivation of male embryonic stem cells from the rat strain WKY/Ztm. Springerplus 3, 588.