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Cell lines from the Egyptian fruit bat are permissive for modified vaccinia Ankara

Ingo Jordan[a,*], Deborah Horn[a], Stefanie Oehmke[a], Fabian H. Leendertz[b], Volker Sandig[a]

[a] ProBioGen AG, Goethestr. 54, 13086 Berlin, Germany
[b] Research Group Emerging Zoonoses, Robert Koch-Institute, Nordufer 20, 13353 Berlin, Germany

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ABSTRACT

Bats are reservoir hosts for a spectrum of infectious diseases. Some pathogens (such as Hendra, Nipah and Marburg viruses) appear to use mainly fruit bats as reservoir. We describe designed immortalization of primary fetal cells from the Egyptian fruit bat (*Rousettus aegyptiacus*) to facilitate isolation and characterization of pathogens associated with these mammals. Three cell lines with different properties were recovered and successful immortalization was confirmed by continuous cultivation for over 18 months. Surprisingly, the cell lines are fully permissive for a highly attenuated poxvirus, modified vaccinia Ankara (MVA). MVA is a safe and well characterized vaccine vector that cannot replicate in most mammalian cells. High permissivity of Rousettus cell lines could justify testing bats for susceptibility to MVA as a replication competent vector with low zoonotic potential to induce herd immunity in bat colonies against viruses causing rabies or haemorrhagic fevers.

1. Introduction

Bats (Chiroptera) are classified into suborders that separate frugivorous from insectivorous and hematophagous bats (with some overlap in the two groups, reviewed in Jones and Teeling, 2006). Species from both groups are implicated as reservoirs for a diverse spectrum of zoonotic pathogens (reviewed in Calisher et al., 2006). Wide distribution and genetic diversity of some of the viruses including lyssaviruses (Arguin et al., 2002; Badrane and Tordo, 2001; Messenger et al., 2002; Van der Poel et al., 2005) and coronaviruses (Chu et al., 2006; Li et al., 2005) suggest a long history of endemic presence in bat populations. For Nipah and Hendra viruses bats appear to function as both reservoir and vector (Chua et al., 2002; Halpin et al., 2000; Hayman et al., 2008; Reynes et al., 2005; Yob et al., 2001). Bats are furthermore investigated as reservoir to filoviruses as animals positive for RNA sequences from or antibodies against filoviruses have been found in regions where outbreak of Marburg or Ebola hemorrhagic fever has been recorded (Leroy et al., 2005; Swanepoel et al., 2007; Towner et al., 2007). However, live filoviruses have not yet been isolated from wild bats, possibly because bats are not a primary reservoir, or because the window of active shedding of filoviruses is brief and may require activation by physiological stimuli such as endocrine responses to stress (Strong et al., 2008).

A combination of properties of bats contributes to complex epidemiology: many bat species live in large colonies at high population densities where spread and maintenance of pathogens could be facilitated. Fluctuations in body temperature and metabolic rates to conserve energy for self-powered flight may help viruses to establish subclinical persistence. Self-powered flight also allows for wide dissemination of pathogens. Finally, bats comprise a very old order of the mammalia class, branching into the various bat species 89 million years ago in the late Cretaceous period (Bininda-Emonds et al., 2007). Their innate and adaptive immune system may react to certain pathogens very differently compared to the immune system of most extant mammalia, and some zoonotic agents may have decreased pathogenicity towards bats due to co-evolution with the flying mammals as reservoir (Badrane and Tordo, 2001).

To facilitate research on possible contributions by the host cell to these phenomena we have immortalized primary cells from the old world fruit bat *Rousettus aegyptiacus*. We describe generation and characterization of designed cell lines and infection experiments that reveal a surprising permissivity for modified vaccinia Ankara, a pox virus highly attenuated for mammalia by adaptation to avian host cells.

2. Materials and methods

2.1. Cell lines

Fetuses from *R. aegyptiacus* were a generous gift from the Wilhelma Zoo in Stuttgart, Germany. A total of five pregnant ani-

* Corresponding author. Tel.: +49 30 924 006x72.
E-mail address: ingo.jordan@probiogen.de (I. Jordan).

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mals were identified by palpation and deeply anaesthesized with isofluran. The uteri were surgically removed and the animals were euthanized by debleeding. The uteri were approximately 2 cm in diameter; they were placed into PBS immediately after removal for transport at ambient temperature to the cell culture laboratory. Six hours after sample removal, the fetuses were excised from the uteri. Amniotic fluid, cells from brain, liver, and the vertebrate column were carefully aspirated under sterile conditions with 18G syringes and transferred (separately, not as pool) into culture medium. Body segments without further attempt at obtaining specific tissues were briefly treated with TrypLE (Gibco) prior to tituration into culture medium. Culture medium is DMEM/F12 (Invitrogen) containing 5% gamma-irradiated fetal calf serum (Biochrom AG); only for the initial five passages the medium was also supplemented with antibiotic and antimycotic (Gibco/Invitrogen), long-EGF (SAFC) to 20 ng/ml and long-R3IGF-I (SAFC) to 10 ng/ml. Cells from a total of 14 preparations were seeded into 6-well culture plates and transfected using effectene (Qiagen) within 7–9 days after plating. Cultivation in all of the described experiments was performed at 37 °C and 8% CO_2.

The plasmid used for transfection and immortalization expresses E1A and E1B from the promoters for human phosphoglycerate kinase and thymidine kinase of herpes simplex virus, respectively (Jordan et al., 2009). Prior to transfection the expression plasmid was cut with ApaLI, RsrII and ScaI restriction enzymes to linearize the plasmid within the bacterial selection markers. No selection markers are expressed in eukaryotic cells. Transfection of primary cells was performed with 2 μg of linearized plasmid DNA and 16 μl enhancer in Qiagen-provided EC-buffer. After 5 min of incubation, depending on confluency of the cell layer, 16–20 μl of effectene reagent was added and this suspension was incubated for further 10 min. Thereafter, the transfection mix was applied to the cell monolayer in a final volume of 1 ml of medium. After 3 h additional 1.5 ml medium were added, the following day the medium was replaced against culture medium. Expression of E1 genes induced foci of early-immortalized clones in 7 of the transfected cultures. Permanent cell lines could be rescued from 3 cultures by continuous passaging until primary cells were lost to senescence.

Already established cell lines used here are AGE1.CR and AGE1.CS (derived from retina and somites, respectively, of muscovy duck embryos, Jordan et al., 2009), Vero (African green monkey kidney cells; ATCC CCL-81), and BHK-21 (baby hamster kidney cells). These cells were cultivated in DMEM/F12 medium containing 5% FCS.

2.2. Immunofluorescence and transient expression of GFP

Cultures of immortalized cells were seeded on glass slides and allowed to proliferate for several days before fixation with ice-cold methanol for 10 min. The fixed cells were incubated according to standard immunofluorescene methods with antibodies against E1A protein (Becton Dickinson, UK; diluted 1:30), secondary antibodies directed against mouse and conjugated to biotin (Jackson Immuno Research, USA; diluted 1:80), and fluorescent dye Texas Red conjugated to streptavidin (Jackson Immuno Research, USA; diluted 1:100). CR cells that stably express the Ad5 E1-region served as positive control, BHK cells as negative control. DAPI (4′,6-diamidino-2-phenylindol; Sigma, USA) to 1 μg/ml was added in the final incubation step to stain the nuclei of the cells for orientation purposes.

The pEGFP-N1 (Clontech) plasmid allows deletion of the strong hCMV promoter using AflIII and BglII restriction enzymes. The GFP reporter gene was then coupled to hPGK and tk promoters from the immortalizing plasmid. Transfection into the Rousettus cell lines was performed with effectene as described above.

2.3. Modified vaccinia Ankara (MVA)

Cells were seeded into 6-well plates and infected with MVA (ATCC #VR-1508) at multiplicity of 0.1 infectious units per cell after 24 h of culture. MVA used in the described experiments was isolated from infected CR cells. Infection was performed by adding MVA directly to the culture medium.

All infections were performed in triplicates. For titration, infected cells were lysed by three cycles of freeze/thawing directly in the culture plates to also harvest virus from within the cells. The suspension was cleared by centrifugation and titration was performed on Vero cells (Vero cells do not replicate MVA but they are susceptible and can be infected for titration purposes), briefly: Vero cells were seeded in 96-well plates at 2×10^4 cells per well and infected with serial 10-fold dilutions of MVA-containing suspension on the following day. Two days thereafter, the cultures were fixed with methanol and incubated with polyclonal vaccinia virus antibodies (Quartett, Germany; at 1:1000 dilution in PBS containing 1% fetal calf serum) for 1 h at 37 °C. Two wash steps were performed with PBS containing 0.05% Tween 20 (Sigma Corp., USA) and secondary antibody to the vaccinia-specific antibody is added at 1:1000 dilution in PBS containing 1% fetal calf serum. This secondary antibody is coupled to the peroxidase enzyme that catalyzes a color reaction upon incubation with AEC reagent (3-amino-9-ethyl-carbozole; 0.3 mg/ml in 0.1 M acetate buffer pH 5.0 containing 0.015% H_2O_2). Infected foci are identified by light microscopy and plaque forming units are calculated from the maximum dilution of MVA suspension that yields a positive dye reaction.

2.4. Adenovirus vector (AdGFP)

To generate AdGFP the gfp gene driven by the human CMV immediate early promoter followed by the bovine growth hormone polyadenylation signal was cloned in antiparallel orientation to E1 region into a shuttle vector containing adenovirus 5 sequences from 1 to 409 and 3510–5120. Using homologous recombination in *E. coli* (strain BJ5183BJ5183 recBCsbcBC) (Chartier et al., 1996), the cassette was integrated into a plasmid containing the complete adenovirus 5 genome. After liberation of the vector sequences from the plasmid backbone by digestion with PacI the vector genome was transfected into HEK293 cells. Plaques were isolated from cell monolayers overlaid with agarose and amplified on HEK 293 cells. Infection with AdGFP was performed in minimal amount of medium (300 μl DMEM/F12) without FCS for 3 h. AdGFP was given to cell monolayers either as crude thaw/freeze lysates from infected HEK 293 cells cleared by brief ultracentrifugation, or purified by ultracentrifugation through a CsCl gradient. CsCl-purification removes cellular debris from a freeze/thaw lysate by clarification at $5000 \times g$; by incubation with 10 U/ml benzonase for 30 min; by banding with $105{,}000 \times g$ in CsCl gradient consisting of layers of 1.5 g/cm^3, 1.35 g/cm^3 and 1.25 g/cm^3 in 10 mM Tris–HCl and 1 mM EDTA, pH 7.5; followed by a desalting step with a BioRad DG10 column.

3. Results

3.1. Cell line derivation

Primary cells prepared from *R. aegyptiacus* fetuses were transfected with an expression plasmid for adenovirus serotype 5 E1A and E1B open reading frames. Transfection efficiency was satisfactory (see parallel GFP control in Fig. 1A) and first foci of clonal cells with less pleomorphic appearance than primary cells were visible within 2 weeks of transfection. Primary cells died from senescence with repeated passaging of the cultures. Senescence describes a

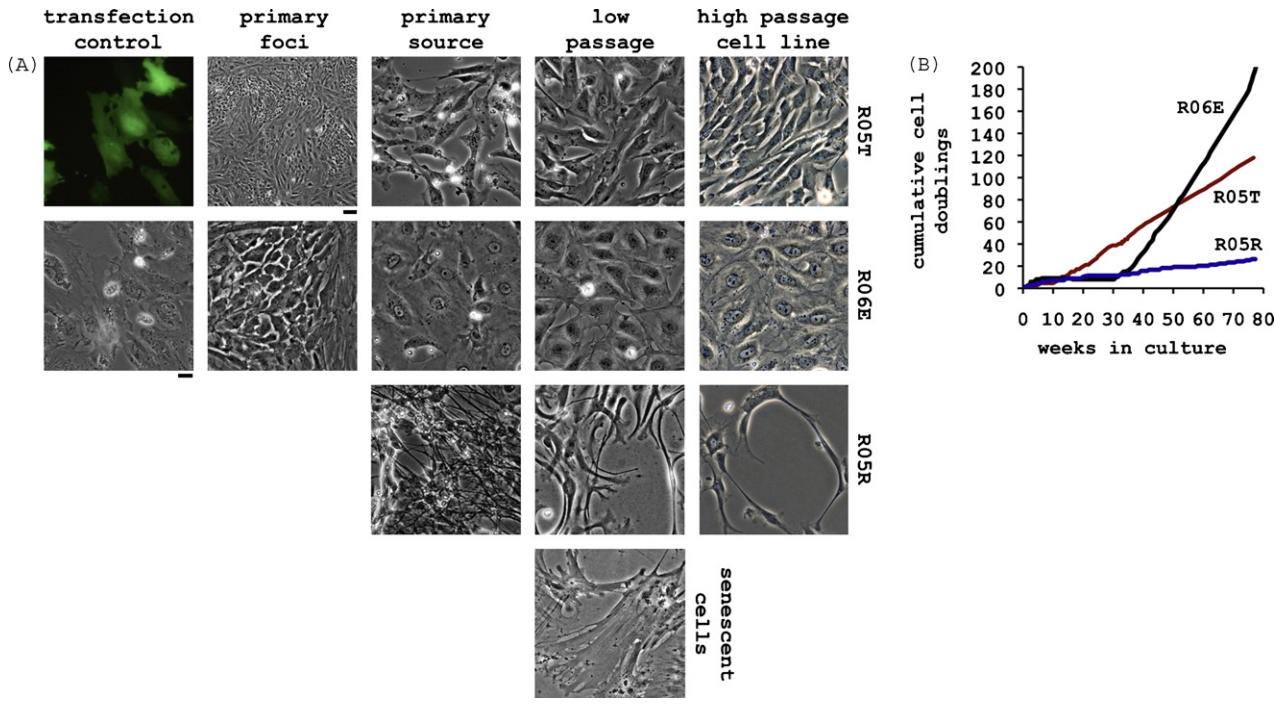

Fig. 1. Cell line generation and passage diagram. (A) GFP positive control in upper panel demonstrating transfection rate in primary Rousettus cells in the first column (scale bar is 20 μm for all images with 20× initial magnification). Foci of immortalized cells still embedded in monolayers of primary cells is shown in the second column (scale bar is 100 μm). Source primary cultures for three cell lines are shown in the third column. Within 10 passages primary cells undergo senescence and are lost leaving low passage cell lines (shown here at week 40) that at low confluency in appearance are surprisingly similar to the source. There are also only few changes in phenotype after 80 weeks of continuous culture. (B) Passage diagram demonstrating stable proliferation rates with a doubling time of approximately 50 h for R05T (after a lag phase of 10 weeks) and 120 h for R05R. R06E cell line exhibits a long lag phase for 30 weeks and thereafter sudden increase in doubling time to approximately 24 h.

cellular state with decreased proliferation rates until complete stasis or cell death due to telomere erosion of the chromosomes and accumulation of signal proteins important in cell cycle control. Senescent cells usually are large and pleomorphic with striated cytoplasm of low phase contrast. Fig. 1A shows three of the final cell lines after 40 weeks (low passage) and after 80 weeks (high passage cell lines) of continuous culture. Surprisingly, even after 35 weeks of continuous culture morphologies of the immortalized cells at subconfluency highly resemble morphology of the source culture (cell lines R05T was derived from the head of the fetus from animal #15, R06E from the body of the fetus from animal #12 and R05R from the vertebrate column of the fetus from animal #12). Doubling time of R06E and R05T is approximately 50 h, of R05R approximately 6 days. Because proliferation of the R05R cells is so slow most experiments described here were performed with R06E and R05T.

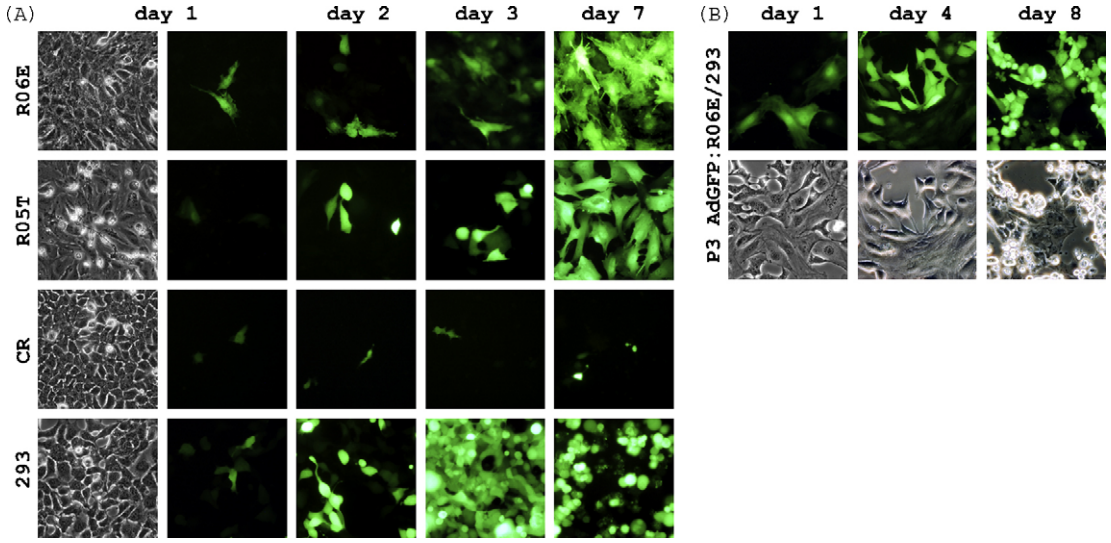

Fig. 2. Spread of AdGFP in cell monolayers and transfer to HEK 293. AdGFP spreads slowly in monolayers of R06E and R05T cells without cytopathic effect. Avian CR cells can be infected but vector does not spread. AdGFP spreads rapidly with fulminant CPE in 293 cells. (B) AdGFP is lost in Rousettus cells within 5 passages. Infected R06E cells at passage 3 were mixed with an equal number of 293 sentinel cells. AdGFP infrequently is transferred from R06E (weak GFP signal) to 293 cells (bright GFP signal without CPE at day 4) by cell–cell contact but not via supernatant. Fulminant CPE in 293 cells at day 8, except for weak GFP expression no effect on Rousettus cells.

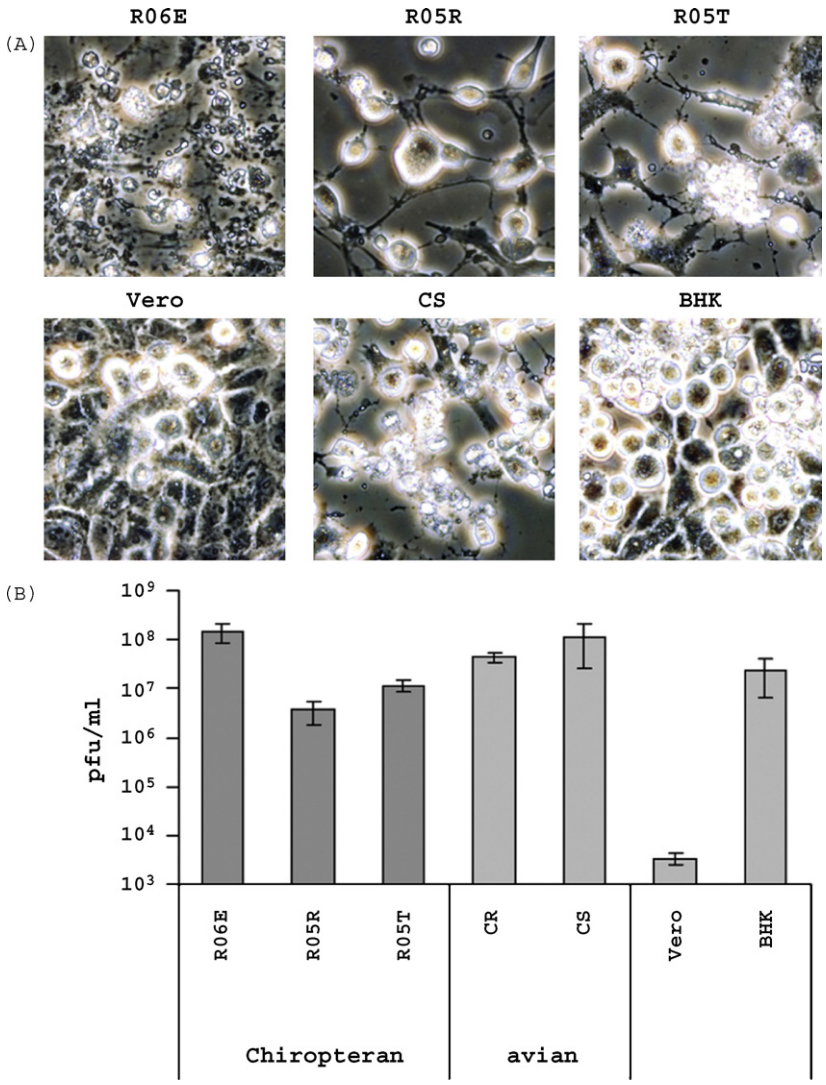

Fig. 3. High permissivity for modified vaccinia Ankara (MVA). (A) All Rousettus cell lines are permissive for highly attenuated MVA. Shown here is CPE 48 h after infection with a MOI of 0.1. (B) Yields of MVA in Rousettus lines are similar to yield obtained by infection of avian cell lines CR and CS. Vero is shown as negative control. This experiment was performed after 35 weeks of continuous cultivation.

3.2. Infection with AdGFP

To examine susceptibility of the cell lines to a common vaccine vector the R06E and R05T lines were infected with E1-deleted human adenovirus vector that expresses GFP (AdGFP). Infection was performed with an MOI of less than 0.1 to determine spread of virus in the culture. As controls, HEK 293 cells (a human cell line known to be highly permissive for adenovirus) and duck CR cells (not permissive for adenovirus) were infected in parallel. Common to all cell lines examined here is expression of human adenovirus serotype 5 E1-region so that the E1-deleted vector is trans-complemented in all hosts. The Rousettus cells appear not to be fully permissive for AdGFP vector: GFP signal spreads initially in the culture but there is no cytopathic effect (Fig. 2A). In the CR negative control there is no spread of GFP beyond occasional limited mitotic division of AdGFP-positive cells.

Because cells are not killed by the AdGFP vector they can be subpassaged, but AdGFP is lost within 5 passages. The vector appears to spread by cell–cell contact only as AdGFP could not be transferred with supernatant from infected Rousettus cells to a HEK 293 cell monolayer. However, if infected Rousettus cells within four passages are co-cultivated with HEK 293 cells the sentinel cells become infected, amplify AdGFP and allow fulminant infection (Fig. 2B). For this experiment, R06E cells were infected with AdGFP and passaged at least three times to remove any lingering input virus. After the third and fourth passage the infected R06E cells were mixed with an equal number of HEK 293 cells. The two cell lines can be differentiated by size and morphology: HEK 293 are smaller than R06E, more compact with more distinctive phase contrast and better discernible nuclei.

3.3. Infection with MVA

We next examined replication of a highly attenuated virus on the cell lines. Adherent cell monolayers (within 35 weeks of cell line inception) were infected with MVA at multiplicity of 0.1 infectious units per cell. As positive controls CR and CS (avian cell lines derived from retina and somites) and as negative control Vero (African green monkey kidney cells) were infected in parallel. MVA is a highly attenuated poxvirus adapted to replication in avian cells. The only mammalian cell line known to be fully permissive for MVA are BHK cells; infection of BHK therefore was performed as reference. 48 h post-infection a strong cytopathic effect was evident in the positive controls and surprisingly also in the Rousettus cell lines (Fig. 3A). As expected, no spread of virus and no or minimal cytopathic effect was visible in the Vero cell line that is refractory for MVA.

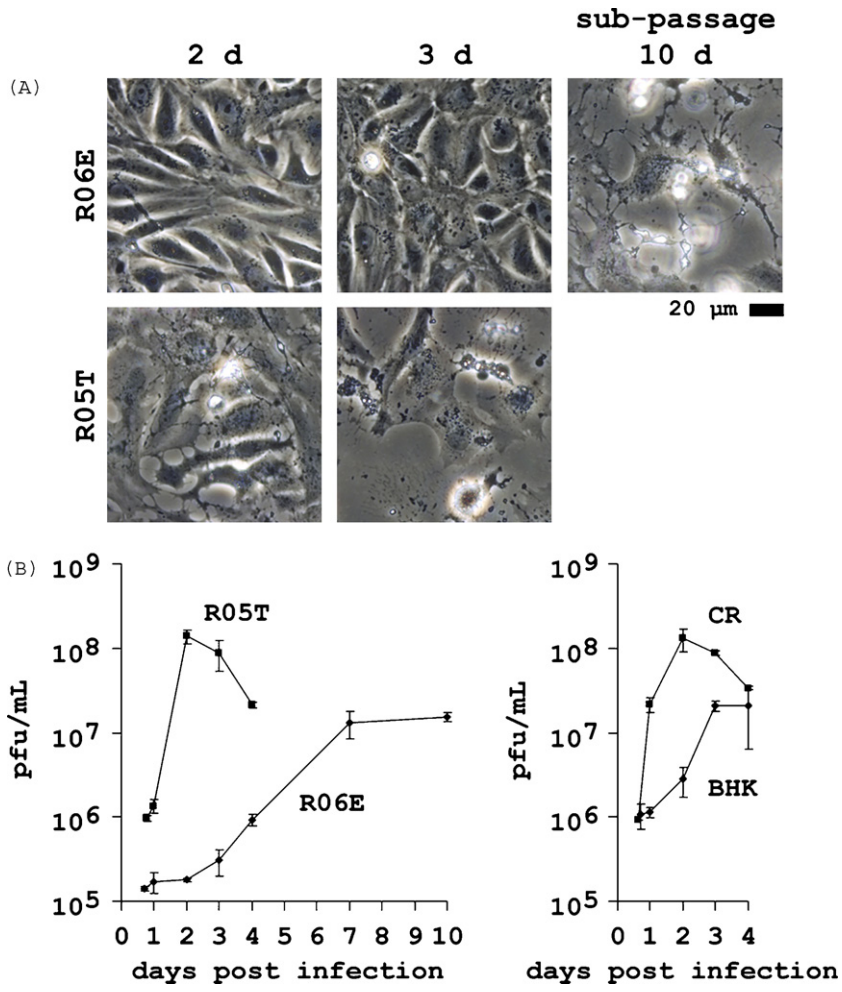

Fig. 4. Kinetic for MVA replication declines in R06E at high passage. (A) Appearance of MVA cytopathic effect is delayed in high passage R06E (but not in R05T) and cultures of R06E have to sub-passaged until infection is visible. (B) Replication of MVA is rapid in R05T and CR, slower in BHK and clearly delayed in the high-passage R06E cell line. This experiment was performed after approximately 65 weeks of continuous cultivation. Infection was performed with a MOI of 0.1 corresponding to an input virus concentration of 2×10^4 pfu/ml in all cultures at day 0.

Titration confirmed productive replication in Rousettus cells. Especially for R06E number of produced infectious units was very high, similar to values obtained with the avian CR and CS cell lines and exceeding values obtained with BHK cells (Fig. 3B). Yields in the negative control corresponds to input virus.

MVA infection experiments were repeated 15 months after cell line generation. At higher passage numbers of R06E, but not R05T, kinetic of MVA replication is delayed but maximum yields remain high (Fig. 4).

3.4. High passage cell lines

The R06E cell line suddenly increased proliferation rates with increasing passage number. We therefore re-examined some properties of the cell lines. Immunofluorescence analysis indicates that at low passage all cells are positive for E1A expression as expected from a cell line obtained by designed (rather than spontaneous) immortalization. However, signal intensity was extremely weak (compare nuclear staining of Rousettus lines to signal obtained with CR cell line as positive control in Fig. 5). Transient transfection of plasmids that express the EGFP reporter gene under control of the promoters used to generate the cell lines indicates surprisingly low expression rates with all promoters compared to the CR lines (Fig. 5C). Even hCMV promoter activity appears to be repressed in Rousettus cells. At higher passage levels (15 months post cell line generation) E1A expression cannot be detected by PCR or immunofluorescence in R06E but is maintained in R05R and R05T (Fig. 5B).

As E1A is involved in the adenoviral response to anti-viral defenses by the host cell we determined susceptibility of early and late passage R06E cells to dsRNA as a common inducer of the innate immunity (Weber et al., 2006). The synthetic analogue polyinosinic–polycytidylic acid (Alexopoulou et al., 2001; Kato et al., 2006) was added directly to the medium of the culture with Vero as a negative control (Emeny and Morgan, 1979) and CS as a positive control (Jordan et al., 2009). Fragmentation of nuclei consistent with induction of apoptosis and intact TLR-3 signaling into the interferon pathways (Dewitte-Orr et al., 2005) was determined by staining with Hoechst 33342 dye. We observed no change in susceptibility of R06E and R05T with passage number (data not shown); both cell lines are refractory to induction of apoptosis by dsRNA.

4. Discussion

Epidemiology of pathogens in reservoirs and vectors is influenced by many factors, from environmental fluctuations (Fogarty et al., 2008) to properties of host or virus. Although evidence of infection with important zoonotic pathogens, including Nipah and Hendra viruses (Hayman et al., 2008; Yob et al., 2001), Ebola and

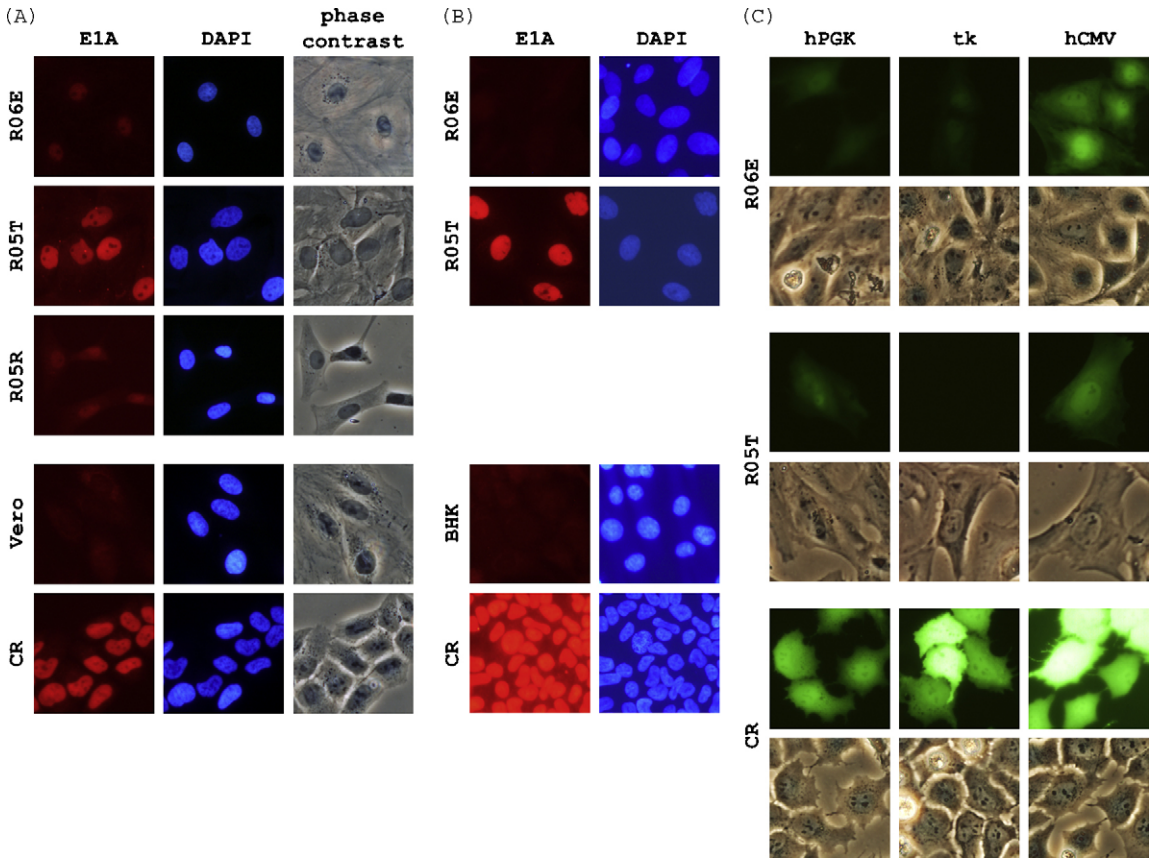

Fig. 5. Immunofluorescence for immortalizing E1A protein and transient transfection to examine expression plasmid promoter activity. (A) All cells are positive for E1A in early passage Rousettus cell lines but signal intensity is close to the detection limit in R06E and R05R. CR cells serve as positive control, Vero as negative control. (B) In high passage cell lines signal intensity remains high in R05T but was lost in R06E. (C) Chiropteran and avian cell lines were transiently transfected with an expression plasmid where GFP reporter is under control of the promoters used in the immortalizing plasmid. hPGK, human phosphoglycerate kinase promoter; tk, herpes simplex virus thymidine kinase promoter. Note extremely low signal strength for hPGK and tk promoters in Rousettus cells but not in CR cells. Even the usually very strong hCMV promoter appears to be repressed in Rousettus cells. Images shown here were taken 24 h post-transfection.

Marburg viruses (Leroy et al., 2005; Swanepoel et al., 2007; Towner et al., 2007), coronaviruses (Chu et al., 2006; Li et al., 2005), and lyssaviruses (Amengual et al., 2007; Arguin et al., 2002; Van der Poel et al., 2005) have been found in apparently healthy bats virus isolation has not always been straightforward. For example, active henipaviruses have been isolated from wild fruit bats with low efficiencies (Chua et al., 2002; Reynes et al., 2005) confirming that transmission may also depend on intricate host-virus processes (Halpin et al., 2000; Middleton et al., 2007). Regarding properties of the viral strain, most lyssavirus genotypes are considered to be highly pathogenic (Badrane et al., 2001). Live virus has been isolated from sick rabid bats (Field et al., 1999; Johnson et al., 2003) but asymptomatic endemic persistence of a wild lyssavirus strain related to genotype 1 has also been detected (in Serengeti spotted hyenas, East et al., 2001).

To investigate contributions at the cellular scale to possible balances of virus susceptibility and resistance we immortalized cells from the fruit bat *R. aegyptiacus* for further research. To our knowledge only two cell lines from insectivorous bats are available (TB-1 Lu, ATCC number CCL-88, isolated in 1965 from the lung of *Tadarida brasiliensis* and Mvi/It, ATCC number CRL-6012, from a skin tumor of *Myotis velifer incautus*) but no cell line from a fruit bat. As fruit bats (but not insectivorous bats) are implicated as reservoir for henipaviruses, Ebola and Marburg viruses a cell line from this genus may provide new and important insights.

Rousettus primary cells were immortalized by liposomal transfection of an expression plasmid for E1A and E1B of human adenovirus 5 in non-cognate expression cassettes. Human adenovirus 5 causes common cold in humans and is not associated with induction of tumors. However, because quiescent host cells are not permissive for a full viral life cycle adenoviruses have evolved mechanism to force cells into S-phase: the E1A proteins disrupt RB/E2F complexes and after dissociation from the repressor, the E2F transcription factors induce progression of the cell cycle. However, E1A also interferes with other transcriptional co-activators and by doing so induces apoptosis via several pathways (Frisch and Mymryk, 2002; Putzer et al., 2000). The adenoviral E1B gene encodes two open reading frames on a bicistronic mRNA, the 21K and 55K proteins, that protect the cells against apoptosis induced by E1A (Putzer et al., 2000; Yew et al., 1994).

Transfection of the expression plasmid for E1A and E1B induced clonal expansion of some cells in the monolayer of primary cells. With continuous passage the majority of the cells in the culture suffered senescence and died. There was no artificial selection pressure applied for integration or maintenance of the E1 plasmid and yet immunofluorescence against E1A protein revealed that only cells that express this nuclear protein survived beyond senescence. Surprisingly, morphologies of the obtained cell lines (R06E, R05T and R05R) and of the source culture that was transfected are very similar. R05R is a cell line with neuronal appearance (doubling times of 6 days, long projections towards other cells at lower cell densities, Brewer and Cotman, 1989) and was obtained from the vertebrate column of one fetus.

Considering the importance of bats as disease reservoir bat cells may have unexpected permissivity for viruses. In addition, for disease reservoirs and carriers it would be desirable to have vaccine vectors available to induce herd immunity against zoonotic pathogens. To release replication-competent vaccine viruses into the wild life is an extremely complex decision but not without precedence (Brochier et al., 1991; Faber et al., 2005). We therefore tested replication of common laboratory viruses and vaccine vectors (human adenovirus 5 vector and modified vaccinia Ankara) on the cells.

R06E and R05T lines were infected with E1-deleted adenovirus vector that expresses GFP (AdGFP). This vector was derived from adenovirus serotype 5 (Ad5) that normally replicates in human cells. Although cells from many species are susceptible to Ad5 infection only cells from swine (Jogler et al., 2006) and the BHK-21 cell line (Doerfler, 1969) were shown to be fully permissive for Ad5 replication. We observed very slow spread of AdGFP in R06E and R05T monolayers (Fig. 2). Perhaps surprising, however, is initial AdGFP replication without any cytopathic effect and decline of the GFP signal intensity within 3–4 passages. Thus, virus after strong initial spread appears to be cleared from the culture.

Consistent with lack of CPE, virus is released with very low efficiency into the supernatant and only in co-cultivation experiments AdGFP can be reliably transferred to HEK 293 cells (Fig. 2B). Transfer of AdGFP by co-cultivation demonstrates that the latently infected Rousettus cells release viral vector. AdGFP levels decline in the infected R06E monolayer and consequently efficiency of transfer decreases with further sub-passage. Co-cultivation also revealed that even at the tremendous MOIs expected when virus is released from neighboring HEK 293 the Rousettus target cells maintained full proliferative potential, did not succumb to cytopathic effect and started to spread into the areas liberated by infected HEK 293 cells. GFP expression did increase in most Rousettus cells but even there not to levels comparable to refractory CR cells.

Rousettus cells were found fully permissive for MVA, a very surprising observation. MVA was generated from vaccinia (cowpox) virus by 574 serial passages in primary chicken fibroblasts near the end of the WHO smallpox eradication program. During the process a number of mutations and deletions in the viral genome accumulated resulting in severe host restriction (Meyer et al., 1991). MVA is a highly promising tool for therapeutic and protective vaccine applications (Sutter and Moss, 1992; Weyer et al., 2007): it can be made to express recombinant vaccine antigens in cells not permissive for a full infectious cycle and combines highest level of safety even for immunocompromized human patients with induction of a robust and broad immune response in the organism. Among mammalian cells only the BHK-21 line (from Syrian hamster kidney) has been shown to be fully permissive for MVA (Carroll and Moss, 1997; Drexler et al., 1998). In the R06E line MVA replicates to levels beyond 10^8 pfu/ml (Fig. 3) that rival avian cells, exceeding the published and our parallel values in the range of 10^6 pfu/ml for replication in BHK (Drexler et al., 1998). The R05T cell line is less efficient than R06E but with 3.3×10^7 pfu/ml in the expected range for a fully permissive host cell. Nonpermissive (but susceptible) Vero cell line provides a base line where yields correspond to input virus.

Surprisingly, kinetic of MVA replication and appearance of fulminant CPE was delayed at higher passages (65 weeks versus 35 weeks of continuous cultivation) of R06E but not of R05T (Fig. 4); peak yields were not affected by high passage. We re-examined the cell lines for E1A expression and observed loss of expression in R06E but not in R05T (Fig. 5). Loss of E1 gene in R06E (and maintenance in R05R) was also confirmed by PCR (data not shown).

Immortalization with E1-genes from human adenovirus 5 has been performed with human cells (HEK 293 and PER.C6) (Fallaux et al., 1998; Graham et al., 1977) and with avian cells (AGE1.CR and CS) (Jordan et al., 2009). In all of these cell lines strong E1 expression is maintained without selection, in the venerable HEK 293 cell line in many laboratories since 1977, in the avian CR cell lines for more than 260 passages that include several cloning steps and adaptation to proliferation in suspension in chemically defined medium. In the Rousettus cell lines E1A and E1B expression is extremely weak especially in R06E. Transfection with expression plasmids for the GFP reporter gene under control of the same promoters used to drive E1A and E1B expression yields low GFP signal intensity in Rousettus cells but not in the avian reference cells. E1 expression close to the detection limit in the R06E cell line possibly was insufficient for a complete immortalization explaining a long lag phase for 30 weeks, during which a subpopulation of R06E evolved into a different lineage with very short doubling times. Serendipitously weak expression of E1A in all Rousettus cell lines may explain the low level of differentiation into epithelial phenotype that is expected in an E1A-positive cell line (Frisch, 1994). Sudden proliferation after a long lag phase in R06E (Fig. 1B) is fully compatible with the model that immortalization occurs in consecutive steps (Hahn and Weinberg, 2002; Ye et al., 2008). If the biochemical pathways for cell cycle progression are not clearly defined the cell population will eventually shunt into alternative routes. Even for stem cell lines with potential for unlimited proliferation culture conditions exert selective pressure such as overexpression of NANOG to relieve dependence on feeder layers (Darr et al., 2006). In R05T E1 expression is stronger and this cell line displayed only a short lag phase (probably adaptation of the nascent cell line to culture conditions ex vivo) after which proliferation rates remain stable also at high passages. R06E is an impressive example for the kinetic with which E1 expression is lost if it is not required by a cell line.

But even more interesting and unexpected is the effect of loss of E1 expression (from human adenovirus) on the kinetic of MVA replication (avian-attenuated poxvirus) in the permissive Rousettus cell line. Parallel experiments with R05T matched in passage number to R06E serve as reference highlighting the role of E1 for our observation. E1A has been implicated in the response of adenovirus against the anti-viral mechanism within the host cell, including inhibition of interferon cascades (Anderson and Fennie, 1987; Leonard and Sen, 1997). The importance of the interferon response feeding into recognition of viral RNA as a pathogen-associated pattern has recently been demonstrated in persistent infection of Ebola virus (Strong et al., 2008). It is possible that the anti-antiviral effects of E1A in the Rousettus cell lines lower the defenses of the producer cell for MVA leading to faster replication. We should be careful in extrapolating from replication of MVA in Rousettus cell lines to other systems. However, in the light of the results by Strong et al. (2008) we speculate that an E1A-positive R05T cell line would be preferred over the late-passage R06E for pathogen isolation as the R05T cell line may be less prone to cryptic infection. To examine field samples for pathogens it may also be helpful to add low levels of mitogens such as phorbol esters to obtain higher levels of virus or better detectable CPE.

In conclusion, compared to avian cells immortalized by identical methods, the Rousettus cells exhibit extremely low levels of gene expression from viral and cellular promoters. Low levels of gene expression may affect virus replication or expression of genes involved in inflammation and innate immunity. The Rousettus cell lines appear not to be notably resistant to viruses: they are partially permissive for a human adenovirus vector and fully permissive for MVA, a highly attenuated virus that does not replicate in most mammalian cells. Permissivity for such a selective virus suggests that the Rousettus cell lines may allow isolation of pathogens where suitable host cells have previously not been available. Also, if MVA can be shown to replicate and spread in bats without causing significant disease this virus has the potential to provide a vaccine vector

that should remain confined to the treated colonies and yet induces herd immunity.

Note added in proof

Since the above was written Towner et al. (2009 in PLoS Pathogens 5, e1000536) have successfully isolated live Marburg virus from wild Rousettus aegyptiacus. Frequency of active virus infection in the investigated colony suggests that the Egyptian fruit bat may provide a primary reservoir for this filovirus.

Conflicts of interest

I.J. and V.S. have applied for patent protection for the described cell lines.

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