

A novel bromodeoxyuridine-resistant wild boar lung cell line facilitates generation of African swine fever virus recombinants

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Abstract Manipulation of African swine fever virus (ASFV) genomes, in particular those from field strains, is still a challenge. We have shown recently that generation of a green-fluorescent-protein-expressing, thymidine-kinase-negative (TK^-) mutant of the low-pathogenic African swine fever virus field strain NHV was supported by a TK^- Vero cell line. Since NHV, like other ASFV field strains, does not replicate well in Vero cells, a bromodeoxyuridine (BrdU)-resistant cell line derived from wild boar lung (WSL) cells, named WSL-Bu, was selected. WSL cells were used because they are suitable for productive replication of NHV and other ASFV field strains. Here, we show that WSL-Bu cells enable positive selection of both TK^- and TK^+ ASFV recombinants, which allows for novel strategies for construction of ASFV mutants. We further demonstrate for a low-pathogenic ASFV strain that TK expression is required for infectious replication in macrophages infected at low multiplicity and that vaccinia TK fully complements ASFV TK in this respect.

Introduction

African swine fever virus (ASFV) is the causative agent of African swine fever (ASF). Isolates of different virulence exist in nature, leading to different outcomes of disease. Clinical signs range from unapparent or chronic forms to a very virulent hemorrhagic disease with high mortality rates in domestic and feral pigs and European wild boars (summarized in ref. [4]). Infection of African wild suids

(warthogs, bushpigs) with ASFV, however, results in an asymptomatic carrier state. Soft ticks (*Ornithodoros erraticus*) in Spain and Portugal and members of the *Ornithodoros moubata* complex in Africa also serve as a natural reservoir for ASFV and a source of transmission to suids [6, 17].

ASF constitutes a major threat to pig husbandry worldwide. This became particularly evident during an outbreak of ASFV in the Georgian Republic in 2007 and its subsequent spread into other Caucasian countries, the Russian Federation, Ukraine and Belarus [9, 14, 22, 24]. So far, no efficacious vaccine or treatment has been developed (for review, see ref. [26]). Thus, disease control is based on culling of infected herds, sanitary measures, and restriction of animal movements and trading [10], which can entail serious economic and social consequences in affected countries. ASFV, or as suggested recently, African swine fever asfivirus [27], is classified as the type, and hitherto only member of the family *Asfarviridae* in the nucleocytoplasmic large DNA virus (NCLDV) superfamily or – as proposed lately – order *Megavirales* [8, 12, 13]. Depending on the virus isolate, the size of its double-stranded DNA genome ranges from 170 kbp to 190 kbp, and it contains more than 150 open reading frames (ORFs), which encode proteins involved not only in viral replication and morphogenesis but also in functions that interfere with host response to infection [29], accounting for the high complexity of virus-host interactions. Viral functions determining pathogenesis and virulence are largely unknown. Thus, development of novel strategies to facilitate the study and genetic manipulation of this complex virus is required.

The main natural target cells for replication of ASFV are porcine monocyte-macrophage cells, which, however, are difficult to work with. Propagation in established cell lines, which are more suitable for experimentation and easier to maintain than pig macrophages, is also possible, and most

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of the *in vitro* studies and ASFV genome manipulations were achieved using Vero cells. However, virus replication in Vero cells requires several passages for adaptation resulting in changes in the viral genome such as the loss of multigene family genes that are involved in virulence and are necessary for viral replication in macrophages and in the tick host vector [5, 19, 30]. Therefore, the development of new protocols and tools for manipulating natural ASFV isolates is of utmost importance, in particular also in view of future vaccine development and functional studies. Recently, two new cell lines were described to be directly susceptible to infection by both field strains and cell-adapted ASFV: the primate-derived COS-1 cell line and the wild-boar-lung-derived cell line WSL [11, 15, 21].

Using WSL cells for infection and recombination, we recently created a recombinant TK⁻, green fluorescent protein (GFP)-expressing ASFV [21] directly from the field strain NHV, a low-virulent, non-haemadsorbing strain, isolated from a chronically infected pig [28]. Generation of the recombinant virus could be detected easily by fluorescence microscopy. Selection of TK⁻, GFP⁺ recombinants was supported by using a TK⁻ Vero cell line, since cells lacking this enzyme activity allow positive selection for mutants of normally TK-expressing viruses by addition of BrdU to the cell culture medium during virus propagation. However, infectious replication of NHV and the NHV-derived recombinant in Vero TK⁻ cells was not efficient and required amplification of the mutant virus in WSL cells after each round of plaque purification in Vero TK⁻ cells, which increased the number of cell culture passages, the likelihood of acquiring fortuitous mutations, and the time for reaching homogeneity of the mutant virus.

Here, we report a significant improvement of this selection system through the isolation of the BrdU-resistant cell line WSL-Bu and show that this cell line accelerates purification of ASFV recombinants with gene insertions in the TK locus. We also show that WSL-Bu cells are suitable for segregating TK⁺ ASFV from a large excess of TK⁻ virus, which opens new possibilities for creating ASFV mutants. In addition, we demonstrate that vaccinia virus TK functionally complements ASFV TK in porcine macrophages and that, as has been reported before for the highly virulent ASFV strain Malawi, the low-pathogenic strain NHV also requires expression of a functional TK for productive low-multiplicity infection of macrophages.

Materials and methods

Cells and viruses

Wild boar lung cells (kindly provided by Roland Riebe, The Collection of Cell Lines in Veterinary Medicine, FLI-

Insel Riems) were maintained in a 1:1 (v/v) mixture of Ham's F12 medium and Iscove's modified Dulbecco's medium, pH 7.2, supplemented with 10 % fetal bovine serum (FBS), 2.4 mM L-glutamine, and 100 units of penicillin and 100 µg of streptomycin per ml. Cell cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO₂. NHV [28], a non-haemadsorbing, low-virulence, wild-type strain of ASFV, was kindly provided by Carlos Martins (FMV, Lisbon, Portugal).

To obtain cell line WSL-Bu, WSL cells were first adapted to growth in cell culture medium containing 0.5 µg ml⁻¹ BrdU followed by a consecutive increase of the inhibitor concentration until cultures maintained in the presence of 100 µg ml⁻¹ BrdU grew comparably to the parental cells. Since infectious replication of wtASFV was completely inhibited already at 25 µg ml⁻¹ BrdU, this concentration was used in selection experiments. Porcine blood macrophages [21] were kindly prepared by Sandra Blome and her team (FLI-Insel Riems).

Construction of plasmids

All cloning procedures were carried out according to standard methods [25]. To obtain plasmid pRFP-vaccTK, the vaccinia virus strain WR TK-ORF (GenBank accession number NC_006998) was isolated from purified viral DNA by PCR using primers VaccTK+ (TAGAAGCTTGTCA TCATGAACGGCGGACATA TTCAG) and VaccTK- (TAGAAGCTTATGAGTCGATGTAACACTTTCTACA CACC). The recognition sequence for *Hind*III is shown in bold. Amplicons were cloned via *Hind*III cleavage sites into the plasmid vector psp73. After sequence verification, the vaccTK ORF was inserted into the *Sma*I-cleaved plasmid pASFVdTK [21]. The resulting plasmid, pASFVdTK_ORFvaccTK, was cleaved with *Acc*65I and used for the integration of an *Acc*65I-cleaved DNA fragment, which was amplified from ASFV DNA using primers ASFVp30pA+ (TAAGGTACCGAGCTC AGCGGCCGCATGTTTTTTT TTATTTGTACTTTGC) and ASFVp30pA- (TAAGGTACC GTATACATATATTTAAAATAAAACCAATTC). The recognition sequence for *Acc*65I is shown in bold. The amplicon encompassed nt 108,565 to nt 108,808 (GenBank accession number U18466) and contained a synthetic polythymidilate tract (typed in italics) and the ASFV p30 promoter. The resulting plasmid was cleaved with *Sac*I and *Not*I to insert a fragment with the ASFV p72 promoter followed by the ORF for the red fluorescent protein (RFP), which was obtained after *Sac*I and *Not*I cleavage of pASFVp72_dsRed, in which the ORF for the *Discosoma sp* red fluorescent protein (RFP) is preceded by the ASFV p72 promoter. A diagram of the resulting plasmid pRFP-VaccTK is depicted in Fig. 2.

Generation of ASFV recombinants

Semi-confluent WSL cells in 6-well plates (approximately 10^6 cells per well) were transfected with 4 μg of recombination plasmid using the FuGene HD transfection reagent as recommended by the supplier (Roche, Mannheim, Germany). The medium was removed 5 h after transfection and the cells were infected with the appropriate ASFV at an MOI of 2. The inoculum was removed 1 h later. Cultures were monitored daily for appearance of autofluorescing plaques. Infected cells from these plaques were isolated by aspiration, and progeny viruses were purified to homogeneity as described in the results section.

Indirect immunofluorescence assay

Cells were fixed with 3 % paraformaldehyde in PBS for 20 min, subjected to membrane permeabilization with 0.2 % Triton X-100, and sequentially incubated with p30-specific MAb C18 (kindly provided by Linda Dixon, Pirbright, UK) and Alexa 488-conjugated rabbit anti-mouse immunoglobulin G (Invitrogen). Fluorescing cells were documented using a Nikon T100 fluorescence microscope with a CCD camera and NIS software, respectively.

Growth kinetics

For growth curves, WSL cultures were infected at the given multiplicities. At 1 h p.i., the inoculum was replaced by fresh culture medium and cells were incubated until the times indicated. Supernatants and cells were harvested and stored at -70°C . Serial dilutions were titrated on WSL cells in 96-well plates, and titres were determined according to the method of Reed and M \ddot{u} rch [23].

Results

Development of a BrdU-resistant WSL cell line

We had shown recently that isolation of a TK^- , GFP^+ recombinant (NHV-dTK-GFP) of the ASFV strain NHV [28] was supported by the availability of a TK^- Vero cell line. Since ASFV field strains do not replicate well in Vero cells, the time needed for plaque formation and, consequently, plaque purification of the recombinant was long in comparison to plaque formation on WSL cells [21]. To enhance the efficacy of TK^- virus isolation out of a large excess of wild-type ASFV virions, a TK^- cell line named WSL-Bu, based on WSL cells, in which both field and laboratory ASFV strains replicate efficiently [11, 21], was selected by consecutively increasing the BrdU concentration in the cell culture medium until cultures maintained in

the presence of 100 $\mu\text{g BrdU ml}^{-1}$ had growth rates similar to that of the parental cells.

To test for the specifics of WSL-Bu cells with regard to replication of TK-expressing and TK-deficient ASFV, cultures were infected with appropriate dilutions of wild-type ASFV strain NHV or recombinant NHV-dTK-GFP [21]. ASFV-NHV expresses a functional TK and should be able to productively replicate and form plaques in WSL-Bu cell cultures maintained in normal culture medium or in hypoxanthine-aminopterin-thymidine (HAT) medium. In the latter, aminopterin blocks dihydrofolate reductase and thus generation of thymidine monophosphate from deoxyuridine monophosphate. Only in the presence of a functional TK is thymidine monophosphate, which is essential for *de novo* viral DNA synthesis, synthesized when thymidine and hypoxanthine are provided. In contrast, in the presence of BrdU, a thymidine analog that induces improper base pairing during DNA replication, resulting in genome inactivation, plaque formation should be inhibited. On the other hand, recombinant NHV-dTK-GFP should productively replicate in the presence of BrdU and fail to form infectious progeny in HAT medium. Figure 1 shows that strain-NHV-induced plaque formation was not affected by incubation in HAT medium but was sensitive to BrdU, since only single infected cells were detectable when BrdU was present. In contrast, NHV-dTK-GFP proved to be resistant to BrdU but sensitive to HAT. From these results and the observation that WSL-Bu cells cannot be propagated in HAT medium (not shown), we conclude that WSL-Bu cells do not express a functional thymidine kinase.

WSL-Bu cells as a new tool to isolate ASFV recombinants

To verify that WSL-Bu cells improve isolation of TK-negative ASFV mutants, WSL cells in 6-well plates were transfected with 4 μg of recombination plasmid pEGFP-BAC-Lox [21] and infected 5 h later with strain NHV at a multiplicity of infection (MOI) of 2 as described [21]. At 4 days p.i., cells from autofluorescing plaques were picked by aspiration and added to WSL-Bu cells after one freeze/thaw cycle followed by incubation in presence of 25 $\mu\text{g ml}^{-1}$ BrdU for 4 days. Cells from 4 fluorescing plaques, harvested as described above, were frozen and thawed twice and adjusted with medium to a final volume of 10 ml, and all wells of a 96-well plate with WSL-Bu cells were inoculated with 100 μl of this suspension. After 6 days of incubation in the presence of 25 $\mu\text{g ml}^{-1}$ BrdU, supernatants and cells from one infected well from each plate were harvested. Analysis of the virus progeny by PCR and immunofluorescence assays [21] demonstrated the homogeneity of all isolates (data not shown), showing that

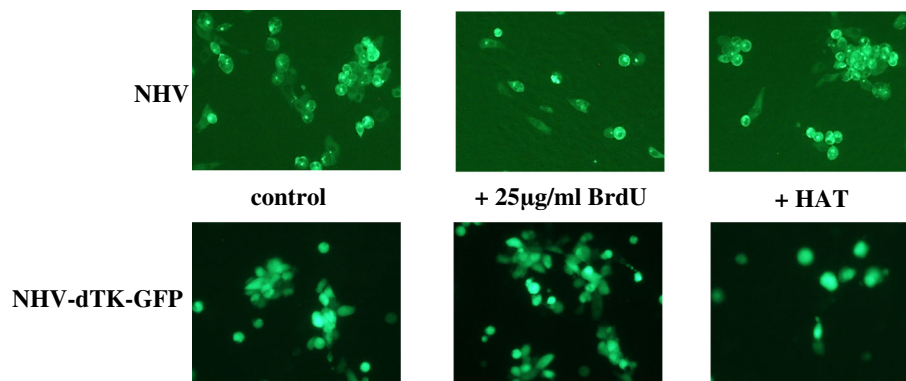


Fig. 1 Specifics of WSL-Bu cells. WSL-Bu cells in 24-well plates were infected with appropriate dilutions of wild-type ASFV strain NHV or recombinant NHV-dTK-GFP in normal cell culture medium (control), in medium containing $25 \mu\text{g ml}^{-1}$ BrdU or in HAT

medium. Cultures were fixed 4 d p.i., and infected cells were visualized either by indirect immunofluorescence using anti-p30 monoclonal antibody C18 (NHV) or by GFP-autofluorescence (NHV-dTK-GFP)

efficient and rapid positive selection for TK-negative ASFV recombinants in WSL-Bu cells significantly reduces the number of consecutive plaque purifications and thus the time needed. One progeny isolate, named NHV-dTK-GFP / WSL-Bu, was chosen for further experiments.

Complementation of TK-negative ASFV by expression of heterologous vaccinia virus TK

As shown in Fig. 1, replication of TK⁻ ASFV in WSL-Bu cells is inhibited in the presence of HAT. This property provides the opportunity to select TK⁺ ASFV from a large excess of TK⁻ viruses. To generate a TK⁺ revertant of NHV-dTK-GFP / WSL-Bu, recombination plasmid pRFP-VaccTK (Fig. 2) was constructed. In this plasmid, transcription of the vaccinia virus TK ORF (vacc-TK ORF) is directed by the ASFV p30 promoter and terminated by the authentic TK polythymidilate tract within the TK-R segment. The vacc-TK expression unit is preceded by an expression cassette for the *Discosoma sp.* red fluorescent protein (RFP) under transcriptional control of the ASFV p72 promoter and nine thymidines (T9 in Fig. 2) to test whether a “cargo” expression cassette can be introduced into the ASFV genome by this approach and to provide the possibility to monitor growth of recombinant virus by RFP autofluorescence. The vacc-TK ORF was chosen to elucidate whether the encoded enzyme functionally complements ASFV-TK in this viral background and thus might be suitable for genetic labeling of respective recombinants.

WSL cells in 6-well plates were transfected with $4 \mu\text{g}$ of recombination plasmid pRFP-VaccTK and infected 5 h later with strain NHV-dTK-GFP/WSL-Bu at an MOI of 2. At 4 days p.i., cells from red autofluorescing plaques (Fig. 3a, b) were picked by aspiration and, after one freeze/thaw cycle, added to WSL-Bu cells in 96-well plates that had been incubated in HAT medium for 8 h. Cultures were maintained in HAT medium to inhibit replication of NHV-

dTK-GFP for 6 days. Cells from four red fluorescing plaques from wells with only few GFP-positive single cells (Fig. 3c, d) were harvested and distributed on WSL-Bu cells in 96-well plates. After 6 days of incubation in HAT medium, supernatants and cells from one well per plate showing only RFP-expressing plaques (Fig. 3e, f) were harvested. Analysis of the virus progeny by testing autofluorescence and sensitivity to BrdU (Fig. 4), as well as sequencing of PCR products encompassing the entire recombination cassette, demonstrated the homogeneity of all isolates, showing that selection of TK-positive ASFV recombinants on WSL-Bu cells using HAT medium is highly efficient. One of the isolates, named NHV-VaccTK-RFP, was selected for further experiments. We conclude that vaccinia TK functionally complements ASFV TK, since BrdU inhibited plaque formation by NHV-VaccTK-RFP on WSL-Bu cells (Fig. 4), whereas replication in HAT medium was not affected (Fig. 3).

To determine whether the expression of vaccinia TK influences growth of NHV-VaccTK-RFP in WSL cells, cultures were infected with this recombinant or NHV-dTK-GFP/WSL. The latter replicates in WSL cells comparably to the wt strain NHV (not shown). Culture supernatants and cells were harvested at the times indicated in Fig. 5 and titrated on WSL cells. The result revealed no significant differences between TK⁻ and vaccinia TK-expressing ASFV recombinants, which confirms that TK expression is neutral with regard to replication in WSL cells [21]. This also demonstrates that expression of vaccinia TK does not affect infectious replication on WSL cells.

Vaccinia TK complements ASFV TK function for replication in macrophages after low-MOI infection

In a previous publication, Moore et al. [18] reported that two TK-deleted ASFVs that had been generated from the cell-culture-adapted pathogenic ASFV isolates Malawi and

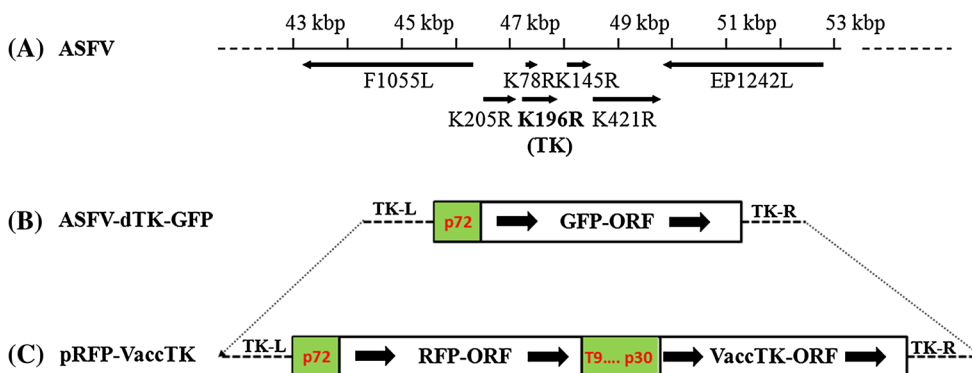


Fig. 2 Construction of recombinant ASFV. A) Schematic representation of the ASFV genome region encompassing the TK ORF. Names and directions of transcription of the ORFs are indicated. Nucleotide numbers (GenBank accession no. U18466.1) are given in kilobase pairs (kbp). The location of the TK ORF (K196R) is shown in bold. B) The TK locus of the TK-negative recombinant ASFV-dTK-GFP is depicted. Only relevant features are shown. C) Schematic diagram of pRFP-VaccTK. The left (TK-L) and right (TK-R)

segments of the ASFV TK gene are contained in pRFP-VaccTK and serve as recombination sequences. The ORF encoding the *Discosoma sp.* RFP is flanked by the p72 promoter and a polythymidylate tract (T9) for transcription termination [2] and followed by the p30 promoter to control expression of the vaccTK-ORF, which is followed by the ASFV TK transcription termination signal located in TK-R

Fig. 3 Isolation of NHV-VaccTK-RFP. WSL cells were transfected with 4 µg of recombination plasmid pRFP-VaccTK and infected 5 h later with strain NHV-dTK-GFP/WSL-Bu at an MOI of 2. GFP (a) and RFP (b) autofluorescence was photographed at 4 days p.i. Virions contained in cells of red autofluorescing plaques were added to WSL-Bu cells that were pre-incubated in HAT medium for 8 h. GFP (c) and RFP (d) autofluorescence in infected cultures, maintained in HAT medium, were photographed after 6 days (first round of HAT selection). GFP (e) and RFP (f) autofluorescence, photographed at 6 days after infection of WSL-Bu cells with first-round virions

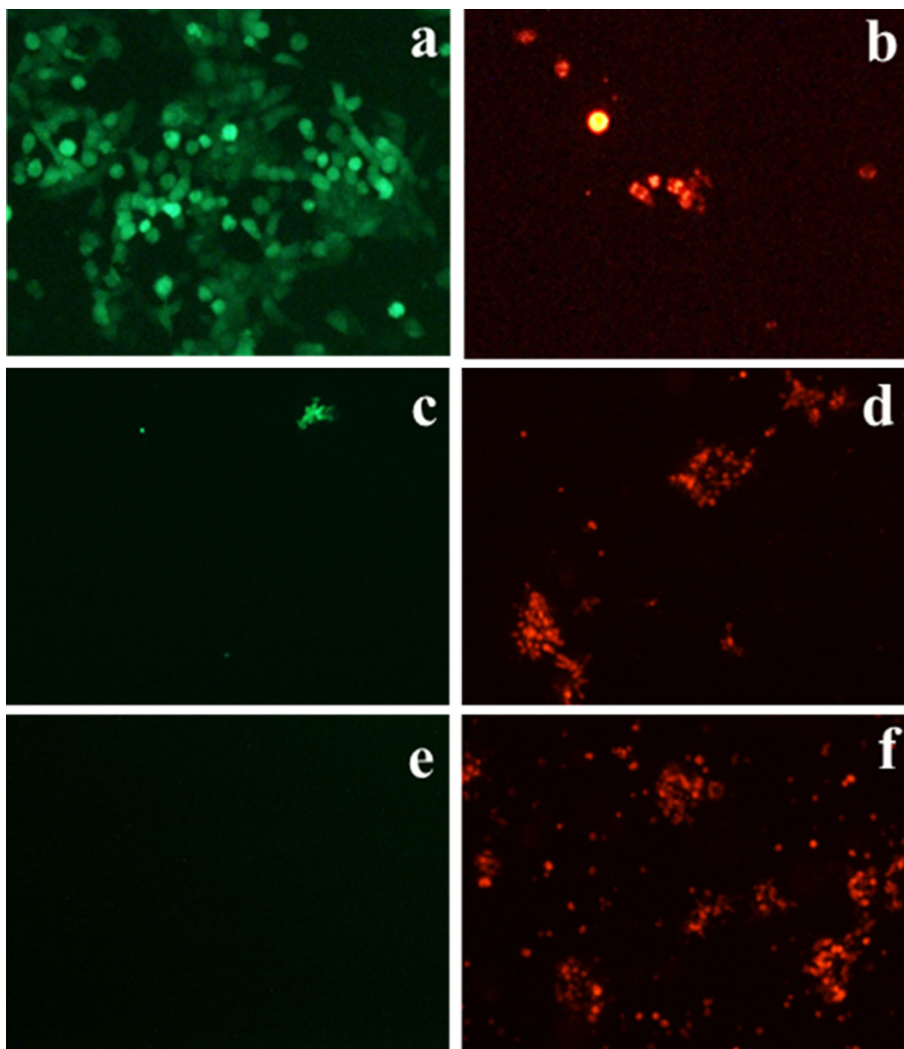


Fig. 4 NHV-VaccTK-RFP is sensitive to BrdU. WSL-Bu cells were infected and incubated without (left) or with $25 \mu\text{g ml}^{-1}$ BrdU (right). RFP autofluorescence was photographed 5 days later

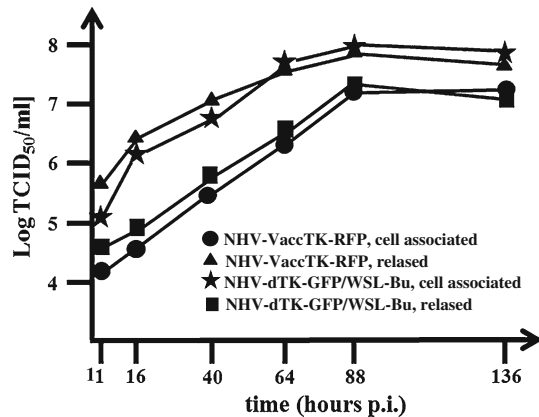
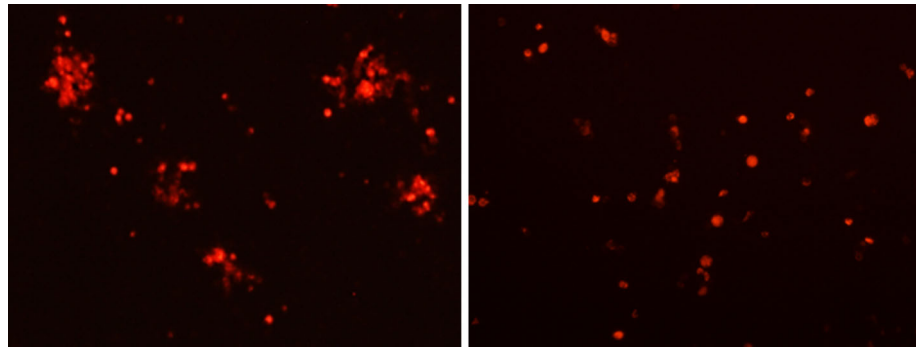


Fig. 5 Growth kinetics of NHV-VaccTK-RFP and NHV-dTK-GFP/WSL-Bu. WSL cells were infected with NHV-VaccTK-RFP (circles and triangles) and NHV-dTK-GFP/WSL-Bu (squares and asterisks) at an MOI of 2. Cells were washed with medium at 1 h p.i. Cells (circles and asterisks) and supernatants (triangles and squares) were harvested at the times indicated and titrated on WSL cells. Results are mean values from two experiments. Deviations were negligible and, for simplicity, not included

Haiti, grew well on Vero cells. However, both mutants had a growth defect in swine macrophages at low multiplicities of infection. To clarify whether the non-pathogenic ASFV strain NHV and its TK⁻ derivative share these properties and to elucidate whether expression of vacc-TK complements ASFV TK function in this respect, swine macrophage cultures were infected with NHV-dTK-GFP/WSL-Bu or NHV-VaccTK-RFP at MOIs between 10^0 and 10^{-4} (Fig. 6A). Cultures were harvested at 1 h p.i. to assess the remaining input virus titers, and at 5 days p.i. to determine the levels of infectious virus progeny production. The respective samples were titrated on WSL cells after one freeze/thaw cycle. As shown in Fig. 6A, NHV-VaccTK-RFP replicated productively in swine macrophage cultures after infection at all multiplicities tested, whereas infection with NHV-dTK-GFP/WSL-Bu did not yield detectable infectious progeny at multiplicities below 10^{-2} . This is also shown in Fig. 6B by comparison of the number of autofluorescing cells in macrophage cultures 1 day and 5 days after infection with NHV-dTK-GFP/WSL-Bu and

NHV-VaccTK-RFP at an MOI of 10^{-3} , demonstrating that the TK⁻ mutant failed to spread within the culture, in contrast to the TK⁺-revertant.

To further corroborate the functional complementation of the defect by vaccinia TK, porcine macrophage cultures were infected with wild-type (wt) NHV, NHV-dTK-GFP/WSL-Bu or NHV-VaccTK-RFP at an MOI of 10^{-2} . The growth kinetics (Fig. 7) showed that the parental strain NHV and the vaccTK-expressing revertant reached titers close to 10^6 TCID₅₀/ml, whereas the TK⁻ mutant replicated poorly, with maximum titers of only about 10^4 TCID₅₀/ml. Thus, for efficient productive replication in macrophages infected at a low MOI, TK expression is also essential for the low-virulent ASFV strain NHV.

Discussion

For viruses that express their own thymidine kinase (TK), cells lacking this enzyme activity allow selection for TK-negative virus mutants by addition of BrdU to the cell culture medium during virus propagation. This approach has been used before, e.g., for obtaining recombinant vaccinia viruses [7, 20] or bovine herpesvirus 1 mutants [3]. Screening of about 60 cell lines, provided by “The Collection of Cell Lines in Veterinary Medicine FLI-Insel Riems”, showed that WSL cells, derived from wild boar lung cells, are permissive for cell-culture-adapted virus and, in contrast to Vero cells, also for field strains of ASFV [21]. To isolate TK⁻ ASFV mutants, a BrdU-resistant variant was selected by adapting the cells to growth in successively increasing BrdU concentrations, as has been described by Kit et al. [16] for HeLa cells and Bello et al. [3] for MDBK cells. Cells resistant to $100 \mu\text{g}$ of BrdU/ml were designated WSL-Bu. Propagation on BrdU-containing media but inhibition of growth in HAT medium provides conclusive evidence for their TK-negative phenotype. This is also substantiated by the growth inhibition of TK⁺ ASFV by BrdU and sensitivity of TK⁻ ASFV mutants to HAT in these cells.

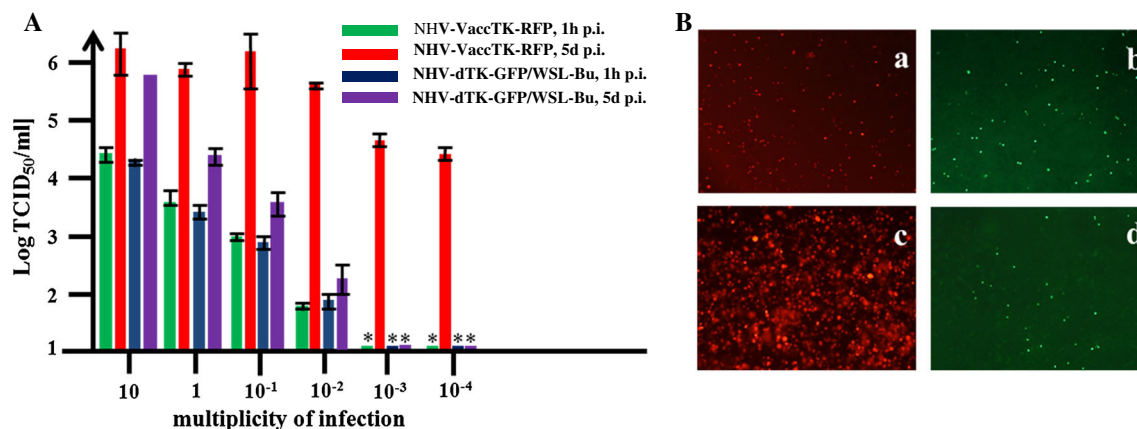


Fig. 6 Expression of TK is required for productive replication of ASFV strain NHV mutants in primary swine macrophages. A) Cells were infected with NHV-VaccTK-RFP (green and red bars) and NHV-dTK-GFP/WSL-Bu (blue and purple) at the MOIs indicated. Cultures were harvested at 1 h (green and blue bars) and 5 d (red and purple bars) p.i. and titrated on WSL cells. Results are mean

values from two experiments. Deviations are depicted. Asterisks indicate samples that were below the detection level of 10 TCID₅₀ ml⁻¹. B) RFP (a and c) and GFP (b and d) autofluorescence images from macrophage cultures infected at an MOI of 10⁻³ at 1 day p.i. (a and b) and 5 d p.i. (c and d)

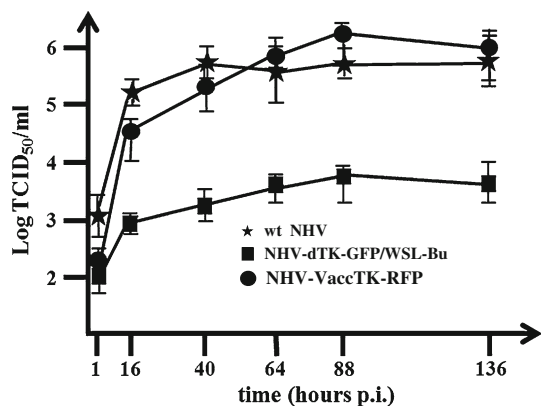


Fig. 7 Growth kinetics of ASFV strain NHV and TK mutants on primary swine macrophages after low-MOI infection. Cells were infected with wild-type NHV (asterisks), NHV-VaccTK-RFP (circles) and NHV-dTK-GFP/WSL-Bu (squares) at an MOI of 0.01. Cultures were harvested at the indicated times and titrated on WSL cells. The graph shows mean values from two experiments. Deviations are depicted

Analysis of the effect of TK deletion and replacement of the NHV-TK by vaccinia TK on infection of macrophages revealed that the heterologous enzyme fully complements the requirement for TK expression after low-multiplicity infection of macrophages by ASFV. We assume that this also applies to *in vivo* infection, since Moore et al. [18] showed that TK of highly pathogenic ASFV is required for efficient replication in swine.

The currently prevailing methodology to study ASFV gene function in the viral context is replacement or partial deletion of ASFV genes by insertion of ASFV p72/polythymidilate-flanked expression cassettes for β -glucuronidase- or β -galactosidase (reviewed in ref. [11]). This

approach enables identification and separation of recombinants from parental virus by chromogenic plaque assays. However, eight or more consecutive rounds of plaque purification are required for purification to homogeneity [1, 11], which extends the time needed to obtain the recombinant and carries the risk of introducing unwanted secondary mutations elsewhere into the genome. The use of WSL-Bu cells for selection of recombinants greatly reduced the number of passages and, consequently, the time needed for purification. A further advantage of the strategy described here arises from the use of open reading frames for autofluorescing reporter proteins. Since the efficiency of recombinant virus generation after transfection and superinfection cannot be predicted and is considerably dependent on the number of cells containing both recombination plasmid and viral DNA, monitoring the respective cell cultures for the presence of autofluorescing cells can provide an indication of the prospect of success of the approach as early as 16 hours after infection.

In addition, the availability of autofluorescing-protein-expressing ASFV variants facilitates at least *in vitro* applications for testing of antiviral molecules, preparation of diagnostics and determination of ASFV survival rates under varying environmental conditions (data not shown).

In summary, we have demonstrated that isolation of ASFV field strain mutants by targeting the viral TK gene is significantly improved in WSL-Bu cells and that TK⁺ viruses can be rapidly isolated out of an excess of TK⁻ viruses. These advances significantly improve the possibility of genetically manipulating ASFV in order to determine gene and protein functions. Furthermore, our results show that integration of the vaccinia TK ORF, accompanied by the RFP expression cassette, if desired,

into the NHV genome does not detectably interfere with NHV replication, that the low-pathogenic NHV strain requires TK expression to grow in low-multiplicity-infected macrophages, and that heterologous vaccinia TK can functionally complement the authentic ASFV enzyme.

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Conflict of interest The authors declare that they have no conflict of interest.

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