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Original Article

Direct cloning of a herpesvirus genome for rapid generation of infectious BAC clones

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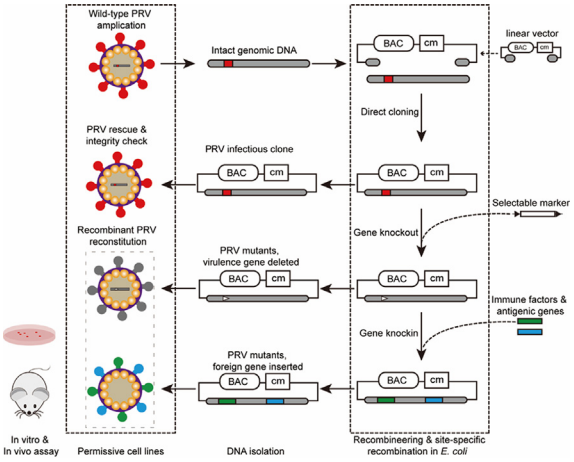
HIGHLIGHTS

- Direct cloning of the isolated herpesvirus genome by ExoCET without the multiple rounds of plaque purification.
- Cloning method avoiding the potential for attenuating mutations to occur during serial passage of the virus in cells.
- Rapid reconstitution of genetically indistinguishable virus upon acquiring the intact viral genome by restriction endonuclease digestion.
- Viral BAC being stable for genetic manipulation in *E. coli*.
- A streamlined approach to develop vector vaccines based on the attenuated recombinant PRV.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: The herpesviridae are DNA viruses with large and complicated genomes. The herpesvirus bacterial artificial chromosomes (BACs) have been useful for generating recombinant viruses to study the biology and pathogenesis. However, the conventional method using homologous recombination is not only time consuming but also prone to accumulate attenuating mutations during serial passage of the virus in cells. Elimination of the BAC vector from the recombinant viral genome requires additional step for phenotypically consistence with the original strain.
Objectives: To generate a streamlined approach for generating infectious BAC clones of herpesvirus.
Methods: The 142-kb pseudorabies virus genome was directly cloned into a bacterial artificial chromosome (BAC) in *Escherichia coli* by Exonuclease Combined with RecET recombination (ExoCET). Placement of the BAC vector at the terminus of the linear virus genome enabled excision of the BAC backbone from the viral genome by restriction endonuclease for delivery into mammalian cells, with the subsequent rapid rescue of virus that was genetically identical to the original strain.
Results: This new approach for molecular cloning of the genome from a large DNA virus and isolation of pure virus lacking the BAC vector from transfected mammalian cells bypass the tedious and time-consuming method of multiple rounds of plaque purification. The viral BAC was stable in E. coli, allowing further mutagenesis mediated by the Red system or various site-specific recombination methods.

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Conclusion: An efficient method for construction of infectious clones of herpesvirus was established. It is expected to be potentially useful for other viruses with large double-stranded DNA genomes.

Introduction

The Herpesviridae is a large family of viruses with double-stranded DNA genomes that range in size from 125 to 240 kilobase pairs. Generating recombinant viruses to study the biology of these viruses and to develop vaccines against them requires precise and effective genetic manipulation methods. Since the construction of the bacterial artificial chromosome (BAC) for murine cytomegalovirus (MCMV) as the first herpesvirus infectious clone [1], numerous herpesvirus genomes have been cloned into BAC vectors. Viral BACs have been established for every human herpesvirus except HHV-7 [2]. More than a dozen animal herpesviruses that are often used as models for studying viral pathogenesis or as vectors for vaccine and gene therapy have been cloned as viral BACs, including Marek's disease virus (MDV), pseudorabies virus (PRV), turkey herpesvirus (HVT), feline herpesvirus type 1, murine gammaherpesvirus 68, rhesus rhadinovirus (RRV), and herpesvirus saimiri [3]. BAC technology has been proven to be an invaluable tool for functional delineation of these viral genomes.

The conventional method for constructing herpesvirus BACs involves direct insertion of the BAC vector into the viral genome via homologous recombination [1,4–7]. The linear BAC vector, flanked on each side by 500–1000 base pairs of viral DNA homologous to the target genome, and the purified viral genomic DNA are cotransfected into virus-permissive cells. Homologous recombination takes place in the cell, producing recombinant virus carrying the BAC vector. Viral plaques are purified using selection based on an antibiotic resistance gene, beta-galactosidase, green fluorescent protein (GFP) or a metabolic gene from the BAC vector. Since the viral genome becomes circularized once during replication in the nucleus, the recombinant viral DNA containing the BAC vector is isolated from infected cells and transformed into *Escherichia coli* for screening using the antibiotic marker of the BAC vector, usually for chloramphenicol resistance. The DNA of the infectious clone is prepared from *E. coli* for restriction enzyme analysis, and once the integrity of the viral BAC is confirmed, the BAC DNA can be prepared and transfected into mammalian cells for reconstitution of the virus.

Other approaches for generating herpesvirus BACs have also been used [8–11]. A critical factor in using infectious clones is that the virus rescued after delivery of the BAC DNA into mammalian cells should be phenotypically consistent with the wild-type parent. One approach to addressing this issue involves insertion of the BAC vector into a gene dispensable for viral growth and pathogenesis, for example the gG gene of PRV and the US2 locus of MDV [12,13]. However, the presence of the BAC vector backbone can cause instability of the PRV genome, resulting in spontaneous deletion of the BAC vector together with the surrounding viral sequences. This finding motivated removal of BAC vector sequences from viral genomes, with three approaches explored to achieve this goal. One approach incorporated duplicated viral sequences flanking both sides of the BAC vector, which led to homologous recombination between the viral sequences upon delivery to mammalian cells and resulted in excision of the BAC vector for reconstitution of a genotypically wild-type MCMV [14]. A slightly modified alternative involved inserting the BAC vector into the terminal repeat (TR) region; the BAC vector was excised upon reconstitution in cells through TR-mediated homologous recombination to rescue wild-type RRV [15]. The second approach utilizes site-specific recombination, with expression of Cre from the BAC vector as a good example [16]. To avoid instability of the viral BAC in *E. coli*, the cre/loxP recombination can be completely abolished by splitting the Cre coding sequence with an artificial intron. After delivery of the viral BAC into mammalian cells, the intron is spliced out to restore Cre activity, and pure virus lacking the BAC vector backbone can be isolated [16]. The third approach involves deletion of mini-F sequences with a repair vector or PCR product in eukaryotic cells by homologous recombination [17]. To increase the efficiency, the viral BAC, in this case HVT BAC, was linearized by the homing endonuclease I-SceI cutting at one end of the vector backbone before transfection together with a repair plasmid. Enhanced GFP (eGFP) expression from the BAC vector backbone facilitated the screening [18].

Herpesvirus genomes are tightly packed in virions in a linear form, a form that we exploited in our approach. Using PRV as an example, we present here an efficient approach to construct infectious herpesvirus clones by placing the BAC vector at the linear terminus of the viral genome via Exonuclease Combined with RecET recombination (ExoCET)-mediated direct cloning [19]. The Rac prophage proteins RecE/RecT mediate highly efficient linear–linear homologous recombination that is mechanistically distinct from recombineering mediated by Redα/Redβ from lambda phage. Using RecET direct cloning, 50 kb fragment of bacterial genomic DNA were captured with a linear plasmid vector [20]. However, the capacity of RecET direct cloning has been limited by co-transformation efficiency, i.e., the efficiency of obtaining the BAC vector and target DNA in the same *E. coli* cell. Therefore, direct cloning of DNA fragments larger than 50 kb by RecET is inefficient. Incubation of linear DNA with T4 polymerase as the in vitro exonuclease enables annealing of some of the linear DNA partners at one of their complementary ends. The BAC vector and target DNA has joined in one piece, breaking the bottleneck of low co-transformation efficiency of two DNA molecular entering in the same *E. coli* cell. The other complementary end can be recombined after pre-annealed linear DNA has been electroporated into the *E. coli* expressing RecET. This ExoCET system was shown to be a simple and efficient direct cloning method to obtain 106 kb of DNA from a prokaryotic genome (4×10^6 bp) and 53 kb of DNA from a more complex eukaryotic genome (3×10^9 bp) [19].

Unlike the methods previously described for viral clone construction and BAC vector excision, direct cloning of the herpesvirus genome via ExoCET offers several advantages. First, co-transformation of the BAC vector backbone and the isolated viral genome DNA into RecET-expressing *E. coli* directly yields the infectious BAC clone, not only bypassing the need for multiple rounds of plaque purification but also avoiding the attenuating mutations that can occur during these rounds of purification. Additionally, linear viral genome is acquired by removing the BAC vector with a restriction endonuclease, followed by delivery into mammalian cells for rapid rescue of the virus, which is genetically identical to the wild-type parent, without any purification process. Finally, the viral BAC, which has the BAC vector backbone incorporated at the viral terminus, is stable in *E. coli* for mutagenesis using the Red system or various site-specific recombinase-mediated methods.

Materials and methods

Ethics statement

All experiments were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The use of animals in this study was approved by the South China Agricultural University Committee of Animal Experiments (approval ID: SYXK2019-0136).

Cells and viruses

The PRV DCD-1 strain (GenBank accession no. OL639029), which is highly pathogenic to pigs, was isolated in Guangzhou, China, in March 2017. The highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) and NADC30-like porcine reproductive and respiratory syndrome virus (NADC30-like virus) were isolated in Guangzhou, China, in January 2019.

African green monkey kidney cells (Vero) and porcine kidney epithelial cells (PK15) were cultured in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal bovine serum and 1% (vol/vol) penicillin–streptomycin. Vero cells were used to rescue PRV DCD-1-derived recombinants and to propagate HP-PRRSV and NADC30-like virus. PK15 cells were used to determine the titer of PRV DCD-1-derived recombinants.

E. coli strains and plasmids

E. coli GB05-dir harboring the expression plasmid pSC101-BAD-ETgA-tet was used for ExoCET direct cloning of the genomic DNA of PRV DCD-1. *E. coli* GB08-red was used for deletion of the virulence genes and insertion of genes for fluorescent reporters and immune factors and antigen genes. *E. coli* GB2005 harboring pSC101-BAD-Cre-tet, pSC101-Rha-Dre-tet or pSC101-BAD-Flp-tet was used to remove the resistance genes after recombineering. pBeloBAC11-cm-ccdB-hyg was used to amplify the linear pBeloBAC11-cm vector for construction of the intermediate plasmid pBeloBAC11-cm-PRV-pBR322-amp-ccdB**.** The pBeloBAC11-cm-PRV-pBR322-amp-ccdB was propagated in *E. coli* GBred-gyrA462. pR6K-kan-ccdB was used as template to amplify the kanamycin resistance gene. The plasmids and bacterial strains for ExoCET direct cloning and Red recombineering can be freely requested from our laboratory (Shandong University) for academic research.

Construction of PRV DCD-1 infectious clones and virus rescue

PRV genomic DNA was extracted from PK15 cells infected with DCD-1 using a QIA-amp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The pBeloBAC11-cm-pBR322-amp-ccdB plasmid was constructed by linear plus linear recombination in GB05-dir (Table S1). The pBeloBAC11-cm linear cloning vector was purified from a gel after electrophoresis of the BamHI-digested pBeloBAC11-cm-pBR322-amp-ccdB. The PRV genomic DNA and pBeloBAC11-cm linear vector were incubated with T4 polymerase according to the described protocol for the ExoCET method [21]. The optimal ratio of the amounts of genomic and vector DNA was titrated and was highly dependent on the molarity and quality of the DNA. The 20 μl reactions consisted of 2 μl of 10 × NEBuffer 2.1 and 0.13 μl of 3 U μl^{-1} T4 polymerase (NEB, cat. no. M0203). The in vitro assembly reactions were prepared in PCR tubes and cycled in a thermocycler as follows: 25℃ for 1 h, 75℃ for 20 min, 50℃ for 30 min, and then held at 4℃. After dialyzing, the DNA mixture was electroporated into L-arabinose-induced *E. coli* GB05-dir harboring pSC101-BAD-ETgA-tet. The infectious clone pBeloBAC11-cm-DCD1 was selected on LB plates containing 15 μg/ml chloramphenicol. The correct recombinants were identified by PvuII restriction enzyme analysis. Sequencing of BAC clones was conducted by PacBio. Two PmeI sites were placed on both ends of the BAC backbone. The PmeI-digested infectious clone was transfected into Vero cells using Lipofectamine 3000 (Thermo Fisher Scientific, Shanghai, China) to rescue the virus rDCD-1.

Knockout of the virulence genes

Using the infectious clone pBeloBAC11-cm-DCD1, the gE-gI, thymidine kinase (TK) and gG genes were deleted by Redαβ recombineering in *E. coli* GB08-red. The kanamycin resistance gene flanked by flippase recognition target (FRT) sites was used to target the gE-gI locus. The homologous arms were incorporated in the oligonucleotides (Table S1). The recombinants were selected on LB plates containing 15 μg/ml kanamycin, and after confirmation by restriction enzyme analysis, the correct clones were transformed into *E. coli* GB2005 harboring pSC101-BAD-Flp-tet for FLP recombination. Removal of the selectable marker was validated by double-streaking on a kanamycin-containing LB plate and a chloramphenicol-containing LB plate.

Using the gE-gI-deleted recombinant pBeloBAC11-cm-DCD1-ΔgEgI, the TK and gG genes were sequentially targeted with the gentamicin resistance gene flanked by lox66 and lox71 sites. The lox66 had mutation at the left inverted repeat and the lox71 had the mutation at the right inverted repeat. Recombination between the lox71 and lox66 sites resulted in a double-mutant lox72 site, which could not be further catalyzed by Cre [22]. The recombinants were selected on LB plates containing 2 μg/ml gentamicin. Then the recombinant BACs were transformed into *E. coli* GB2005 harboring pSC101-BAD-Cre-tet for Cre recombination. Removal of the selectable marker was validated by double-streaking on a gentamicin-containing LB plate and a chloramphenicol-containing LB plate. The modified BACs were named pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK and pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-ΔgG.

The above BACs were transfected into Vero cells using Lipofectamine 3000 to rescue the attenuated mutants rDCD1-ΔgEgI, rDCD1-ΔgEgI-ΔTK and rDCD1-ΔgEgI-ΔTK-ΔgG. The recombinant virus was verified by PCR using primers flanking the FRT or lox sites (Table S1).

Knockin of the fluorescent reporter and immune factor genes and antigen genes

The cassettes mNeonGreen-lox66-genta-lox71, mCherry-FRT-Kan-FRT and enhanced blue fluorescent protein (eBFP)-rox-Kan-rox, flanked with the homologous arms to the TK, gG or gE locus, respectively, were assembled by linear plus linear recombination in *E. coli* GB05-dir to generate three intermediate plasmids (Fig. S7). The cassette was released from its plasmid backbone using the designed restriction enzyme recognition sites and electroporated into *E. coli* GB08-red containing pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-ΔgG. The recombinant BACs with gentamicin or kanamycin resistance genes were transformed into *E. coli* GB2005 harboring pSC101-BAD-Cre-tet, pSC101-BAD-Flp-tet or pSC101-BAD-Dre-tet for site-specific recombination to remove the selectable markers. The final BACs with the fluorescent reporter genes were pBeloBAC11-cm-DCD1-ΔTK-mNeonGreen, pBeloBAC11-cm-DCD1-ΔgG-eBFP and pBeloBAC11-PRV-DCD1-ΔgEgI-mCherry. The mNeonGreen, eBFP and mCherry coding regions were placed under the endogenous promoters of TK, gG and gE respectively. Additionally, pBeloBAC11-cm-DCD1-ΔTK-CMV-mNeonGreen and pBeloBAC11-cm-DCD1-ΔTK-CAG-mNeonGreen were constructed

to investigate the CMV and CAG (CMV early enhancer/chicken β actin) promoter at the TK locus.

Using the same approach as the fluorescent reporter gene knockin, the pBeloBAC11-cm-DCD1-ΔgG-IL18-γ was constructed. The construct contained a P2A sequence, which encodes a self-cleaving peptide. Genes for the immune factors IL18 and IFN-γ were fused via the P2A sequence and placed under the gG promoter. The antigen genes GP3, GP5 and M were knocked in at the TK locus; the cDNA for these genes was obtained by RT-PCR from the genome of HP-PRRSV or NADC30-like virus. The three genes were fused via the P2A sequence and placed under the TK, CMV or CAG promoters.

Analysis of exogenous gene expression

Fluorescent protein expression in the recombinant PRV DCD-1 was visualized by confocal microscopy. Vero cells were seeded onto a slide in a six-well plate and then inoculated with the recombinant virus. After 12 h, the plate was examined using a confocal laser scanning microscope.

For qRT-PCR analysis, total RNA was extracted with TRIzol (Sigma), and reverse transcription was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara China). The quantity of cDNA was determined by SYBR Green I qRT-PCR performed with a Light Cycler 1.5 (Roche, Basel, Switzerland). The primers for qRT-PCR are listed in Table S1. The reaction mixture for qRT-PCR was as follows: 10.0 μl of 2 × SYBR Green Premix ExTaq II (TaKaRa Biotech, Dalian, China); 0.4 μl of each primer (25 μM); 2.0 μl 1 × cDNA template; and 7.6 μl nuclease-free water, with a total volume per reaction of 20 μl. The reaction procedure included pre-denaturation at 95 °C for 10 min, followed by 40 cycles of a melting step at 95 °C for 10 sec and annealing at 60 °C for 1 min. The fragments for quantification were cloned into a pBR322 vector to prepare a standard plasmid. Serial standard plasmid dilutions (10^2 to 10^8 copies/μl) were used to optimize the qRT-PCR reaction and determine the standard curve. Final concentrations were calculated per microliter of DNA.

For Western blots, Vero cells were inoculated with the DCD-1 mutants for 16 h in a six-well plate. Twenty microliters of each of the virus-infected cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto nitrocellulose membranes. Western blotting was carried out using monoclonal anti-HA mouse antibody (1:500 dilution, Shanghai, China) for IL18-γ and monoclonal anti-Flag mouse antibody (1:500 dilution, Shanghai, China) for HP-PRRSV GP3-GP5-M as the primary antibodies, and then horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution, Southern Biotechnology, USA) as the secondary antibody.

One-step growth curves (TCID50)

The in vitro replication capacity of the rescued viruses was assessed in PK15 cells. Briefly, PK15 cell monolayers in 6-well plates were infected with the fourth passage of wild-type PRV DCD-1, the rescued DCD-1 and the various mutants at 1.5 × 10^3 TCID50 (multiplicity of infection ≈ 0.01), and each virus was harvested at the indicated times post-infection (p.i.). The median tissue culture infective dose (TCID50) of the harvested viruses was determined using 96-well plates. Then, PK15 cells in 96-well plates were inoculated with 10-fold diluted viral stocks and incubated at 37 ℃ with 5% CO2 for six days. The TCID50 values were calculated by observing the cytopathic effect, according to the Reed and Muench method [23].

Animal experiments

To evaluate the virulence of the attenuated virus in vivo, three groups (5 mice per group) of four-week-old BALB/c mice were infected orally with 0.1 ml of 10^6 TCID50 of virus (DCD-1, rDCD-1, or rDCD1-ΔgEgI-ΔTK-ΔgG). One group of mice was inoculated with DMEM without virus as a negative control. The infected and control groups were separately housed in different negative-pressure isolators and monitored daily for 14 days while the morbidity and mortality of the animals were recorded.

Statistical analysis

The data were shown as mean ± standard deviation (SD). Statistical analysis was performed using the one-way ANOVA in the GraphPad Prism package. Significant differences were determined as $^*p < 0.05$ (significant) or $^{**}p < 0.01$ (highly significant).

Results

Generation of PRV infectious clone by direct cloning

The approach for constructing the PRV DCD-1 infectious clones and mutants is illustrated in Fig. 1. To clone the entire PRV DCD-1 genome, we employed the ExoCET method to ensure successful cloning of a double-stranded stretch of DNA larger than 100 kb into a BAC vector [21]. The intermediate plasmid pBeloBAC11-cm-PRV-pBR322-amp-ccdB was constructed by RecET-mediated linear plus linear homologous recombination, incorporating 80 bp homology arms (HA) from the termini of the linear PRV genome (Fig. S2). The intermediate plasmid pBeloBAC11-cm-PRV-pBR322-amp-ccdB was propagated in *E. coli* GBred-gyrA462, which has an Arg462Cys mutation in gyrA that confers CcdB resistance [34]. However, the direct cloning was conducted in *E. coli* GB05-dir, which has the wild-type GyrA that is sensitive to CcdB. The toxin-encoding gene ccdB selected against background DNA of the intermediate plasmid remaining due to incomplete BamHI digestion. After BamHI digestion, the HA were exposed at the linear BAC vector. The DCD-1 genomic DNA and the linear vector were firstly incubated in the exonuclease reaction system for in vitro assembly and then co-electroporated into RecET-expressing *E. coli* strain to complete direct cloning. The optimal ratio of linear vector to DCD-1 genomic DNA was titrated. In a 20 μl reaction, 0.5 μg or 1 μg of linear vector was tested with 1 μg, 2 μg, 5 μg, or 10 μg of DCD-1 genomic DNA. With 0.5 μg linear vector and 2 μg or 5 μg of DCD-1 genomic DNA, three correct infectious clones were identified from 72 colonies. With 1 μg linear vector and 2 μg DCD-1 genomic DNA, two correct infectious clones were identified from 72 colonies. However, results were suboptimal with 1 μg or 10 μg DCD-1 genomic DNA, leading to one or zero infectious clones from 72 colonies. Incorrect clones were mainly empty vector resulting from self-circularization. The correct infectious clones of pBeloBAC11-cm-DCD1 were screened by restriction enzyme analysis (Fig. 2A). Following the cloning experiments, which were completed within one week, whole BAC sequencing was conducted on four BAC clones with different restriction enzyme digestion patterns. One clone was identical to the viral genome sequence, and the other three clones had deletions in the Us region which is, bracketed by the inverted repeats. After PmeI digestion, the BAC DNA was transfected into Vero cells. Cytopathic effects were observed at 2 to 4 days post transfection, suggesting that the infectious clones gave rise to viable viruses (Fig. 2B and 2C). Absence of the BAC vector backbone was confirmed by PCR (Fig. S6). The stability of the rescued DCD-1 was validated by PCR analysis after three passages in PK15 cells (Fig. 2D).

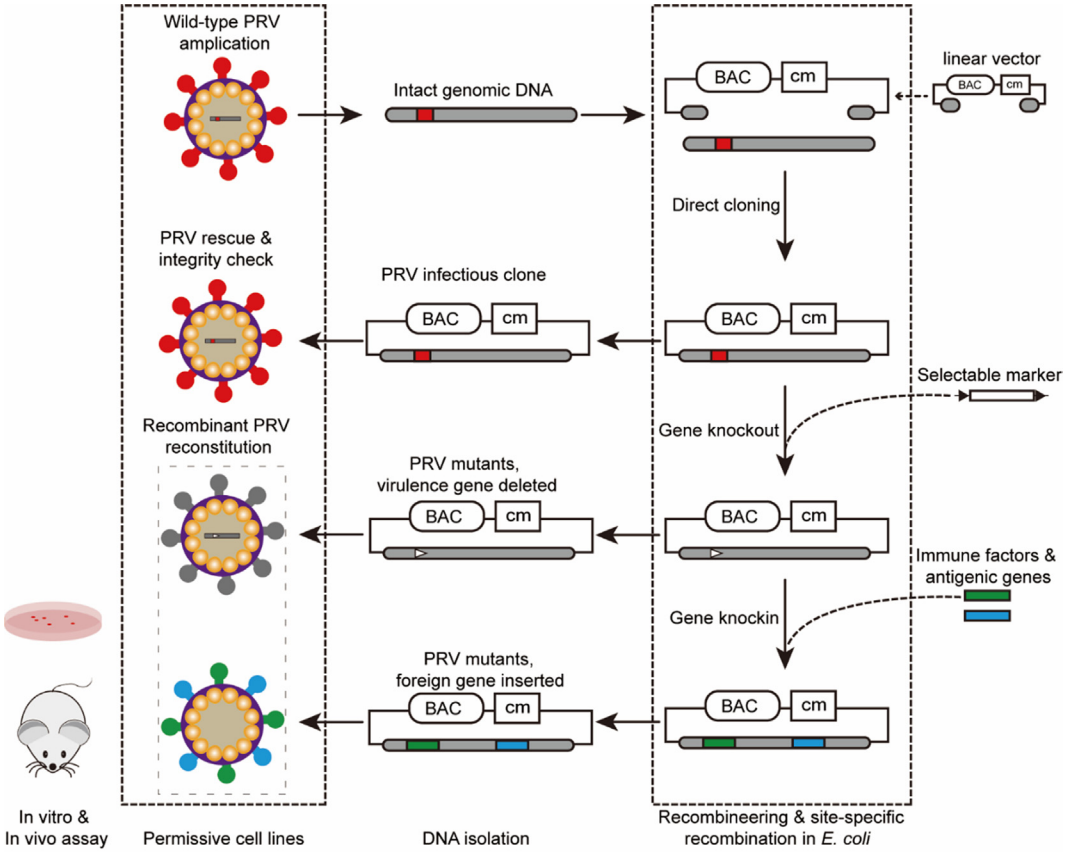

Fig. 1. Construction of pseudorabies virus infectious clone. After amplification and purification of the highly pathogenic PRV isolate DCD-1, intact viral DNA was isolated. The intermediate plasmid pBeloBAC11-cm-PRV-pBR322-amp-ccdB, which contained the 80 bp end sequences of the PRV genome, was constructed. BamHI digestion of the intermediate plasmid released the linear vector pBeloBAC11-cm and exposed homology arms. The viral DNA and the linear vector were co-electroporated into a RecET-expressing *E. coli* strain for direct cloning. The infectious clone was validated by restriction enzyme analysis, a rescue experiment and integrity check. Knockout of the virulence gene (represented by the red box in the genome) and knockin of the immune factor genes and antigen genes were achieved by Redαβ-mediated deletion or insertion followed by site-specific recombination to remove the selectable marker. The recombinant virus was rescued for in vitro and in vivo characterization.

The recombinant attenuated DCD-1 was generated by deletion of the virulence genes UL23 (TK) and US8 (gE)-US7 (gI), as well as the dispensable gene US4 (gG) (Fig. S3). The kanamycin resistance gene flanked by FRT sites was used for Redαβ-mediated deletion of gE-gI, with the same region deleted as in the natural gE-gI deletion in the attenuated PRV vaccine strain Bartha-k61 [24]. The selectable marker was removed by Flp recombination, and the TK and gG genes were sequentially replaced by the lox66-genta-lox77 cassette and Cre recombination to eliminate the gentamicin resistance gene. BAC clones derived by site-specific recombination were confirmed by restriction enzyme analysis and sequencing (Fig. 2A, Fig. S4). After rescue of the recombinant virus, the stability of the attenuated DCD-1 was characterized by PCR analysis after multiple passages (Fig. 2D). The titers of wild-type DCD-1, BAC-derived DCD-1 and various attenuated DCD-1 strains showed no obvious differences (Fig. 2E). Immune factor and antigen gene knockins were also conducted by BAC engineering.

The virulence of the attenuated recombinant PRV DCD-1 was tested in SPF mice. Mice inoculated with the parental DCD-1 exhibited 100% mortality. In contrast, mice inoculated with rDCD1-ΔgEgI-ΔTK-ΔgG exhibited no mortality (Fig. S8). This result is consistent with a previous study showing that gE-gI and TK are vital for viral virulence but dispensable for viral replication [25–27].

Promoter assessment at the TK, gG and gI loci

Attenuated DCD-1 has the potential to not only be a vaccine but also to be a live vaccine vector for expressing foreign antigens. Since the deletion of the three virulence factors did not alter the stability or the titer of the DCD-1, we examined the expression profile of foreign genes at these three loci using various fluorescent proteins. The mNeonGreen, eBFP and mCherry genes were expressed from the endogenous promoters of the TK, gG and gI genes, respectively. In addition, we also investigated CMV and CAG promoters driving mNeonGreen expression at the TK locus (Fig. 3A). Recombinant DCD-1 viruses expressing these fluorescent proteins could be rescued from the infectious clones without any difficulty. Expression of the fluorescent proteins did not change the propagation properties when compared to the wild-type virus (Fig. 3B). The gG promoter has been reported to be one of the strongest promoters of PRV [28–30]. However, according to the qPCR results, it was not stronger than the TK promoter or the CMV and CAG promoters at the TK locus (Fig. 3C). At the TK locus, the CAG promoter was slightly stronger than the other promoters. Based on fluorescence intensity, live cell imaging of recombinant viruses under the confocal microscope showed that the gI promoter was not as strong as the others (Fig. 3D and 3E), which was consistent with the qPCR data (Fig. 3C).

Expression of cytokines and antigen genes in recombinant DCD-1

To develop the attenuated DCD-1 strain as a viral vector vaccine, genes for the cytokines IL18 and IFN-γ were inserted at the gG locus, aiming to enhance the immune response to the virus. The two cytokines were co-expressed from the gG promoter using the 2A self-cleaving, peptide-based, multi-gene expression system.

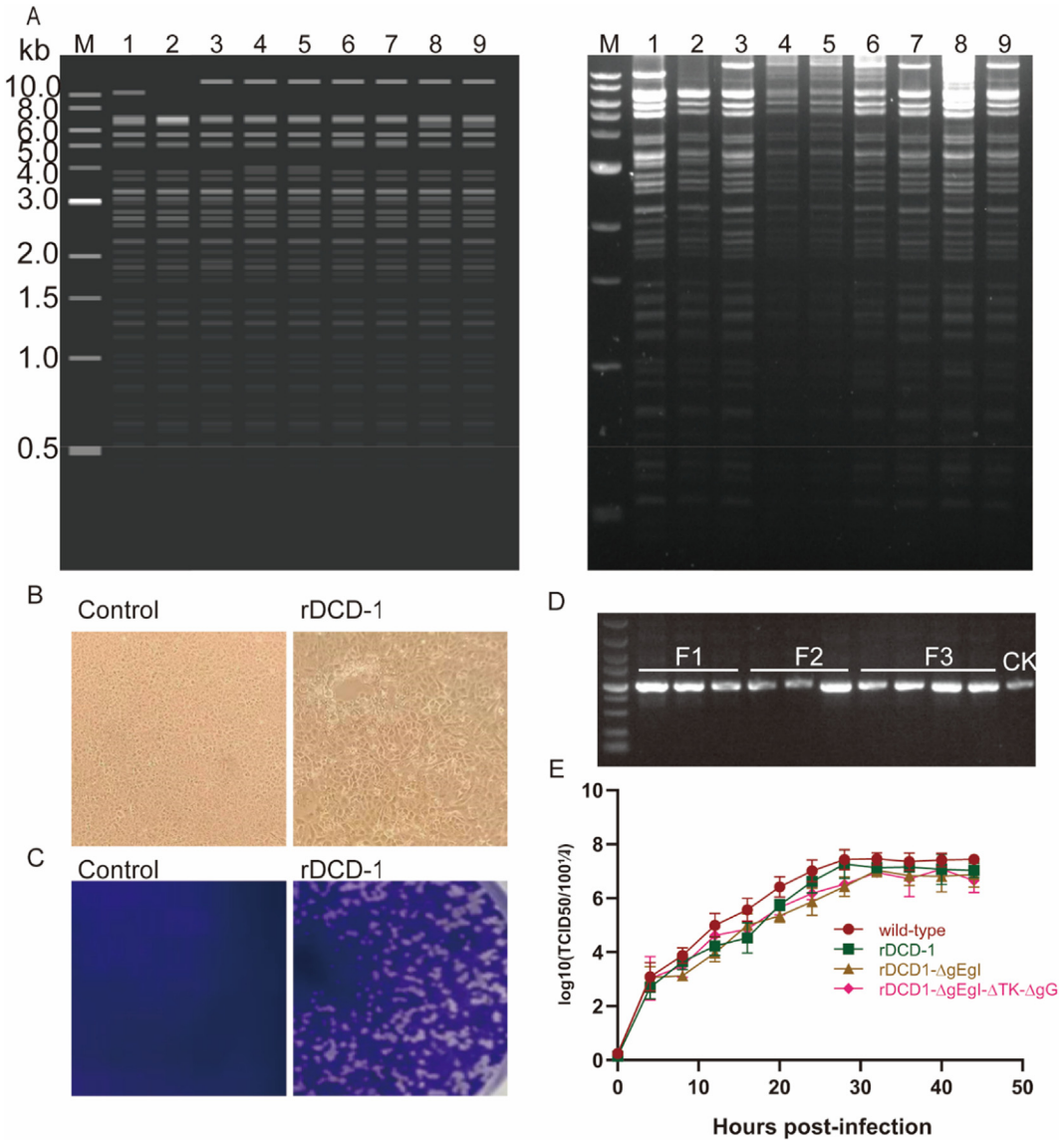

Fig. 2. Validation and characterization of the infectious clones. (A) NotI restriction enzyme analysis of the wild-type DCD-1 BAC clone and the mutants. Left panel, pattern from in silico prediction (SnapGene); right panel, agarose gel picture after electrophoresis. M, 1 kb DNA ladder (NEB); lanes 1–9, pBeloBAC11-cm-DCD1, pBeloBAC11-cm-DCD1-ΔgEgI, pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-ΔgG-IL18-γ, pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-HPPRRSV-ΔgG-IL18-γ, pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-NADC30like-ΔgG-IL18-γ, pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-CMV-HPPRRSV-ΔgG-IL18-γ, pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-CMV-NADC30like-ΔgG-IL18-γ, pBeloBAC11-cm-DCD1-ΔgEgI-ΔTK-CAG-HPPRRSV-ΔgG-IL18-γ, and pBeloBAC11-cmDCD1-ΔgEgI-ΔTK-CAG-NADC30like-ΔgG-IL18-γ (Fig. S4). (B) Rescue experiment. The linearized BAC DNA of pBeloBAC11-cm-DCD1 was transfected into Vero cells, showing typical cytopathic effects (CPE) of PRV infection in cell culture. Left, mock infection; right, CPE of rescued viruses in Vero cells. (C) Validation of the rescued DCD-1. The rescued DCD-1 was used to infect Vero cells, plates were fixed at 48 h post-infection and stained with crystal violet. Left, mock infection; right, plaques formed in monolayer Vero cultures. (D) Stability of the rescued virus. After serial passages in PK15 cells, the cell lysates were subjected to PCR analysis with primers listed in Table S1. F1, passage 1; F2, passage 2; F3, passage 3; CK, control using DCD-1 virus as template. (E) Titer comparison using the wild-type DCD-1, BAC-derived DCD-1 and BAC-derived mutants. Vero cells were infected with rescued wild-type DCD-1 and the mutants at a multiplicity of infection (MOI) of 0.01 PFU/cell. Each virus was harvested at the indicated time points and titrated as described. Total viral titers were determined in three technical replicates and expressed as the median tissue culture infectious dose (TCID50/100 μl). wild, wild-type DCD-1; rDCD-1, BAC-derived DCD-1; rDCD1-ΔgEgI, gE/gI deletion mutant; rDCD1-ΔgEgI-ΔTK-ΔgG, gE/gI, TK and gG deletion mutant.

HP-PRRSV has become widely spread in China since 2006, and there was an outbreak of the NADC30-like virus in 2015, with this virus becoming locally dominant in some provinces. Although NADC30-like virus is as not pathogenic as HP-RRRSV, it has a high incidence of recombination with other virus strains, which can result in changes in virulence. The current commercial PRRSV vaccines cannot provide complete protection against infection with HP-PRRSV and NADC30-like virus. Using the IL18- and IFN-γ-expressing mutants, we cloned genes GP3, GP5 and M, which are antigen genes of the HP-PRRSV and NADC30-like viruses, and expressed them under the endogenous TK promoter, CMV promoter or CAG promoter. The GP3, GP5 and M genes were co-expressed using P2A peptide linkers (Fig. 4A). The titers of the modified viruses containing the GP3, GP5 and M gene were determined with single-step growth curve analysis. All of the recombinant viruses replicated to titers comparable to that of rDCD-1 (Fig. 4B).

To analyze the expression of cytokines and the antigen genes, PK15 cells were infected with the rescued recombinant PRV DCD-1 strains and harvested at 12 hpi. RNA in cell lysates was analyzed by qRT-PCR. The transcription of IL18 and IFN-γ reached the same level, because they were in the P2A system. Furthermore, the

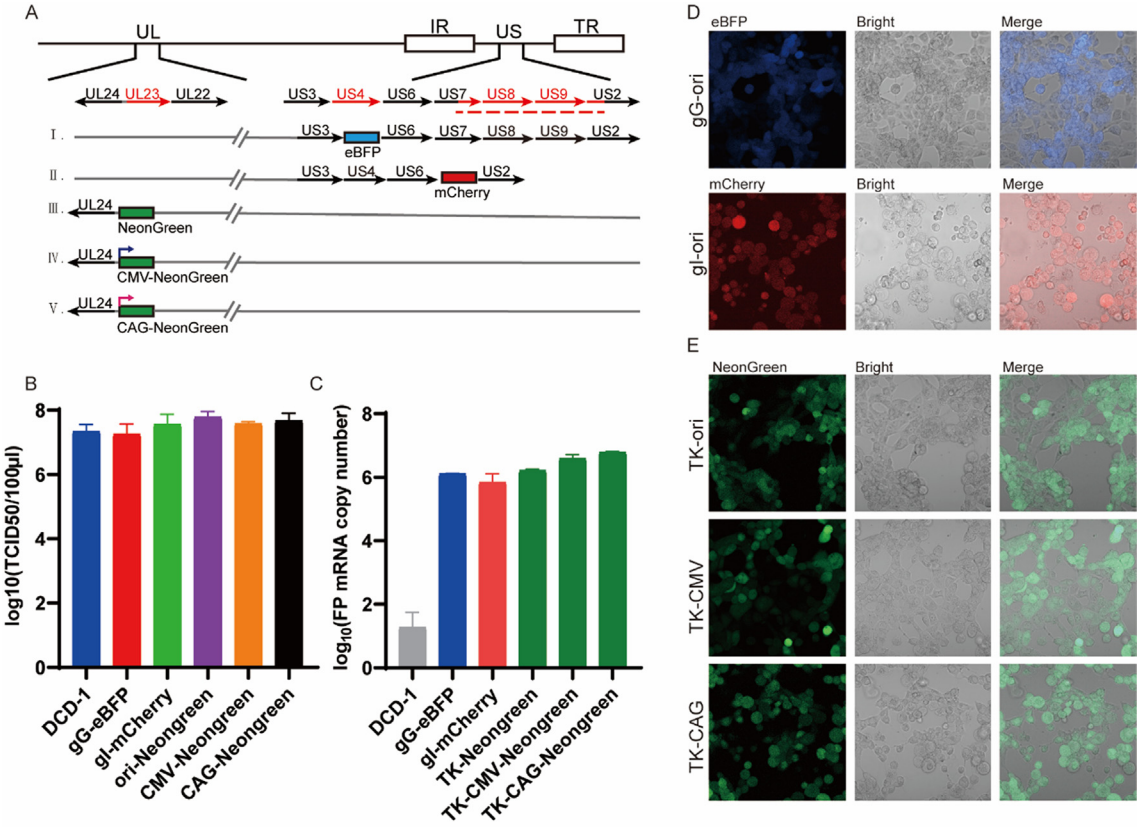

Fig. 3. Fluorescent protein expression at the TK, gG and gI locus. (A) Schematic map of the PRV genome. The mNeonGreen, eBFP and mCherry genes were inserted at the TK (UL23), gG (US4) and gI (US7) loci, respectively. The fluorescent protein coding genes were driven by the endogenous promoter, CMV promoter or CAG promoter. (B) Propagation of the fluorescent protein expression mutants. Vero cells were infected at a MOI of 1 PFU per cell and harvested at 24 h postinfection (hpi). The titer of virus produced from infected cells after a single cycle of growth was enumerated by plaque assay. The results were obtained from two independent infections. (C) Analysis of mRNA. Cells were harvested at 12 hpi. The mRNA levels in cell lysates were quantified by SYBR Green I quantitative real-time PCR using the gB gene as an indicator for the presence of viral DNA. (D) and (E) Live cell imaging of recombinant viruses expressing fluorescent proteins. Vero cell monolayers were infected at an MOI of 1. The images were taken at 72 hpi with 20 × objective lenses by confocal microscopy.

transcription of IL18 and IFN-γ was not influenced by the different expression cassettes at the TK locus (Fig. 4C). The transcription of the three PRRSV antigen genes at the TK locus remained at similar levels under the same promoter. The antigen genes were transcribed at a higher level from the CAG promoter, which was consistent with the previous fluorescent marker gene expression experiment (Fig. 4D). The expression of the five foreign genes was validated by Western blot, using anti-HA-tag antibody for IL18 and IFN-γ and anti-Flag-tag antibody for GP3, GP5 and M (Fig. 4E and 4F, Fig. S5).

Biological stability of the recombinant virus in vitro

Total DNA was extracted from cytopathic Vero cells, and the integrity of the recovered viral DNA was verified by PCR and sequencing (sequence data are available upon request), demonstrating the biological stability of replication (Table S1; Fig. 5A and 5B). After 15 passages, fluorescent proteins were expressed from the recombinant viruses (Fig. 5C). The results demonstrated that the cloned foreign genes were biologically stable in the recombinant virus.

Discussion

Viral BACs provide a solution for maintenance and manipulation of large DNA virus genomes as they can efficiently be modified using well-established mutagenesis techniques in *E. coli*. Recombineering is a very popular method for generating recombinant DNA using homologous recombination; the homologous recombinases Redα/Redβ of bacteriophage λ can efficiently mediate recombination between homologous arms as short as 35–50 bp and precisely modify circular DNA molecules of virtually any size [31–33]. Negative selection markers such as *rpsL* and *ccdB* are used to achieve traceless modification and point mutations [34–36].

The RecET recombination system, derived from the *E. coli* Rac prophage, is more efficient than Redα/Redβ at mediating recombination between linear DNA substrates and has become a powerful tool for direct cloning [20]. The first human adenoviral DNA library covering 34 genotypes was constructed using full-length, RecET-mediated direct cloning to incorporate viral genomes into a p15A plasmid [37]. Adenoviruses are double-stranded DNA viruses with genomes between 26 and 46 kb in length, which is within the cloning capacity of the RecET system, i.e., around 50 kb. To directly clone DNA with larger sizes or from complex genomes, the ExoCET system was developed, which combines an in vitro exonuclease and annealing with the in vivo RecET homologous recombination method to break through the bottleneck of low co-transformation efficiency [21].

In this study, we demonstrated a streamlined approach for creating infectious BAC clones of PRV by ExoCET. The linear BAC vector flanking the homologous sequences was released from an intermediate plasmid containing a pBR322 replication origin and the *ccdB* gene. The high-copy replicon ensured good yield and high DNA quality for production of the linear BAC vector. The toxin-encoding gene *ccdB* selected against background DNA of the

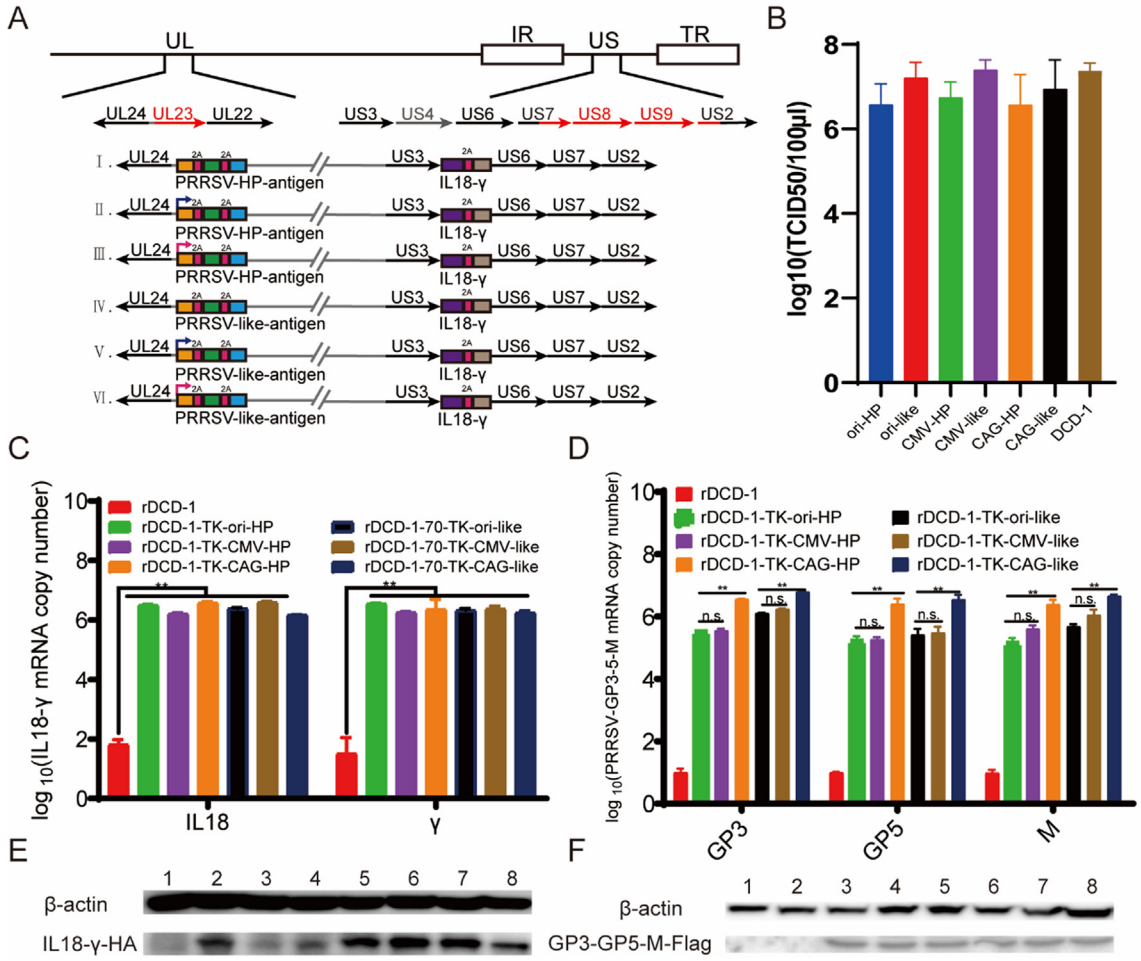

Fig. 4. Recombinant PRV DCD-1 expressing cytokine and antigen genes. (A) Schematic map of the recombinant DCD-1 genome. The HP-PRRSV or the NADC30-like virus antigen proteins were inserted at the TK (UL23) locus. The IL18 & IFN-γ genes were inserted at the gG (US4) locus. (B) Single-step growth curve analysis was performed for each mutant virus expressing PRRSV antigens and compared with that of DCD-1 (WT). Vero cells were infected at a MOI of 1 PFU per cell and harvested at 24 hpi. The titer of virus produced from infected cells after a single cycle of growth was enumerated by plaque assay, and the results were plotted on the graph. Data are an average of two independent infections. Error bars represent the mean ± S.D. (C) and (D) Analysis of mRNA. PK15 cells were infected by the DCD-1 mutant at a MOI of 0.01 PFU/cell and harvested at 24 hpi. To evaluate the transcription of GP3, GP5 and M at the TK locus and IL18 and IFN-γ at the gG locus, the mRNA levels in cell lysates were quantified by SYBR Green I quantitative real-time PCR. Relative threshold cycle (CT) values for each sample were calculated. The final concentration was calculated as copy numbers per 100 μl. Results are presented as the mean ± S.D. Asterisks represent significant differences between groups ($P \leq 0.05$). (E) and (F) Western blot of the foreign proteins expressed from recombinant DCD-1. Western blot analysis was performed using anti-HA-tag antibody for IL18 and IFN-γ and anti-Flag-tag antibody for GP3, GP5 and M. Lanes 1–8, rDCD-1, rDCD1-ΔgG-IL18-γ, rDCD1-ΔTK-HPPRRSV, rDCD1-ΔTK-CMV-HPPRRSV, rDCD1-ΔTK-CAG-HPPRRSV, rDCD1-ΔTK-NADC30like, rDCD1-ΔTK-CMV-NADC30like, rDCD1-ΔTK-CAG-NADC30like.

intermediate plasmid remaining due to incomplete digestion by restriction enzymes. After the viral genomic DNA was isolated, it was incubated with the linear BAC vector for exonuclease-mediated in vitro assembly. The assembly products were then electroporated into the RecET-expressing *E. coli* strain to finalize the incorporation of the BAC vector backbone at the terminus of the linear PRV genome. Generation of the PRV BAC is straightforward and requires only several days to complete. Compared to the conventional method of inserting the BAC vector into the viral genome via rare recombination events in susceptible eukaryotic cells, the direct cloning method completely bypasses the extremely tedious purification method, which often requires several passages of the virus. Constant selective pressure during serial virus passage can often result in compensatory mutations in the viral genome. When a mini-F sequence was introduced into the US2 locus of the Meleagrid herpesvirus 1 (MeHV-1 or turkey herpesvirus) genome by homologous recombination to obtain infectious MeHV-1 clones, the in vitro properties of viruses recovered from these clones were consistent with those of the parental MeHV-1. However, the rescued MeHV-1 viruses were significantly attenuated in vivo. Complete sequencing of the infectious clones identified eight missing or defective genes [38]. Accumulation of attenuating mutations was systematically analyzed in a high passage of Gallid herpesvirus type 2 [39].

In the infectious PRV BAC that we generated using ExoCET, restriction endonuclease recognition sites were placed at the junction of the BAC vector backbone and the linear terminus of the viral genome. Therefore, it was possible to extract the intact viral genome from the infectious clone by simply cutting off the BAC vector backbone with the restriction enzymes. The virus, which was genetically indistinguishable from the wild-type strain, was directly reconstituted by delivery of the restriction endonuclease-digested BAC DNA into mammalian cells without any selection and purification. Other methods for BAC vector backbone excision, such as homologous recombination between duplicated sequences flanking the mini-F replicon, require multiple passages and plaque purification in mammalian cells. The excision of the BAC vector cassette via homologous recombination between the introduced

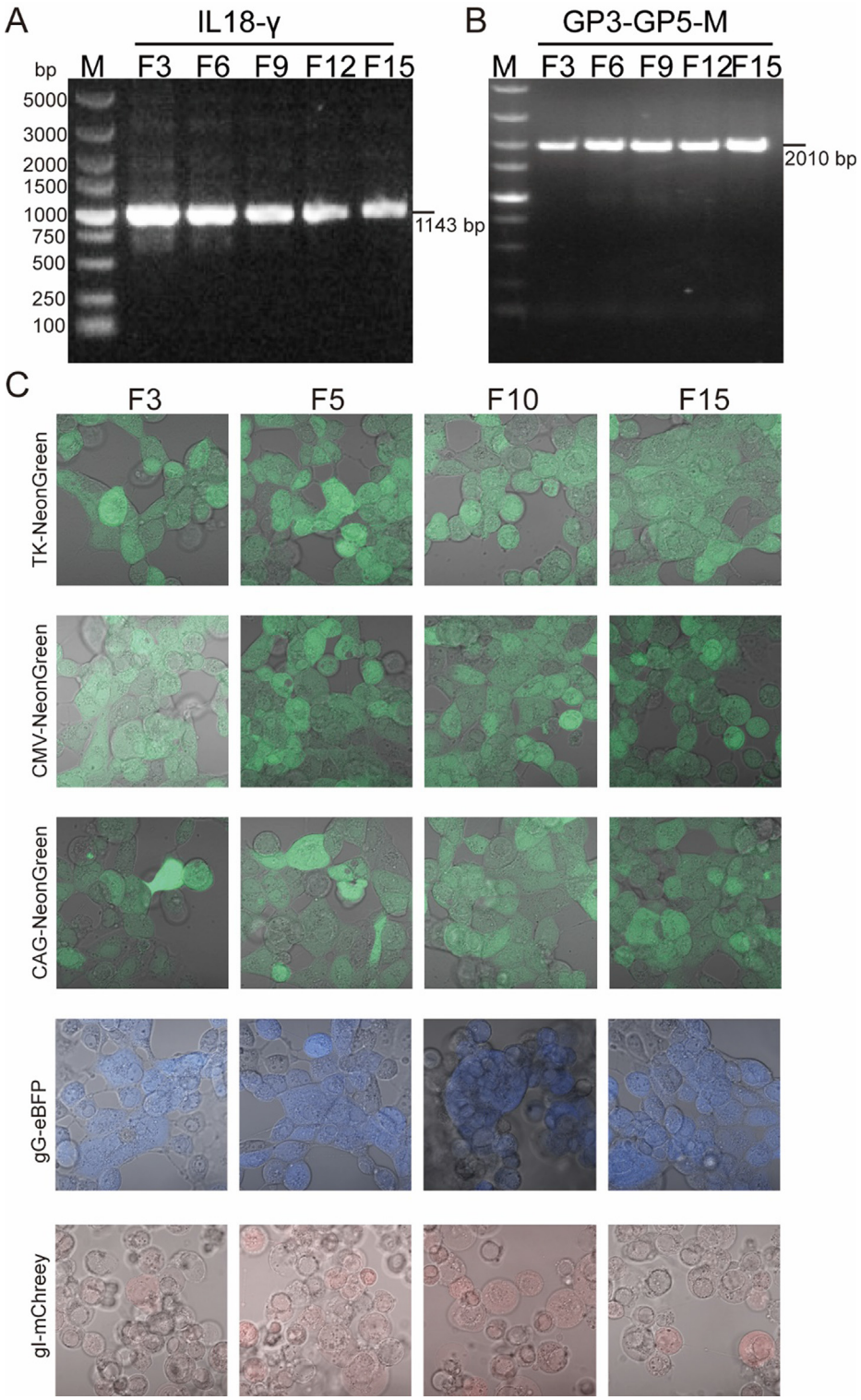

Fig. 5. Stability characterization of the recombinant viruses by PCR and laser confocal analysis. (A) PCR verification of the 1143 bp IL18-γ fusion fragment after serial passages of the virus in PK15 cells. (B) PCR verification of the 2010 bp GP3-GP5-M fusion fragment after serial passages of the virus in cells. (C) The rescued rDCD1-TK-NeonGreen, rDCD1-TK-CMV-NeonGreen, rDCD1-TK-CAG-NeonGreen, rDCD1-gG-eBFP and rDCD1-gI-mCherry viruses were cultured by serial passages in PK15 cells, and the samples were collected at passages 3, 5, 10 and 15. Images of the samples were obtained by confocal laser scanning microscopy.

duplicated sequences or the terminal repeats required five virus passages [14,15]. Moreover, the duplicated sequences flanking the mini-F replicon can cause difficulties in creating mutations at loci adjacent to the BAC vector backbone [10]. Although the completion of Cre-mediated excision of a BAC vector only needed one round of viral growth in cultured cells [16], the remaining loxP sequence was capable of causing phenotypic alterations to the virus [40].

PRV, a member of the Alphaherpesvirinae, is a swine virus with a linear DNA genome of approximately 143 kb [41]. Infections with PRV cause nervous disorders, dyspnea, weight loss, abortion and piglet death [27]. Many nonessential genes, including the

well-studied virulence factor glycoprotein gE-gI complex and TK, can be deleted without deleterious effects on virus propagation [25,26]. Moreover, attenuated PRV strains, such as the TK and glycoprotein mutants, can be used to express heterologous antigens for development of multivalent PRV vector vaccines [42]. In this study, we used an infectious BAC clone that was generated from the highly pathogenic PRV isolate DCD1, which emerged in several pig farms in Guangdong, China, in 2010. We started by knocking out the TK and glycoprotein virulence genes using Red system-mediated recombineering. A systematic expression profile assessment for optimal loci and exogenic promoters was then conducted by insertion of fluorescent reporter genes. The selection markers were removed by Cre, Flp or Dre recombination [43]. Our infectious BAC, which has the mini-F cassette incorporated at the terminus of the linear viral genome, was shown to be stable for highly efficient mutagenesis in *E. coli* using the Red system and various site-specific recombinases, including Cre, Flp and Dre.

Using the attenuated recombinant PRV DCD-1, our next goal is to develop vector vaccines against PRRSV, which has been epidemic in China for more than 20 years and which causes tremendous economic losses. Towards this goal, at one locus, we co-expressed the cytokines IL18 and IFNγ as vaccine adjuvants, given that expression of these genes was shown to potentiate the immune response to vaccination in various experimental models [44]. At other loci, we co-expressed the GP5, GP3 and M proteins from HP-PRRSV and NADC30-like virus, which are highly prevalent strains in China [45–47]. The NADC30-like virus strains have been prevalent in China since 2013 and reported in more than nine provinces. However, there are no vaccines available.

In summary, we established a novel methodology for the efficient generation of infectious BAC clones of herpesviruses. Virus that is genetically indistinguishable from the original strain can be rapidly rescued upon acquiring the intact viral genome by restriction endonuclease digestion. This new approach for construction of infectious clones could potentially be used for other viruses with large double-stranded DNA genomes, such as Poxviridae.

Institutional Review Board Statement

Not applicable.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

CRediT authorship contribution statement

Hengxing Yuan: Visualization, Investigation, Formal analysis, Software, Validation, Data curation, Writing – original draft. **Yaoyao Zheng:** Investigation, Formal analysis, Validation. **Xiaoling Yan:** Investigation, Formal analysis. **Hailong Wang:** Methodology, Funding acquisition. **Youming Zhang:** Conceptualization, Supervision, Funding acquisition. **Jingyun Ma:** Conceptualization, Methodology, Supervision, Funding acquisition. **Jun Fu:** Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Compliance with Ethics Requirements

All experiments were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The use of animals in this study was approved by the South China Agricultural University Committee of Animal Experiments (approval ID: SYXK2019-0136)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.02.012.

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