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Live attenuated pseudorabies virus developed using the CRISPR/Cas9 system

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ABSTRACT

Currently, pseudorabies virus (PRV) variant strains are outbreaking in China; these variants belong to genotype II PRV. The traditional Bartha-K61 vaccine has failed to provide complete protection against the emergent variant strains. Therefore, rapid attenuation of current epidemic strains is needed for effective PRV control. In this study, we report a rapid method for editing the PRV genome using the CRISPR-Cas9 system. We developed a triple gE/gI/TK gene-inactivated HeN1 PRV strain, because mice were more susceptible to PRV infection, we then evaluated the attenuation of PRV in the mice and demonstrated that modified PRV was fully attenuated. Furthermore, the attenuated strain also induced immune protection in response to a parental PRV challenge. Overall, we showed that PRVs can be rapidly attenuated using CRISPR-Cas9 technology, which will be critical for PRV control, especially when new variant PRV strains emerge.

1. Introduction

Pseudorabies virus (PRV) is a double-stranded DNA virus of approximately 150 kb in length that belongs to the Alphaherpesvirinae subfamily (Mettenleiter, 1991). PRV can reportedly infect most livestock and wild animals, although swine are the natural host. PRV-infected swine manifest different clinical symptoms depending on age. In growing pigs, respiratory symptoms and growth retardation are always observed; in adult swine, PRV can cause reproductive failure; and in suckling pigs, the virus can cause a fatal infection (Mettenleiter, 1991; Muller et al., 2011; Pomeranz et al., 2005). Consequently, PRV causes large economic losses within the swine industry. Previously, the Bartha-K61 vaccine has been used to protect swine from PRV. However, PRV variants have recently emerged in pigs vaccinated with the Bartha-K61 vaccine in China (An et al., 2013; Luo et al., 2014). This suggests that the Bartha-K61 vaccine fails to provide complete protection against variant strains. In a recent study, according to genome sequence analysis Ye et al. demonstrated that PRV can be divided into 2 genotypes. The emergent strain belongs to genotype II, which is distinct from the European and American strains that belong to genotype I (Ye et al., 2015). The Bartha-K61 vaccine was derived from genotype I, which exhibits marked sequence divergence compared to the emergent strains. This lack of homology could explain the low efficacy of the Bartha vaccine in protecting pigs from emergent strains (An et al., 2013; Ye et al., 2015).

CRISPR/Cas9 has recently been developed as a powerful DNA editor (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013; Ran et al., 2013). The CRISPR/Cas9 system has successfully been used to edit HIV-1 (Ebina et al., 2013; Liao et al., 2015), HSV-1 (Bi et al., 2014) and PRV (Liang et al., 2016; Xu et al., 2015). The method is simple, requiring only the design of an effective single guide RNA (sgRNA) that is specific to a given target gene. DNA viruses can easily be edited, and the only delay involves determining the targeted sequence. In this study, we attempted to attenuate a current epidemic PRV variant strain using the CRISPR/Cas9 system.

2. Materials and methods

2.1. Cell lines and viruses

Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) in a humidified incubator under 5% CO_2 at 37 °C. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Life Technologies) and

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Table 1
sgRNAs used in this study.

sgRNA-gE1	5′- CACCGgggcaggaacgtccagatcc-3′
	5′- AAACggatctggacgttcctgcccC-3′
sgRNA-gE2	5′- CACCGgctgcgcgccgcgcagctcc-3′
	5′- AAACggagctgcgcggcgcgcagcC-3′
sgRNA-gE3	5′- CACCGgggccgcgcctgcgtccccg-3′
	5′- AAACcggggacgcaggcgcggcccC-3′
sgRNA-gI1	5′-CACCGgtcgggggcgtcctcttcag-3′
	5′-AAACctgaagaggacgcccccgacC-3′
sgRNA-gI2	5′-CACCGgcgcccaacctgacgataga-3′
	5′-AAACtctactgtcaggttgggcgcC-3′
sgRNA-gI3	5′-CACCGagacgggacgctgctgtttc-3′
	5′-AAACgaaacagcagcgtcccgtctC-3′
sgRNA-TK1	5′-CACCGcatcagcgcggcggccttcg-3′
	5′-AAACcgaaggccgccgcgctgatgC-3′
sgRNA-TK2	5′-CACCGgcgcaaacgtctacgccatgc-3′
	5′-AAACgcatggcgtagacgtttgcgcC-3′
sgRNA-TK3	5′-CACCGcacgctgcgctacctgagct-3′
	5′-AAACagctcaggtagcgcagcgtgC-3′

antibiotics (0.1 mg/mL streptomycin and 100 IU/mL penicillin). The PRV HeN1 strain used (GenBank accession number: KP098534.1) has been described previously (An et al., 2013; Ye et al., 2015).

2.2. Antibodies

Antibodies against US3, gE and gI were separately raised in mice. Purified antigens of US3 (from 40 aa to 230 aa), gE (from 24 aa to 331 aa) and gI (from 59 aa to 365 aa) were immunized three times at intervals of approximately 2 weeks, and immunized mouse sera were collected for further studies.

2.3. Generation of CRISPR/Cas9 sgRNA plasmids

All sgRNAs were designed using an online CRISPR Design Tool (https://wwws.blueheronbio.com/external/tools/gRNASrc.jsp) to target the open reading frames of the gE, gI and TK genes. Three sgRNAs were designed for each gene. CRISPR/Cas9 constructs were created as previously described (Tang et al., 2016). All constructs were verified by sequencing (Table 1).

2.4. Effective sgRNA screening

Vero cells were seeded in 12-well plates and transiently transfected 12 h later with the indicated CRISPR/Cas9 plasmids (2 μg per well) using X-tremeGENE HP DNA transfection reagent (Roche, USA) according to the manufacturer's instructions. Twelve hours post transfection, PRV HeN1 was inoculated at a multiple of infection (MOI) of 0.01 and subjected to a plaque-forming assay or Western blotting. For the plaque-forming assay, at 2 hpi, the cells were washed three times with PBS and overlaid with 2% low melting-point agarose (Lonza, USA) in DMEM medium containing 2% FBS; the plates were further incubated at 37 °C for 3–5 days and then stained with 0.5% crystal violet (Baso, China). Western blotting was conducted 48 hpi as follows: cells were collected and washed once with PBS and then lysed in RIPA Lysis Buffer containing a protease inhibitor cocktail (Roche, USA). Proteins in the cell lysates were resolved using SDS-PAGE and transferred to PVDF membranes (Millipore, Germany), which were then probed with the indicated antibodies. The most effective sgRNAs (based on decreasing plaque numbers or levels of protein expression) were chosen for further study.

2.5. Gene inactivation by CRISPR/Cas9

Vero cells were seeded in 12-well plates and transiently transfected 12 h later with the indicated CRISPR/Cas9 plasmids (2 μg per well) using X-tremeGENE HP DNA transfection reagent. Twelve hours post transfection, PRV HeN1 was inoculated at a MOI of 0.01. At 48 hpi, supernatants were collected for plaque purification. Several plaques were randomly selected, and viral DNA was extracted using an EasyPure Viral DNA/RNA Kit (Transgen, China) according to the manufacturer's instructions. Inactivation-specific genes were confirmed by DNA sequencing or Western blotting. For the absence of the TK antibody, TK inactivation was verified by Acyclovir (ACV) (Sigma, USA) treatment.

2.6. In vitro growth properties

Viral titers are recorded as the number of plaque-forming units (PFU) or as the 50% tissue culture infection dose (TCID50) in Vero cells. Vero cells were infected with wild type PRV HeN1 or $gE^-/gI^-/TK^-$ PRV at a MOI of 0.01. The infected cells were collected at 24, 48, 72 and 96 hpi. The viral titers at the various time points are recorded as PFU or TCID50.

2.7. Animals and experimental design

Six- to eight-week-old female BALB/c mice were obtained from and housed at our institute and divided into 5 groups (10 mice per group). To evaluate the pathogenesis of $gE^-/gI^-/TK^-$ PRV, the mice were infected with HeN1 or $gE^-/gI^-/TK^-$ PRV by subcutaneous injection with 2×10^4 PFU of virus or DMEM (100 μL). To evaluate the immune protection of $gE^-/gI^-/TK^-$ PRV, the mice were immunized with 2×10^4 PFU $gE^-/gI^-/TK^-$ PRV (n = 10) or DMEM (n = 10) and were challenged with 2.5×10^3 PFU of virulent HeN1 3 weeks later. All animal experiments were approved by the Animal Ethics Committee of the institute and were performed in accordance with animal use ethical guidelines and approved protocols.

2.8. Statistical analysis

Statistical analyses were performed using ANOVA as implemented in SPSS version 19.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effective sgRNA screening

It has been reported that the absence of glycoprotein E (gE), glycoprotein I (gI) and thymidine kinase (TK) attenuates PRV (Mettenleiter, 2003; Zhang et al., 2015). We first designed and constructed three gE-specific CRISPR/Cas9 constructs. The targeted sequences and protospacer-adjacent motif (PAM) are shown in Fig. 1A. We next tested whether the CRISPR/Cas9 constructs effectively disrupted the PRV genome (thereby inhibiting viral replication) by transfecting the constructs into Vero cells. After 12 h, the cells were inoculated with PRV HeN1 (MOI = 0.01). The number of viral plaques was significantly reduced in the cells that had been transfected with sgRNA-E1 compared with the control group (Fig. 1B), indicating effective PRV genome disruption by sgRNA-E1. Disruption of the PRV genome was also confirmed at the protein level at 48 hpi (Fig. 1C). We further demonstrated that a small amount of each CRISPR/Cas9 construct (0.5 μg) was sufficient to effectively inhibit viral replication (Fig. 1D). The effective sgRNAs for gI and TK were screened using the same method (data not shown).