Live attenuated pseudorabies virus developed using the CRISPR/Cas9 system

Running title: Pseudorabies virus attenuated by CRISPR-Cas9

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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.

Keywords: CRISPR/Cas9, Pseudorabies virus, attenuation, vaccine

1 **1. Introduction**

2 Pseudorabies virus (PRV) is a double-stranded DNA virus of approximately
3 150 kb in length that belongs to the Alphaherpesvirinae subfamily (Mettenleiter,
4 1991). PRV can reportedly infect most livestock and wild animals, although swine
5 are the natural host. PRV-infected swine manifest different clinical symptoms
6 depending on age. In growing pigs, respiratory symptoms and growth retardation are
7 always observed; in adult swine, PRV can cause reproductive failure; and in
8 suckling pigs, the virus can cause a fatal infection (Mettenleiter, 1991; Muller et al.,
9 2011; Pomeranz et al., 2005). Consequently, PRV causes large economic losses
10 within the swine industry. Previously, the Bartha-K61 vaccine has been used to
11 protect swine from PRV. However, PRV variants have recently emerged in pigs
12 vaccinated with the Bartha-K61 vaccine in China (An et al., 2013; Luo et al., 2014).
13 This suggests that the Bartha-K61 vaccine fails to provide complete protection
14 against variant strains. In a recent study, according to genome sequence analysis Ye
15 et al. demonstrated that PRV can be divided into 2 genotypes. The emergent strain
16 belongs to genotype II, which is distinct from the European and American strains
17 that belong to genotype I (Ye et al., 2015). The Bartha-K61 vaccine was derived
18 from genotype I, which exhibits marked sequence divergence compared to the
19 emergent strains. This lack of homology could explain the low efficacy of the Bartha
20 vaccine in protecting pigs from emergent strains (An et al., 2013; Ye et al., 2015).

21 CRISPR/Cas9 has recently been developed as a powerful DNA editor (Cho et
22 al., 2013; Cong et al., 2013; Mali et al., 2013; Ran et al., 2013). The CRISPR/Cas9

1 system has successfully been used to edit HIV-1 (Ebina et al., 2013; Liao et al.,
2 2015), HSV-1 (Bi et al., 2014) and PRV (Liang et al., 2016; Xu et al., 2015). The
3 method is simple, requiring only the design of an effective single guide RNA
4 (sgRNA) that is specific to a given target gene. DNA viruses can easily be edited,
5 and the only delay involves determining the targeted sequence. In this study, we
6 attempted to attenuate a current epidemic PRV variant strain using the CRISPR/Cas9
7 system.

8 **2. Materials and Methods**

9 **2.1 Cell lines and viruses**

10 Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM;
11 Invitrogen) in a humidified incubator under 5% CO₂ at 37°C. All culture media were
12 supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Life
13 Technologies) and antibiotics (0.1 mg/mL streptomycin and 100 IU/mL penicillin).
14 The PRV HeN1 strain used (GenBank accession number: KP098534.1) has been
15 described previously (An et al., 2013; Ye et al., 2015).

16 **2.2 Antibodies**

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

21 **2.3 Generation of CRISPR/Cas9 sgRNA plasmids**

22 All sgRNAs were designed using an online CRISPR Design Tool

1 (<https://www.blueheronbio.com/external/tools/gRNASrc.jsp>) to target the open
2 reading frames of the gE, gI and TK genes. Three sgRNAs were designed for each
3 gene. CRISPR/Cas9 constructs were created as previously described (Tang et al.,
4 2016). All constructs were verified by sequencing.

5 Table 1. sgRNAs used in this study.

sgRNA-gE1	5'- CACCGgggcaggaacgtccagatcc-3' 5'- AAACggatctggacgttctgcccC-3'
sgRNA-gE2	5'- CACCGgctgcgcgccgcgcagctcc-3' 5'- AAACggagctgcgcggcgcgcagcC-3'
sgRNA-gE3	5'- CACCGggggccgcgcctgcgtccccg-3' 5'- AAACcgggggacgcaggcgcggcccC-3'
sgRNA-gI1	5'-CACCGgtcggggcgctctcttcag-3' 5'-AAACctgaagaggacgccccgacC-3'
sgRNA-gI2	5'-CACCGgcgccaacctgacgataga-3' 5'-AAACtctactgtcaggttgggcgcC-3'
sgRNA-gI3	5'-CACCGagacgggacgtgtgtttc-3' 5'-AAACgaaacagcagcgtcccgtctC-3'
sgRNA-TK1	5'-CACCGcatcagcgcggcgcccttcg-3' 5'-AAACcgaaggccgcgcgtgatgC-3'
sgRNA-TK2	5'-CACCGgcgcaaactctacgccatgc-3' 5'-AAACgcatggcgtagacgtttgcgcC-3'
sgRNA-TK3	5'-CACCGcacgtgcgctacgtgagct-3' 5'-AAACagctcaggtagcgcagcgtgC-3'

6

7 2.4 Effective sgRNA screening

8 Vero cells were seeded in 12-well plates and transiently transfected 12 h later
9 with the indicated CRISPR/Cas9 plasmids (2 µg per well) using X-tremeGENE HP
10 DNA transfection reagent (Roche, USA) according to the manufacturer's instructions.
11 Twelve hours post transfection, PRV HeN1 was inoculated at a multiple of infection
12 (MOI) of 0.01 and subjected to a plaque-forming assay or Western blotting. For the
13 plaque-forming assay, at 2 hpi, the cells were washed three times with PBS and

1 overlaid with 2% low melting-point agarose (Lonza, USA) in DMEM medium
2 containing 2% FBS; the plates were further incubated at 37°C for 3–5 days and then
3 stained with 0.5% crystal violet (Baso, China). Western blotting was conducted 48
4 hpi as follows: cells were collected and washed once with PBS and then lysed in
5 RIPA Lysis Buffer containing a protease inhibitor cocktail (Roche, USA). Proteins
6 in the cell lysates were resolved using SDS-PAGE and transferred to PVDF
7 membranes (Millipore, Germany), which were then probed with the indicated
8 antibodies. The most effective sgRNAs (based on decreasing plaque numbers or
9 levels of protein expression) were chosen for further study.

10 **2.5 Gene inactivation by CRISPR/Cas9**

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

20 **2.6 In vitro growth properties**

21 Viral titers are recorded as the number of plaque-forming units (PFU) or as the
22 50% tissue culture infection dose (TCID₅₀) in Vero cells. Vero cells were infected

1 with wild type PRV HeN1 or gE⁻/gI⁻/TK⁻ PRV at a MOI of 0.01. The infected cells
2 were collected at 24, 48, 72 and 96 hpi. The viral titers at the various time points are
3 recorded as PFU or TCID₅₀.

4 **2.7 Animals and experimental design**

5 Six- to eight-week-old female BALB/c mice were obtained from and housed at
6 our institute and divided into 5 groups (10 mice per group). To evaluate the
7 pathogenesis of gE⁻/gI⁻/TK⁻ PRV, the mice were infected with HeN1 or gE⁻/gI⁻/TK⁻
8 PRV by subcutaneous injection with 2×10^4 PFU of virus or DMEM (100 μ L). To
9 evaluate the immune protection of gE⁻/gI⁻/TK⁻ PRV, the mice were immunized with
10 2×10^4 PFU gE⁻/gI⁻/TK⁻ PRV (n=10) or DMEM (n=10) and were challenged with
11 2.5×10^3 PFU of virulent HeN1 3 weeks later. All animal experiments were
12 approved by the Animal Ethics Committee of the institute and were performed in
13 accordance with animal use ethical guidelines and approved protocols.

14 **2.8 Statistical analysis**

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

17 **3. Results**

18 **3.1 Effective sgRNA screening**

19 It has been reported that the absence of glycoprotein E (gE), glycoprotein I (gI)
20 and thymidine kinase (TK) attenuates PRV (Mettenleiter, 2003; Zhang et al., 2015).
21 We first designed and constructed three gE-specific CRISPR/Cas9 constructs. The
22 targeted sequences and protospacer-adjacent motif (PAM) are shown in Figure 1A.

1 We next tested whether the CRISPR/Cas9 constructs effectively disrupted the PRV
2 genome (thereby inhibiting viral replication) by transfecting the constructs into Vero
3 cells. After 12 h, the cells were inoculated with PRV HeN1 (MOI=0.01). The
4 number of viral plaques was significantly reduced in the cells that had been
5 transfected with sgRNA-E1 compared with the control group (Fig. 1B), indicating
6 effective PRV genome disruption by sgRNA-E1. Disruption of the PRV genome was
7 also confirmed at the protein level at 48 hpi (Fig. 1C). We further demonstrated that
8 a small amount of each CRISPR/Cas9 construct (0.5 µg) was sufficient to effectively
9 inhibit viral replication (Fig. 1D). The effective sgRNAs for gI and TK were
10 screened using the same method (data not shown).

11 **3.2 Insertions and deletions (indels) detection and gE⁻ PRV identification**

12 Next, we tested whether the DNA breaks induced inactivating indels at the
13 targeted cleavage site. The site cleaved by sgRNA-E1 contained a *Sau3A* cleavage
14 site (Fig. 1A). However, indels would prevent *Sau3A* digestion. PCR products
15 collected from viruses in the supernatant of the control group were fully digested by
16 *Sau3A*. However, those from sgRNA-E1-treated cells were incompletely digested
17 (Fig. 2A). The PCR products were also cloned for sequencing, and several mutations
18 were found (Fig. 2B); 34.4% of the clones had insertions or deletions in the
19 sgRNA-E1 targeted region (Fig. 2C). Among all 11 clones, 8 were expected to result
20 in truncated proteins or to displace the remaining coding sequence out of frame.
21 Thus, CRISPR/CaS9 was highly effective in disrupting the PRV genome and
22 inducing indels. We further purified gE-disrupted PRV using plaque purification

1 assays and randomly selected two purified isolates for PCR-based sequencing. The
2 gE genes contained deletions of “A” and “GA,” and these strains were successfully
3 cloned (Fig. 2D); the resulting isolates were named PRV HeN1-del-A and PRV
4 HeN1-del-GA, respectively (Fig. 2D). gE protein disruption was confirmed by
5 Western blot analysis using wild type PRV HeN1 and Bartha-K61 (which is known
6 to contain gE, gI, Us9 and Us2 region deletions) strains as positive and negative
7 controls, respectively (Fig. 2E). No disruption was observed in the gI and US3
8 proteins of the HeN1-del-A or HeN1-del-GA strains, indicating that sgRNA-E1 had
9 good target specificity (Fig. 2E).

10 **3.3 Construction and identification of gE⁻/gI⁻/TK⁻ PRV**

11 Using the method described above, we next inactivated the gI gene in the
12 HeN1-del-A strain. We designed three gI-specific sgRNAs, of which sgRNA-I1
13 gave the best results (data not shown). We then completely knocked out gI using
14 sgRNA-I1, as demonstrated by Western blotting and DNA sequencing analysis (Fig.
15 3A and 3B). This gE/gI knockout strain was used for further TK inactivation. Three
16 TK-specific sgRNAs were designed using methods similar to those described above.
17 sgRNA-TK1 showed the greatest inhibitory effect on PRV among the three sgRNAs
18 (data not shown). To confirm the absence of TK, we verified TK inactivation by
19 DNA sequencing. A “T” deletion was found at nt 397; this mutation disrupted the
20 TK ORF and produced a pre-stop codon (Fig. 3C). Acyclovir (ACV) treatment was
21 used to further confirm TK inactivation. ACV inhibits wild-type virus replication but
22 not the replication of TK-defective strains. In the presence of 400 μ M ACV, the

1 HeN1 strain was significantly inhibited by ACV. However, the viral progeny of the
2 gE/gI/TK gene-inactivated PRV strain was not inhibited by ACV (Fig. 3D). Thus, a
3 triple gE/gI/TK gene-inactivated HeN1 PRV strain was successfully developed
4 (hereafter named gE⁻/gI⁻/TK⁻ PRV). To determine whether gE/gI/TK inactivation
5 affected PRV replication, the replication properties of the virus were analyzed.
6 One-step growth curves indicated that the virus replicated more slowly than the
7 parent virus PRV HeN1 at 36 h and 48 h (Fig. 3E). Plaque morphology and size are
8 shown in Fig. 3F; the plaque size of gE⁻/gI⁻/TK⁻ PRV was significantly smaller than
9 parent virus. We further tested the stability of gE⁻/gI⁻/TK⁻ PRV by passaging it 20
10 times in Vero cells. No reversion mutation was found by sequencing (data not
11 shown).

12 **3.4 Evaluation of attenuation and protection efficacy**

13 We used a mouse model which was susceptible to PRV infection to evaluate
14 PRV pathogenesis. To test if PRV gE⁻/gI⁻/TK⁻ was attenuated, 6-week-old female,
15 specific-pathogen free (SPF) BALB/c mice were randomly divided into five groups
16 (n=10/group). The mice were infected with 2×10^4 PFU of HeN1 or gE⁻/gI⁻/TK⁻
17 PRV by subcutaneous injection; DMEM (100 μ L) was used as a control. The
18 HeN1-infected mice had a mortality rate of 100% with serious neurological signs
19 post-infection. However, the gE⁻/gI⁻/TK⁻ PRV-infected group had a 100% survival
20 rate, as did the control group, and no clinical symptoms were observed (Fig. 4A).
21 These results indicate that gE⁻/gI⁻/TK⁻ PRV was fully attenuated. We further
22 determined the protective efficacy of gE⁻/gI⁻/TK⁻ PRV. The mice were immunized

1 with 2×10^4 PFU gE⁻/gI⁻/TK⁻ PRV (n=10), 2×10^4 PFU Bartha-K61 PRV (n=10) as
2 a positive control, or DMEM (n=10). Three weeks later, the mice were challenged
3 with 2.5×10^3 PFU of virulent HeN1. All the mice remained clinically healthy
4 post-vaccination, and none of the mice that were vaccinated with gE⁻/gI⁻/TK⁻ PRV
5 died after the HeN1 challenge. However, 20% (2/10) of the Bartha-K61 mice and
6 60% (6/10) of the control mice died post-challenge, indicating that Bartha-K61
7 failed to provide complete protection against the virulent HeN1 strain.

8 **4. Discussion**

9 Here, we showed that PRV genomes can be rapidly edited using a CRISPR/Cas9
10 system by simple transfection with a specific sgRNA. Different types of mutations,
11 including a single nucleotide substitution, subversion, or insertions or deletions,
12 were introduced into target genes. Most of these mutations destroyed target gene
13 expression. CRISPR/Cas9 can be applied to new isolates as they emerge in nature as
14 soon as a target gene sequence is identified to facilitate the design of guide RNAs.
15 However, CRISPR/Cas9 has some limitations for use in PRV gene inactivation. First,
16 this technique produces a random collection of mutants that must be screened and
17 purified. This includes the need to sequence a collection of isolates, and some
18 isolates may be not the desired recombinants (i.e., in-frame deletions, in-frame
19 insertions, and missense mutations). Second, CRISPR/Cas9 targeting of the 3' region
20 of an ORF will potentially result in the expression of truncated products, whereas
21 targeting of the 5' ORF will create alternative ATG translation start sites that
22 potentially produce protein products. In this study, we targeted the 5' ORFs of target

1 genes and used gE and gI polyclonal antibodies to detect the expression of these
2 genes. These antibodies were raised in mice with antigens spanning the majority of
3 each ORF, and we did not detect any truncated products. Thus, disruption of gE or gI
4 did not produce N-terminally or C-terminally truncated products. Third, we
5 inactivated ORFs by introducing frameshift mutations, although we did not detect
6 reversion; in contrast to deletions, frameshift mutations are prone to reversion of
7 wild-type sequences. To solve these problems, large deletions spanning most of the
8 ORFs could be introduced for gene deletion in future studies.

9 sgRNA efficacy determines the ratio of gene disruption. Although multiple
10 CRISPR sequence design tools exist, many software programs fully rely on
11 computational analysis, resulting in the design of numerous sgRNAs with no
12 mechanism to sort them (Brazelton et al., 2016). In addition, PRV genomes contain
13 high GC content. Therefore, the available tools were limited. To solve these
14 problems, for each gene of interest, we designed three sgRNAs, of which at least one
15 was effective (data not shown). To obtain the best results, we usually selected the
16 most effective sgRNA for further study.

17 Screening to identify effective sgRNAs is a key factor affecting CRISPR/Cas9
18 editing efficacy. Traditional sgRNA screening is validated using amplified fragment
19 length polymorphism (AFLP), a T7 endonuclease I assay (T7E1) and surveyor
20 mismatch cleavage assays or DNA sequencing (Wang et al., 2015). The PRV
21 genome has high GC content, rendering it difficult to manipulate. The methods
22 described above for sgRNA screening are not suitable for PRV. In this study, we

1 hypothesized that PRV genome breaks introduced by effective sgRNAs would
2 inhibit viral replication. Thus, we screened for effective sgRNAs using plaque
3 forming assays and Western blotting. In a previous study, Xu et al. knocked out the
4 PRV EP0 gene using CRISPR/Cas9, but they did not provide a sgRNA screening
5 method; rather, the authors co-transfected the PRV genome with sgRNA (Xu et al.,
6 2015). Obtaining extracted PRV genomes of high quality is laborious and requires
7 an experienced and skillful operator. Therefore, our method is more feasible. In a
8 recent study, Liang et al. combined CRISPR/Cas9 and a Cre/Lox system to knock
9 out a PRV virulence gene—a procedure that required several steps. First, two HR
10 plasmids that included a fluorescent tag were constructed. Recombinant virus was
11 then purified using FACS technology. Finally, the Cre/Lox system was used to
12 remove the fluorescent tags. Although this approach inactivated ORF by creating a
13 large deletion, the method requires high familiarity with the techniques employed
14 and may not be easy to accomplish.

15 In our study, we did not demonstrate attenuation in the natural host, as has been
16 shown by other groups (Cong et al., 2016; Liang et al., 2016; Tong et al., 2016).
17 Cong et al. reported that gE/gI deletion in PRV results in varying PR-specific
18 clinical signs in both mice and sheep, indicating that PRV harboring a gE/gI deletion
19 may not be safe for further application (Cong et al., 2016). Additionally, Tong et al.
20 developed a gE/gI-deleted PRV that was not evaluated in susceptible animals (Tong
21 et al., 2016). In the present study, we created an inactivated gE⁻/gI⁻/TK⁻ PRV that
22 was found to be safe in mice, and this attenuated PRV is therefore likely relatively

1 safe in pigs.

2 In conclusion, we rapidly knocked out three PRV virulence genes using the
3 CRISPR/Cas9 system. This is very important for prompt PRV prevention and
4 control in situations such as the current epidemic of variant PRV in China. This
5 strategy is also suitable for other large DNA viruses that are difficult to manipulate.

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FIGURE LEGENDS

FIGURE 1 CRISPR/Cas9 inhibits PRV replication. (A) CRISPR/Cas9 targeted sequences and PAM (box). The Cas9 digestion sites (arrows) and *Sau3A* site (labeled box) are indicated in the gE-1 sequence. (B) gRNA-gE1 inhibits PRV plaque formation. Vero cells were transfected with 2 µg of gRNA-gE1, 2, or 3, or with control plasmids. Twelve hours later, the cells were infected with HeN1 PRV (MOI=0.01), and a plaque assay was performed. (C) At 48 hpi, the cells were harvested and lysed for Western blot analysis. "Mock" indicates cells that were treated with transfection agent, and "control" indicates Cas9 vector-treated cells. (D) Vero cells were transfected with increasing amounts of the gE1 plasmid (0, 0.5, 1, and 2 µg); 12 h later, the cells were infected with HeN1 PRV (MOI=0.01). Virus production was quantified using Western blot analysis at 48 hpi. The experiment was performed three times, and a representative result is shown.

FIGURE 2 CRISPR/Cas9 induced a high ratio of indel editing in the PRV genome. (A) A 574-bp fragment was amplified from PRV supernatant isolated from gRNA-gE1-treated cells using the primers F: 5'-CCATGCGACCCTTTCTGCTGC-3' and R: 5'-CGTGTAGAGGCCCGTGTCGTTG-3'. The fragment was then digested using *Sau3A*. "Mock" indicates cells that were treated with transfection agent, and "control" indicates Cas9 vector-treated cells. (B) Representative mutations induced by gRNA-gE1. (C) Summary of the indel mutations in the gRNA-gE1-treated and control groups. (D) The gE gene sequence of two purified gE-inactivated viruses. (E)

1 Western blot confirming gE inactivation. HeN1 and Bartha-K61 strains were used as
2 positive and negative controls, respectively.

3 **FIGURE 3 Identification of gI and TK-disrupted PRV. (A)** Western blotting
4 confirmed gI inactivation. **(B)** DNA sequencing used to confirm gI inactivation. The
5 gI region was amplified using the primers F: 5'-CGCCACCGCTCGGTGATC-3' and
6 R: 5'-GCGGAAGGCTTCGTGGTGC-3'. A "T" base was deleted at nt 98 (in the red
7 box) of the gI gene coding sequence. The PAM is shown in the green box. **(C)** DNA
8 sequences confirming TK inactivation. The TK region was amplified using the
9 primers F: 5'-AAGCAGAACGGCAGCCTGAGCG-3' and R:
10 5'-GGGCACGGCAAACCTTTATTGGGAT-3'. A "T" base was deleted at nt 397 (in
11 the red box) of the TK gene coding sequence. The PAM is shown in the green box.
12 **(D)** TK inactivation was confirmed by ACV (400 μ M) treatment. Vero cells were
13 infected with HeN1 or gE/gI/TK gene-inactivated PRV at a MOI of 0.01 in the
14 presence or absence of ACV. At 24 hpi, viral load was titrated (* indicates $P<0.05$).
15 **(E)** Replication kinetics of triple gE/gI/TK gene-inactivated PRV in vitro (*
16 indicates $P<0.05$). **(F)** Plaque morphologies and sizes of wild-type PRV and
17 gE/gI/TK gene-inactivated PRV are also shown.

18 **FIGURE 4 Triple gE/gI/TK gene inactivation attenuated PRV strains**
19 **effectively and prevented epidemic PRV challenge. (A)** Six-week-old, female,
20 specific-pathogen free (SPF) BALB/C mice were randomly divided into three groups
21 (n=10/group). The mice were infected with HeN1 or gE⁻/gI⁻/TK⁻ by subcutaneous
22 injection with 2×10^4 PFU of each virus or DMEM (100 μ L). Survival was recorded

1 up to 15 days. **(B)** Six-week-old, female, SPF BALB/C mice were randomly divided
2 into three groups (n=10/group) and immunized with 2×10^4 PFU of gE⁻/gI⁻/TK⁻,
3 Bartha-K61 or 100 μ L DMEM. Three weeks post immunization, the mice were
4 challenged by subcutaneous injection with 2.5×10^3 PFU of virulent HeN1 PRV, and
5 survival was recorded up to 15 days.

Fig.1, Tang et al., 2016

A

B

C

D

Fig.2, Tang et al., 2016

Fig.2, Tang et al., 2016

D

E

Fig.3, Tang et al., 2016

Fig.3, Tang et al., 2016

C

D

Fig.3, Tang et al., 2016

E

F

Fig.4, Tang et al., 2015

A

B