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CRISPR/Cas9-mediated inhibition of pseudorabies virus and African swine fever virus replication in cell culture

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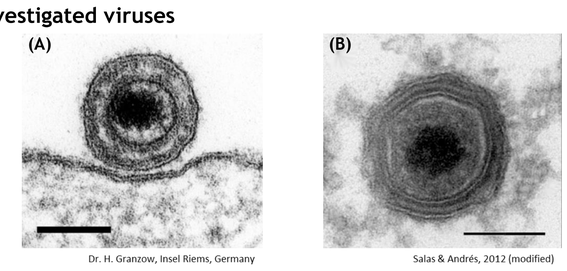
Introduction

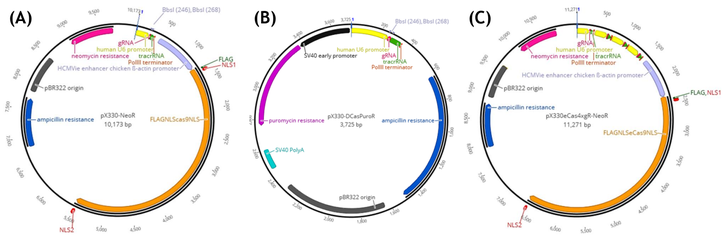
During the last years, specific RNA-mediated prokaryotic defense systems have been converted into powerful tools for genome editing in eukaryotes. This also opens new possibilities for prevention and control of virus infections, i.e. by facilitating targeted viral gene deletions or mutations during development of attenuated live vaccines. On the other hand, resistant host organisms can be generated, either by knocking out cellular virus receptor genes or by constitutive expression of antiviral RNAs by the host. In the present study, we used the CRISPR (clustered regularly interspaced short palindromic repeats) - Cas9 nuclease system from *Streptococcus pyogenes* for targeting two swine pathogens: pseudorabies virus (PrV, *Suid alphaherpesvirus 1*) and African swine fever virus (ASFV). To this end, permissive rabbit kidney (RK13, for PrV) or wild boar lung (WSL, for ASFV) cell lines were modified by stable transfection with plasmids containing neomycin or puromycin resistance genes, together with expression cassettes for Cas9 and guide RNAs with one to four virus gene-specific target sequences of 20 nucleotides. The target sequences were chosen within conserved regions of essential open reading frames, encoding the major capsid protein (UL19), the immediate-early regulatory protein IE180 and envelope glycoprotein gB (UL27) of PrV, or the major capsid protein p72 (B646L), DNA polymerase (G1211R), an inner envelope protein (E199L), or the secreted phosphoprotein p30 (CP204L) of ASFV. To determine inhibition of virus replication, plating efficiencies and growth kinetics of reporter protein (dsRed or mCherry) labeled PrV and ASFV mutants were compared to those observed in the parental cell lines. Genetic stability of the obtained transgenic cell lines over > 30 passages, and sequence alterations of occurring escape virus mutants were also investigated.

1. Investigated viruses



(A) PrV (*Suid alphaherpesvirus 1*): genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae*, order *Herpesvirales*. Causes Aujeszky's disease. The enveloped virion contains an icosahedral capsid and a double-stranded DNA genome of approx. 145 kbp encoding 69 different proteins. Replication in the host cell nucleus.
(B) ASFV (*African swine fever virus*): genus *Asfivirus*, family *Asfarviridae*. The doubly-enveloped virion contains an icosahedral capsid and a double-stranded DNA genome of 170 to 193 kbp encoding 151 to 167 different proteins. Replication in the host cell cytoplasm. Bars: 100 nm.

2. Plasmid vectors



(A) The CRISPR/Cas9 plasmids used in this study were derived from pX330-U6-Chimeric_BB-CBh-hSpCas9 (Cong et al., 2013), which permits the insertion of arbitrary target sequences between two *Bbs*I restriction sites within a guide RNA expression cassette. For selection of transgenic cell lines the original vector was modified by insertion of a neomycin resistance gene, and, for targeting of cytoplasmic ASFV DNA, by removal of sequences encoding nuclear localization signals (NLS), from both ends of the FLAG-tagged Cas9 nuclease. **(B)** A modified vector containing a puromycin resistance gene, and no Cas9 gene, could be used for stable expression of a second guide RNA. **(C)** Usage of a multiplex vector system (Sakuma et al., 2014) resulted in plasmids containing tandem arrays of expression cassettes for up to seven different guide RNAs .

3. Tested viral target sequences

PrV

Major capsid protein pUL19 (1330 amino acids)

UL19gR1	CGTCGCGCGCATGACCCACGCGG (codons 245 – 253)	**–**
UL19gR2	GCGCTGCATGAGCCCGACGAGGG (codons 475 – 483)	**+**
UL19gR3	GCTCTCGTACGCGCTCATGGCGG (codons 988 – 996)	**++**

Immediate early protein IE180 (1450 amino acids)

IE180gR1	CTTTGACTTCATCGAGACCGAGG (codons 5 – 13)	**–**
IE180gR2	CGTGCGCCTCATCTACAGCGGGG (codons 546 – 554)	**++**
IE180gR3	GATGAAGTCAAAGAGATCGTCGG (codons 2 – 9 R)	**+**

Envelope glycoprotein B (920 amino acids)

PgBgR1	GACGGGGTACAGGGCCTTCATGG (codons 835 – 842 R)	**–**

Genome end / concatemeric junction

PCJgR1	GCCCAGCTCTCCCCCGAGCGCGG (nt. 2 – 24 i.a.)	**–**

ASFV

Major capsid protein p72 (646 amino acids)

B646LgR1	TTTTGTCTTATTGCTAACGATGG (codons 7 – 14)	**?**
B646LgR2	CATAAAGCTTGCATCGCAAAAGG (codons 358 – 366)	**–**

DNA-polymerase pG1211R (1206 amino acids)

G1211RgR1	ACCACGTCGTTTGCCTGATTTGG (codons 224 – 232)	**–**
G1211RgR2	ACAAAACTCACCAAAACGATAGG (codons 799 – 807)	**–**

Immunogenic phosphoprotein p30 (201 amino acids)

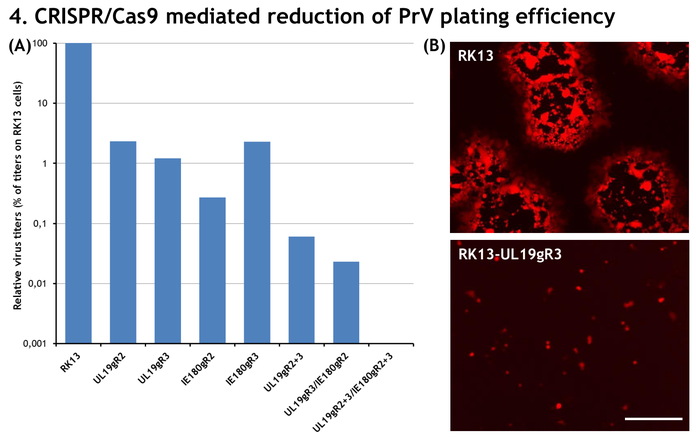
CP204RgR1	GCAAGGGTATACTGAACATCAGG (codons 71 – 79)	**+++**

Inner membrane protein pE199L (199 amino acids)

E199LgR1	GCATTTTTATTTCCATTACACGG (codons 90 – 98)	**+**

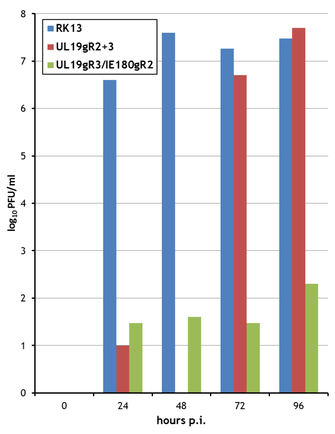
Locations of the target sequences within the respective open reading frames (codons), or within the viral genome (nt.) are indicated. Reverse strand sequences are marked by (R). The protospacer adjacent motifs (PAM : 5'-NGG-3') are highlighted in red, and were not included in the guide RNAs. Effects on virus replication were roughly quantified : no inhibition (-), detectable (+), strong (++), or complete inhibition (+++). (?) indicates ambiguous results.

4. CRISPR/Cas9 mediated reduction of PrV plating efficiency



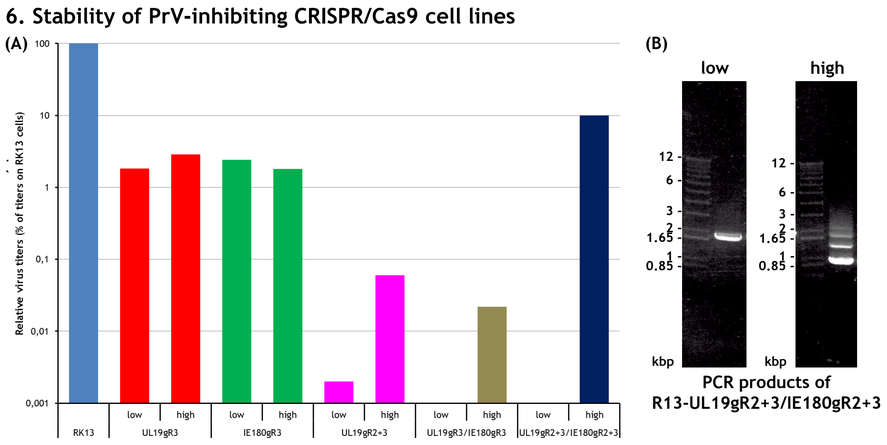
Representative clones of transgenic RK13-UL19gR2, -UL19gR3, -IE180gR2, -IE180gR3, -UL19gR2+3, -UL19gR3/IE180gR2 and -UL19gR2+3/IE180gR2+3 cells, as well as normal RK13 cells were infected with serial dilutions of PrV-KaΔgGmCherry under plaque assay conditions. **(A)** After two days the apparent virus titers (PFU/ml) were determined, and percentages of titers on RK13 cells were calculated. Note that scale is logarithmic. **(B)** Exemplary fluorescence images of identical virus dilutions show large plaques on RK13 cells, and only single infected RK13 -UL19gR3 cells. Bar: 500 µm.

5. PrV growth kinetics on inhibitory cells



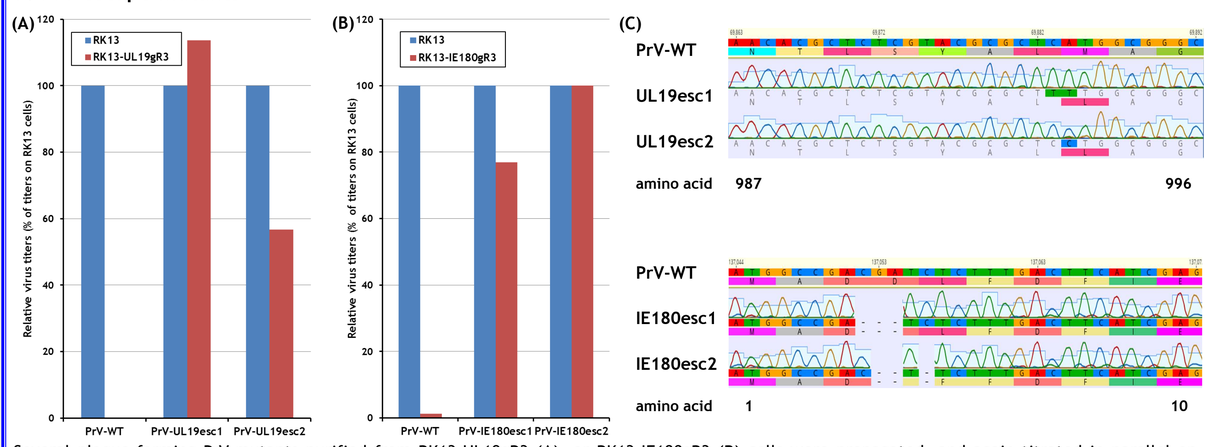
The indicated cell lines were infected with PrV-KaΔgGmCherry at a MOI of 0.05, and total progeny virus titers after 0, 24, 48, 72, and 96 h were determined on RK13 cells.

6. Stability of PrV-inhibiting CRISPR/Cas9 cell lines



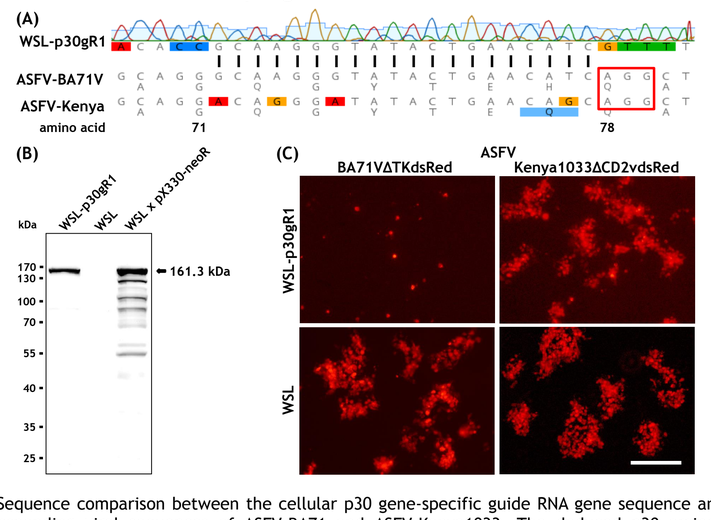
Representative clones of transgenic RK13-UL19gR3, -IE180gR3, -UL19gR2+3, -UL19gR3/IE180gR2 and -UL19gR2+3/IE180gR2+3 cells after low (aprox. 5) and high (> 30) passage numbers, as well as normal RK13 cells were infected with serial dilutions of PrV-KaΔgGmCherry under plaque assay conditions. **(A)** After two days the apparent virus titers (PFU/ml) were determined, and percentages of titers on RK13 cells were calculated. Note that scale is logarithmic. **(B)** PCR amplification and sequencing of the gene cluster encoding the four guide RNAs of RK13-UL19gR2+3/IE180gR2+3) cells indicated a stepwise loss of them during cell passage, presumably due to recombination between different copies of the almost perfect tandem repeats.

7. PrV escape mutants



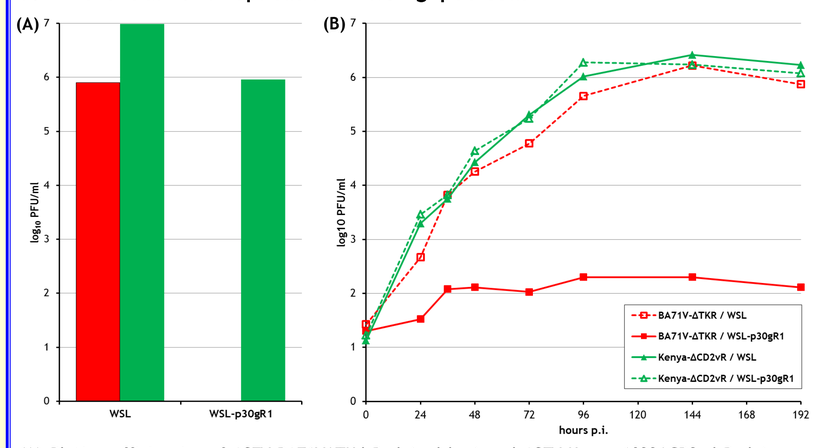
Several plaque-forming PrV-mutants purified from RK13-UL19gR3 **(A)**, or RK13-IE180gR3 **(B)** cells were propagated, and again titrated in parallel on RK13 and the corresponding CRISPR/Cas9 cell lines, demonstrating that, unlike the parental virus (PrV-WT), the escape mutants were not or only marginally inhibited. **(C)** PCR amplification and sequencing of the guide RNA target sequences revealed short in-frame deletions or single nucleotide exchanges leading to amino acid substitutions in pUL19 or IE180, respectively.

8. Characterization of WSL-p30gR1 cells



(A) Sequence comparison between the cellular p30 gene-specific guide RNA gene sequence and the corresponding viral sequences of ASFV-BA71 and ASFV-Kenya1033. The deduced p30 amino acid sequences with position numbers are given below the viral gene fragments. Differences to ASFV-BA71 are colored, and the targeted 20 nt (vertical lines) as well as the following PAM (red rectangle) are indicated. **(B)** Expression of FLAG-tagged Cas9 in WSL-p30gR1 cells, and in WSL cells transfected with pX330-neoR was detected by Western blot analyses using an anti-FLAG monoclonal antibody (Sigma-Aldrich). **(C)** Microscopic autofluorescence images showing single or foci of WSL-p30gR1 and WSL cells infected with the same dilutions of ASFV-BA71VΔTKdsRed and ASFV-Kenya1033ΔCD2vdsRed at day 5 after infection. Bar indicates 200 µm.

9. Differential ASFV replication in WSL-gRp30 cells



(A) Plating efficiencies of ASFV-BA71VΔTKdsRed (red bar) and ASFV-Kenya 1033ΔCD2vdsRed (green bars) on WSL and WSL-p30gR1 cells. Microtiter plates infected in parallel with serial virus dilutions were analyzed for foci of infected cells 5 d p.i. Total apparent titers are indicated. **(B)** Growth kinetics of ASFV-BA71VΔTKdsRed (squares) and ASFV-Kenya 1033ΔCD2vdsRed (triangles) on WSL (dotted lines), and WSL-p30gR1 (continuous lines) cells after infection at an MOI of 0.3. Shown are the mean results of three experiments.

Summary

- We prepared transgenic RK13 and WSL cell lines which constitutively express Cas9 nuclease and one, two, or four guide RNAs targeting essential genes or genome parts of PrV or ASFV.
- Several of the single guide RNAs had no detectable effects on virus replication in the cells, whereas others led to significant (> 100fold) reduction of plating efficiency and progeny virus titers.
- Coexpression of two or four guide RNAs targeting the same or different PrV genes abrogated plaque formation and productive replication almost completely.
- However, whereas virus inhibition mediated by single guide RNAs remained constant over many (> 50) passages of the cell lines, the initially more pronounced effects of the used multiplex systems declined gradually, obviously due to deleterious recombination events between the different guide RNA expression cassettes.
- Propagation of PrV on CRISPR/Cas9 cell lines targeting the major capsid protein (pUL19) or the immediate early protein (IE180) genes reproducibly led to the occurrence of escape virus mutants exhibiting in-frame deletions or nucleotide and deduced amino acid substitutions within the target sequences.
- In contrast, recombinant WSL cell lines which express a single guide RNA targeting codons 71 to 78 of the essential phosphoprotein p30 gene of ASFV inhibited virus spread and productive replication almost completely, and no escape mutations were observed up to now.
- The specificity of these effects could be confirmed by the unaffected replication of an ASFV strain possessing four naturally occurred base substitutions within the target region of the p30 gene.
- Thus, it appears conceivable that Cas9 nuclease and PRV- or ASFV gene-specific guide RNAs can be also permanently expressed in transgenic pigs to make them resistant against the respective pathogens.