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An experimental approach of an *in vivo* pathogen genome targeting strategy to generate African swine fever resistant pigs

THESIS

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In vitro genome editing activity of Cas9 in somatic cells after random and transposon-based genomic Cas9 integration. Plos one (submitted February 2022)

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LIST OF ABBREVIATIONS

ΔTK	delta Thymidine kinase
6-DMAP	6-Di-methylaminopurine
aa	Amino acid
Abs	Antibodies
acr	Anti-CRISPR
AI	Artificial insemination
ANPEP	Aminopeptidase N
ASFV	African swine fever virus
B2M	Beta-2-microglobulin
B4GALNT2	Beta-1,4 N-acetylgalactosaminyltransferase 2
C1-7	Wild-type control pigs 1 to 7 during infection study
CASA	Computer-assisted sperm analysis
cascade	Cas complex for antiviral defence
COCs	Cumulus-oocyte-complexes
CRISPR	Clustered regularly interspaced short palindromic repeats and its associated proteins
crRNA	CRISPR RNA
CSFV	Classical swine fever virus
DMEM	Dulbecco´s modified Eagle's medium
DMSO	Dimethyl sulfoxide
dpi	Days post-infection
DSBs	Double-strand breaks
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FCS	Foetal calf serum
FMD	Foot-and-mouth-disease
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescence protein
GGTA1	alpha-1,3-galactosyltransferase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMO	Genetically modified organism
gRNA	guide RNA
HAD 50	50 % hemadsorbing dose
HDR	Homology-directed repair

HEGs	Homing endonuclease genes
HEPN	Higher eukaryotes and prokaryotes nucleotide-binding
HIV-I	Human Immunodeficiency Virus-I
HPAI	Highly pathogenic avian influenza
ICP-4	Infected-cell polypeptide-4
IMDM	Iscove's Modified Dulbecco's Medium
Indels	Insertions and deletions
ITR	Inverted terminal repeats
IVF	*in vitro* fertilisation
KI	Knock-in
KO	Knockout
LB	Lysogeny broth
MDV	Marek's disease virus
MFI	Median fluorescence intensity
MOI	Multiplicity of infection
NBCS	New born calf serum
NGS	Next generation sequencing
NHEJ	Non-homologous end-joining
NLS	Nuclear localisation sequence
ORF	Open reading frame
PAM	Protospacer adjacent motif
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDCoV	Porcine deltacoronavirus
PEDV	Porcine epidemic diarrhoea virus
Pen/Strep	Penicillin/Streptomycin
PERVs	Porcine endogenous retroviruses
PRRS	Porcine reproductive and respiratory syndrome
PFU	Plaque forming unit
pUC-Rosa26	Vector pUC57-Brick-Rosa26-HA-Cas9-HiFi-Neo-dTK
PXM	Porcine X Media
PZM-3	Porcine-zygote media
RELA	v-rel reticuloendotheliosis viral oncogene homolog A

RI pig	Random integration of Cas9
RMCE	Recombinase Mediated Cassette Exchange
RNAi	RNA interference
RT-qPCR	Reverse-transcription quantitative PCR
SB	Sleeping Beauty
SB pigs	Sleeping beauty transposon based Cas9 integration
SCNT	Somatic cell nuclear transfer
siRNA	short-interfering double-stranded RNA
SpCas9	*Streptococcus pyrogens* Cas9
SRCR5	Fifth scavenger receptor cysteine-rich
T1-7	Transgenic pigs 1 to 7 during infection study
T7 assay	T7 endonuclease-I assay
TALENs	Transcription activator-like nucleases
TCID 50	50 % Tissue Culture Infectious Dose
TG	Transgenic
TGEV	Transmissible gastroenteritis virus
TL	Tyrode's lactate
tracrRNA	Trans-activating CRISPR RNA
WSL	Wild boar lung cells
WT	Wild-type
ZFNs	Zinc-finger nucleases

SUMMARY

An experimental approach of an *in vivo* pathogen genome targeting strategy to generate African swine fever resistant pigs

Jenny-Helena Söllner

The domesticated pig is invaluable as a global protein source. However, pork production frequently faces animal welfare and production challenges induced by viral disease outbreaks. For instance, the African swine fever virus (ASFV) reached eastern Europe in 2014 and in 2020 Germany. The virus causes mortalities of nearly 100 % in domestic pigs and wild boars. Until now, no treatments or vaccines are available, and containment of the virus relies on strict biosecurity measures. Therefore, it is of great importance to understand host-virus interactions to support the development of vaccines and/or anti-viral drugs.

Genome editing tools such as CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats and its associated protein) have made it possible to further understand host-pathogen interactions by altering the genome of the host or the pathogen to study the mechanisms of infections. The CRISPR-Cas9 system is comprised of two main components; the Cas9 endonuclease which induces DSBs (double-strand breaks) in DNA and a guide RNA (gRNA) which will lead Cas9 to its target DNA. One such strategy to investigate host-pathogen interaction, is called '*in vivo* pathogen genome targeting'. Cas9 is integrated into the host genome and programmed to induce DSBs in a vital locus of a certain virus. Upon infection of the host, Cas9 and the gRNA target the viral genome, induce DSBs, and inhibit replication. Such strategy has been successfully employed to generate chickens resistant to Marek's disease. Another study modified wild boar lung cells to express Cas9 and target the CP204L gene of ASFV. CP204L transcribes p30, a protein crucial for ASFV replication, and upon infection the modified cells displayed significantly reduced viral replication.

In this project, we generated pigs based on an *in vivo* pathogen genome targeting strategy targeting the essential CP204L gene of ASFV. Foetal porcine fibroblasts were modified and used as donor cells for somatic cell nuclear transfer (SCNT) followed by embryo transfers. The cloning efficiencies ranged from 4.7 to 13.6 %. In total, 17 pigs were generated which carried the Cas9 and gRNA integrations. While it is essential for the *in vivo* pathogen genome targeting strategy that the integrated Cas9 can induce DSBs, fibroblasts of the transgenic pigs were isolated and subjected to *in vitro* experiments. The Cas9 expressing fibroblasts were transfected with gRNAs targeting different porcine loci (GGTA1, B4GALNT2NT2, B2M). The transfection with gRNAs led to DSBs in the porcine genome of the transfected cells which was confirmed by Sanger sequencing, next generation sequencing and flow cytometry. Therefore, the transfected gRNAs guided the translated Cas9,

which was integrated into the porcine genome to the target sequence. The experiments validated that the Cas9 sequence was not only integrated into the porcine genome but also that the protein was successfully translated. Based on these findings and the inhibition of viral replication in modified wild boar lung cells, the transgenic pigs were admitted to an ASFV infection study. Seven control and seven transgenic pigs were infected, each with approximately 10^5 $TCID_{50}$ (50 % Tissue Culture Infectious Dose) of a highly virulent ASFV isolate. No differences between control and transgenic pigs were observed in terms of disease development, indicating that ASFV was still able to replicate within the transgenic pigs. In conclusion, Cas9 expression in the pigs was sufficient to induce genome edits within the porcine genome but not to protect the animals from ASF infection.

ZUSAMMENFASSUNG

Ein experimenteller Ansatz zur Erstellung Afrikanischer Schweinepest resistenter Schweine, basierend auf einer *in vivo* pathogen-spezfischen Genome Modifizierung.

Jenny-Helena Söllner

Das Hausschwein ist als globale Proteinquelle von unschätzbarem Wert. Die Schweinefleischproduktion steht jedoch häufig vor Herausforderungen, die durch Ausbrüche von Viruserkrankungen verursacht werden. Zum Bespiel hat das Virus, das die Afrikanische Schweinepest (ASP) verursacht, im Jahr 2014 Osteuropa und 2020 Deutschland erreicht. Das Virus verursacht bei Haus- und Wildschweinen eine Sterblichkeitsrate von fast 100 %. Bislang gibt es keine Behandlungsmöglichkeiten oder Impfstoffe, und die Eindämmung des Virus hängt von strengen Biosicherheitsmaßnahmen ab. Daher ist es von großer Bedeutung, die Wechselwirkungen zwischen Wirt und Virus zu verstehen, um die Entwicklung von Impfstoffen und/oder antiviralen Medikamenten zu unterstützen.

Mit Hilfe von Genom-Editierungs Technologien wie CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats und assoziiertes Protein) ist es möglich, die Infektionsmechansisms zwischen Wirt und Erreger gezielt zu erforschen und ein besseres Verständnis zu schaffen, indem das Genom des Wirts oder des Erregers verändert wird. Das CRISPR-Cas9 System besteht aus zwei Hauptkomponenten: der Cas9-Endonuklease, die Doppelstrangbrüche (DSBs) in der DNA induziert, und einer RNA (guideRNA), die Cas9 zu seiner Ziel-DNA führt. Eine dieser Strategien um Infektionsmechanismen zu verstehen, ist das so genannte „*in vivo* pathogen genome targeting". Bei dieser Strategie wird Cas9 in das Wirtsgenom integriert und so programmiert, dass es DSBs in einem lebenswichtigen Gen eines bestimmten Virus induziert. Nach der Infektion des Wirts greifen Cas9 und die gRNA das virale Genom an, induzieren DSBs und hemmen die Replikation. Diese Strategie wurde bereits erfolgreich eingesetzt, um Hühner zu erzeugen, die gegen die Marek-Krankheit resistent sind. Zudem wurden Lungenzellen von Wildschweinen so modifiziert, dass sie Cas9 exprimieren und das CP204L-Gen im ASP Virus angreifen. CP204L, das Protein 30 transkribiert, ist für die Replikation des ASP Viruses von entscheidender Bedeutung. Bei einer Infektion der Lungenzellen zeigten die modifizierten Zellen eine deutlich reduzierte Virusreplikation.

In diesem Projekt haben wir Schweine auf der Grundlage einer „*in vivo* pathogen genome“ Strategie erzeugt, die auf das essentielle CP204L-Gen des ASP Viruses abzielt. Fötale Schweinefibroblasten wurden modifiziert und als Spenderzellen für den somatischen Zellkerntransfer mit anschließendem Embryotransfer verwendet. Die Kloneffektivität lag zwischen

4,7 und 13,6 %. Insgesamt wurden 17 Schweine erzeugt, die die Cas9-Integrationen trugen. Da es für die „*in vivo* pathogen genome targeting“ Strategie wichtig ist, dass das integrierte Cas9 DSBs induzieren kann, wurden Fibroblasten der transgenen Schweine isoliert und *in vitro*-Experimenten unterzogen. Die Cas9-exprimierenden Fibroblasten wurden mit guideRNAs transfiziert, die auf verschiedene bekannte Schweine-Loci abzielen (GGTA1, B4GALNT2NT2, B2M). Die Transfektion mit guideRNAs führte zu DSBs im Genom der transfizierten Zellen, was durch Sanger-Sequenzierung, Next Generation Sequencing und Durchflusszytometrie bestätigt wurde. Die guideRNAs leiteten also das translatierte Cas9, das in das Schweinegenom integriert wurde, zur Zielsequenz. Die Experimente bestätigten, dass die Cas9-Sequenz nicht nur in das Schweinegenom integriert wurde, sondern auch, dass das Protein erfolgreich translatiert wurde. Auf der Grundlage dieser Ergebnisse und der erfolgreichen Hemmung der Virusreplikation in modifizierten Wildschwein-Lungenzellen wurden die transgenen Schweine in eine ASP-Infektionsstudie aufgenommen. Sieben Kontrollschweine und sieben transgene Schweine wurden jeweils mit etwa 10^5 $TCID_{50}$ (50 % Tissue Culture Infectious Dose) eines hochvirulenten ASP-Isolats infiziert. Hinsichtlich der Krankheitsentwicklung wurden keine Unterschiede zwischen Kontroll- und transgenen Schweinen festgestellt, was darauf hindeutet, dass sich das ASP Virus in den transgenen Schweinen weiterhin vermehren konnte. Zusammenfassend lässt sich sagen, dass die Cas9-Expression in den Schweinen ausreichte, um Genom-Editierungen im Schweinegenom zu induzieren, aber nicht, um die Tiere vor einer ASP-Infektion zu schützen.

1 INTRODUCTION

Livestock production is frequently threatened by infectious diseases, causing animal suffering and enormous economic losses. In the meantime, animal production is challenged to become more sustainable while covering the growing food demand (Alexandratos and Bruinsma, 2012). Infectious diseases jeopardize both objectives of livestock production, to become more sustainable and enhance production for a growing world population. Conventionally, infectious diseases are controlled through biosecurity measures, immunisation, hygiene, quarantine, culling, and education (Saegerman, Del Pozzo and Humblet, 2012). By means of these measures, Rinderpest was eradicated in 2011 and other viral diseases such as Classical swine fever (CSF) and Foot-and-mouth disease (FMD) were eliminated in the European Union (OIE World Animal Health Information and Analysis Departmen, 2018; OIE World Animal Health Information and Analysis Department, 2021b). Despite all these efforts, global livestock production still experiences severe outbreaks of infectious diseases, resulting in the culling of millions of animals. The most prevalent examples in the EU are the outbreaks of the Highly pathogenic avian influenza (HPAI) and African swine fever (ASF). HPAI alone caused the culling of more than 246 million animals, between 2005 and 2020 (OIE World Animal Health Information and Analysis Department, 2021a). In 2019, China lost approximately 30 % of their pig population to ASF, resulting in an 8 % decrease of global pork production in 2020 (FAO, 2020). ASF has also been present in eastern Europe since 2014 and reached central Europe in 2020, where the first case of ASF in wild boar was reported in Germany. So far, a total of 2,714 wild boars were found to be infected (28.01.2022) in Germany and four outbreaks in domestic pigs were confirmed (Animal Disease Information System (ADIS), 2020, 2021, 2022). In January 2022 also Italy reported 31 outbreaks (28.01.2022) of ASF in wild boar (Animal Disease Information System (ADIS), 2022; OIE World Animal Health Information and Analysis Department, 2022), highlighting the necessity to contain the disease.

No treatments or vaccines are available for ASF, and the disease causes mortalities of up to 100 % (King *et al.*, 2011). Considering there are no immunisation schemes available for ASF, protection of domestic pigs relies solely on the control of spreading the disease. However, in the last years new technologies have been developed which seem suitable to protect livestock from infectious diseases. Genome editors such as CRISPR-Cas can modify the genome of animals rendering them less susceptible to diseases. The CRISPR-Cas9 system is comprised of two main components; the Cas9 endonuclease which induces DSBs (double-strand breaks) in DNA and a guide RNA (gRNA) which will lead Cas9 to its target DNA (Jinek *et al.*, 2012). In combination with reproductive technologies such as somatic cell nuclear transfer (SCNT) and micromanipulation of

zygotes, animals resistant to specific diseases can be generated. One of the most prominent examples are, pigs resistant against Porcine reproductive and respiratory syndrome (PRRS) (Whitworth *et al.*, 2016; Burkard *et al.*, 2017; Wells *et al.*, 2017; Yang *et al.*, 2018; Guo *et al.*, 2019). CRISPR-Cas9 was employed to knock-out CD163, the porcine receptor necessary for PRRSV infection. Pigs carrying the CD163 KO (knock-out) were able to withstand PRRSV infection. Similarly, essential genes of e.g., a virus can be targeted to investigate whether viral replication would be inhibited. A strategy called '*in vivo* pathogen genome targeting' integrates Cas9 into the host genome and is programmed to induce DSBs in a vital gene of a certain virus. Upon infection with that virus, viral replication may be inhibited. For instance, transgenic chickens were generated expressing the Cas9 protein targeting viral genes of Marek's Disease Virus (MDV). Upon infection, the animals were resistant to MDV, identifying the target gene ICP4 (infected-cell polypeptide-4) essential for disease development (Challagulla *et al.*, 2021).

Such a strategy may also be employed to generate ASF resistant pigs. Several viral proteins of the ASF virus have been shown to be important for virus entry and attachment. The proteins p72, p12, p30/32, and p54 encoded by B646L, O61R, CP204L, and E183L, respectively, have been identified to be essential for virus entry (Dixon *et al.*, 2004, 2013). Previously, modified wild boar lung (WSL) cells expressing Cas9 and a gRNA targeting CP204L of ASFV were generated. These cells were found to have almost no viral replication upon infection with an virulent ASFV strain (Hübner *et al.*, 2018). On the other hand, a strain carrying variation within the target sequence of CP204L showed no inhibition of viral replication, which was the perfect proof-of principle experiment as it shows that the disruption of CP204L was causative for ASFV resilience (Hübner *et al.*, 2018). It was concluded that the production of transgenic pigs expressing Cas9 and gRNA cassette targeting p30 may facilitate *in vivo* pathogen genome targeting against ASFV infection.

Two approaches were designed to modify porcine fibroblasts for *in vivo* pathogen genome targeting. The resilient WSL cells were generated through random integration of a modified Cas9 and gRNA expression vector (Hübner *et al.*, 2018), which is commonly used to induce genome edits in mammalian cells (pX330-U6-Chimeric_BB-CBh-hSpCas9) (Cong *et al.*, 2013; Ran *et al.*, 2013). The vector was modified to express a selection marker to select cells expressing the selection marker. The second approach was based on Sleeping Beauty (SB) transposon transposition. The SB transposon system consists of two parts, the transposon containing the gene to be inserted and a vector encoding the transposase. The transgenes in the transposon vector are flanked by inverted terminal repeats (ITRs), which will be recognised by the SB transposase. When the transposase locates the ITRs, it excises the sequence within the ITRs and the insert is integrated into genomic DNA in a 'cut-and-paste' manner (Ivics *et al.*, 1997; Ivics and Izsvák, 2015). SB transposon-based

transgenesis is highly efficient in pigs (Carlson *et al.*, 2011; Garrels *et al.*, 2011; Jakobsen *et al.*, 2011) and other mammalian species (Mátés *et al.*, 2009; Garrels *et al.*, 2016) and integrated transgenes are stably expressed. Therefore, a SB vector system was built to integrate Cas9 and gRNA transgenes into porcine fibroblasts. After fibroblast were successfully modified, they were used as donor cells for SCNT.

The aim of the study was to explore the potential of a CRISPR-Cas9 *in vivo* pathogen genome targeting strategy to generate ASF resilient pigs. Based on the results of Hübner et. al., (2018) in which ASFV replication was inhibited in Cas9 and gRNA expressing WSL cells, it was hypothesised that an *in vivo* pathogen genome targeting strategy in pigs facilitates resilience to ASF.

The objectives of the project were the following:

1. To generate transgenic Cas9 and gRNA expressing pigs for an *in vivo* pathogen genome targeting strategy against ASFV.
2. To assess the functionality of the Cas9 transgene in the animals.
3. To investigate the susceptibility to ASF of the transgenic pigs expressing Cas9 and gRNAs targeting CP204L of ASFV.

To accomplish the above-mentioned objectives the following steps were performed:

1. Isolated wild-type fibroblasts were modified to express Cas9 and a gRNA targeting CP204L of ASFV based on random integration and transposon-based integration.
2. Modified fibroblasts were used for somatic cell nuclear transfer to generate transgenic pigs.
3. Generated offspring was genotyped.
4. Isolated fibroblasts of the transgenic offspring were transfected *in vitro* with gRNAs to assess Cas9 functionality.
5. Animals were subjected to *in vitro* and *in vivo* ASFV studies.

2 LITERATURE REVIEW

The literature review is based on the previously published work 'Genome Editing Strategies to Protect Livestock from Viral Infections' (Söllner, Mettenleiter and Petersen, 2021).

2.1 GENOME EDITING

Genome editing tools are employed to induce double-strand breaks (DSBs) at specific locations in genomes. Before the discovery of CRISPR-Cas (Clustered regularly interspaced short palindromic repeats and its associated proteins) as genome editing tool, TALENs (transcription activator-like nucleases) and Zinc-finger nucleases (ZFNs) were utilised to induce targeted modifications in DNA. However, due to several limitations TALEN and ZFNs have been widely replaced by CRISPR-Cas. The endonuclease protein of genome editors cleaves the target DNA at specific locations which will be repaired by either non-homologous end-joining (NHEJ) or by homology-directed repair (HDR) (Rouet, Smih and Jasin, 1994) (Figure 1). NHEJ is an error-prone mechanism which leads to insertions and deletions (indels) in the target sequence. The most frequent repair mechanism NHEJ, is desired for generating functional gene knock-outs (KO). HDR on the other hand generates functional knock-ins (KI) when a DNA donor template with homologous arms is available for insertion (Ran *et al.*, 2013). However, the mechanism only occurs in the late S and G2 phase of the cell cycle (Heyer, Ehmsen and Liu, 2010).

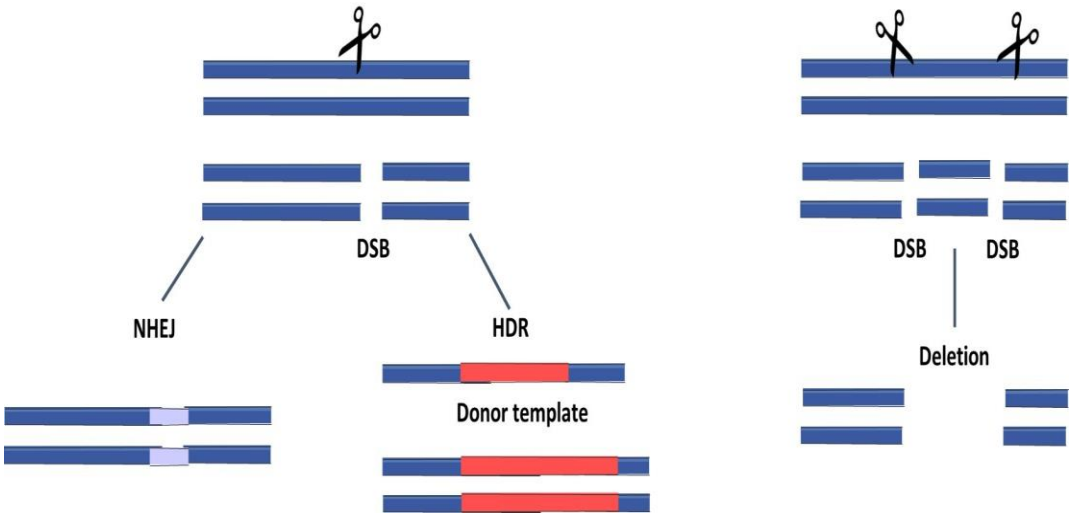

Figure 1: Genome editing strategies: Genome editors induce targeted double-strand breaks (DSBs). Single target sited can be repaired by either non-homologous end joining (NHEJ)-generating inserts and deletions or by homology-directed repair (HDR). By providing a donor template, the desired sequence can be integrated by HDR. Large deletions can be induced by cleaving two target sites in the locus of interest. (Figure retrieved from Söllner et. al., (2021)).

2.2 The CRISPR-Cas System

CRISPR-Cas has been the latest addition to the genome editing toolbox. Its potential to induce directed DSB in the DNA (Jinek *et al.*, 2012) has been exploited and further developed as genome editing tool in the last years. CRISPRs were first discovered in 1987 by Ishino et. al. (1987) in *E.coli* (*Escherichia coli*) (Ishino *et al.*, 1987) and further research revealed that CRISPRs and its associated genes (*cas*) are part of the adaptive immune systems of prokaryotes (Mojica *et al.*, 2000; Makarova *et al.*, 2006). Since then, the CRISPR-Cas systems of archaea and bacteria have been extensively researched, described, and classified (Makarova *et al.*, 2011, 2015, 2020; Koonin, Makarova and Zhang, 2017; Makarova, Wolf and Koonin, 2018). The CRISPR-Cas systems have been classified into two classes, class 1 consisting of type I, II, and IV and their corresponding sub-types, class 2 includes type V and VI and their sub-types. Class 1 contains multiple Cas proteins, a so-called cascade (Cas complex for antiviral defence) and an endonuclease Cas which binds to crRNAs (CRISPR RNA), an RNA displaying homology to the target sequence. Class 2 consists of only one multidomain endonuclease binding to crRNAs.

2.2.1 Cas9 variants repurposed as genome editing tools

The most prominent genome editing variant of the Cas family is the Cas9 protein. Cas9 belongs to the class 2 type II CRISPR system, an endonuclease binding to a targeting crRNA. It is characterised by two nuclease domains, the RuvC and HNH nucleases. The nucleases induce DSBs in DNA by cleaving the complementary and non-complementary strand of the crRNA. CRISPR RNA in

combination with a tracrRNA (trans-activating CRISPR RNA) form the gRNA (guide RNA) and can be synthesised to target specific locations in the genome (Jinek *et al.*, 2012). However, target sites are limited to PAM (protospacer adjacent motif) availability. The crRNA requires a 5' NGG 3' PAM adjacent to the target sequences to induce a DSBs in combination with Cas9, thereby limiting target sites. Nonetheless, since Cas9 was first retrieved from *Streptococcus pyrogens* (SpCas9) CRISPR-Cas has revolutionised the field of genome editing due to its higher specificity, greater efficiency, and easier adaptation compared to ZFN and TALEN (Doudna and Charpentier, 2014). Bioengineering made it possible to diversify PAM requirements and there are now several varieties of Cas9 variants available (Pickar-Oliver and Gersbach, 2019). For example; VQR SpCas9 recognises 5' NGAN 3' and 5' NGNG 3' PAMs and EQR SpCas9 5' NGAG 3' PAMs (Kleinstiver *et al.*, 2015). Recently, a near-PAMless SpCas9 modified within the PAM-interaction site was reported, recognising 5' NRN 3' (R: A or G) and 5' NYN 3' (Y: C or T) PAMs (Walton *et al.*, 2020). Cas9 activity has also been improved further over the years and Cas9 derivatives were engineered to enhance target specificity. In Schmidt-Burgk et al. (2020), the different Cas9 modifications were evaluated by their cleaving activity and on-target specificity. According to their research, WT (wild-type) SpCas9 ranks the lowest in specificity, and modified proteins such as evoCas9 and SpCas9HF1 the highest, though their cleavage activity scores were lower than the WT SpCas9. The variants HiFi Cas9 and eSpCas9 on the other hand, showed high activity as well as specificity (Schmid-Burgk *et al.*, 2020). Therefore, it is now possible to design genome editing experiments to efficiently target almost any locus of a genome with Cas9.

2.2.2 Different Cas variants repurposed as genome editing tools

Not just the development of more efficient Cas9 protein has contributed to the diverse utilisation of the CRISPR-Cas system as genome editing tool, also other proteins of the same family have proven to be useful. Cas12a previously known as Cpf1, targets T-rich PAM sequences (5' TTTN 3') (Zetsche *et al.*, 2015). The endonuclease Cas12a belongs to the class 2 type V of the CRISPR-Cas systems and does not require a tracrRNA unlike Cas9. Its advantage over Cas9 in addition to the T-rich PAM sequence, is that it induces sticky ends after DNA cleavage to facilitate more efficient HDR experiments (Zetsche *et al.*, 2015). While Cas12a and Cas9 target and cleave DNA, Cas13 is able to cleave RNA (Abudayyeh *et al.*, 2016; Cox *et al.*, 2017; Konermann *et al.*, 2018). Cas13 is part of class 2 type VI and consists of two HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domains which will cleave target RNA if guided by a crRNA. A class 1 type 7 Cas protein; Cas3 has also been repurposed for genome editing, the first one of its kind. Cas3 has been shown to induce genomic deletions of up to 100 kb (Dolan *et al.*, 2019; Morisaka *et al.*, 2019).

2.3 GENOME EDITING AS STRATEGY TO CONFER VIRAL RESISTANCE

2.3.1 Disease susceptibility

Genome editing can facilitate the elimination of the genetic susceptibility of livestock to specific diseases. For instance, when viral entry depends on specific receptors, the genetic code of the receptor can be modified, making it impossible for the virus to enter the cells (Whitworth *et al.*, 2016). Also, inter-and intraspecies genetic variation may determine disease outcome of viral infections. As for instance, ASFV causes fatal disease in domestic pigs and wild boars, but remains subclinical in bushpigs and warthogs (Anderson *et al.*, 1998; Oura *et al.*, 1998). Genome editing can be used to introduce or eliminate these variations and render disease resistant animals (Figure 2) (Lillico *et al.*, 2013, 2016; Koslová *et al.*, 2020; McCleary *et al.*, 2020).

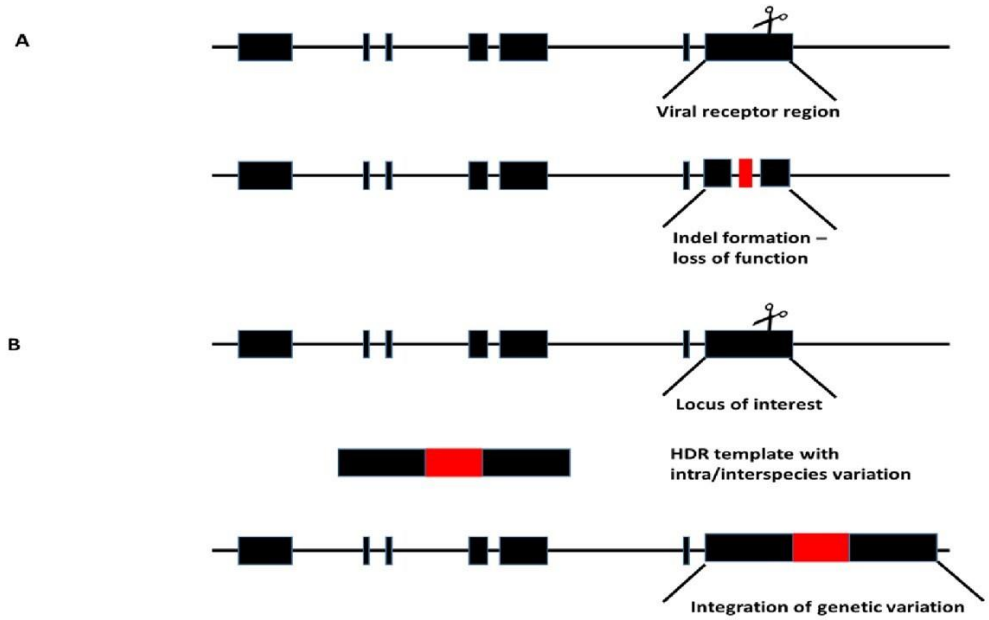

Figure 2: Targeting loci of interest: **A)** Previously identified receptors susceptible to viral infection could be targeted with genome editors, thereby inducing loss of function. **B)** Identified inter/intra-species variation responsible for viral susceptibility can be inserted by homology-directed repair (HDR). (Figure retrieved from Söllner et.al., (2021)).

2.3.2 *In vivo* pathogen genome targeting

Another strategy to induce viral resistance is *in viv*o pathogen genome targeting (Chen *et al.*, 2017), a strategy derived from RNA interference (RNAi). RNAi has emerged as a suitable tool to target RNA viruses in animal cells (Chen *et al.*, 2008; Lyall *et al.*, 2011; Wang *et al.*, 2012). It acts as a post-transcriptional gene silencing mechanism employing short-interfering dsRNA (siRNA) to downregulate viral mRNA expression (Bitko and Barik, 2001). While RNAi acts on a post-transcriptional level, *in vivo* pathogen genome targeting already infers before viral replication when

DNA/RNA viruses enter the cell (Chen *et al.*, 2017). By integrating a Cas9 cassette and gRNAs specific to a virus, into the host's genome, Cas9 will cleave the viral DNA/RNA upon cell entry and inhibit viral replication (Figure 3).

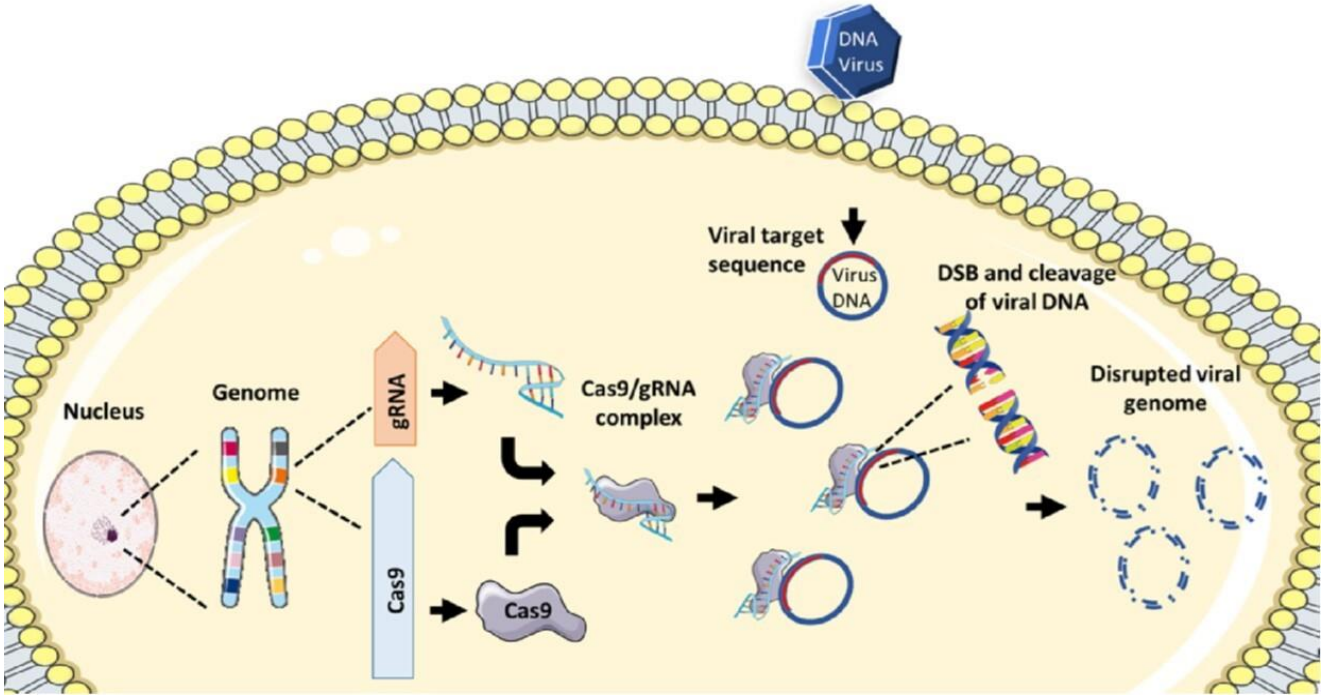

Figure 3: *In vivo* pathogen genome targeting: Cas9 and gRNAs (gRNAs) targeting virus genomes are integrated not the host genome via genome editors or transposon systems. Cas9 and gRNAs are expressed and form a gRNA-Cas9 duplex. The gRNAs target the complementary DNA of the virus genome, and Cas9 induces a double-strand break (DSB) blocking viral replication. (Figure retrieved from Söllner et.al., (2021)).

2.3.3 Gene drives

Disease resistance can be promoted by so-called gene drives, which turn heterozygous alleles into homozygous alleles. Gene drives use site-specific homing endonuclease genes (HEGs), which occur naturally and detect site-specific sequences in the homologous chromosome that does not carry the HEG sequence. HEGs then induce DSBs in the homologous chromosome and integrate via homologous recombination using the chromosome containing the HEG sequences as template (Burt, 2003). The properties of the CRISPR-Cas system make it suitable to generate 'CRISPR alleles' (Hammond *et al.*, 2016), the gRNAs can be designed to induce DSBs at specific locations and also 'copy' the transgene into the homologous chromosome, thereby promoting inheritance (Figure 4). A CRISPR-Cas9 gene drive was designed to establish a sterile female mosquito (*Anopheles gambiae*) population, to potentially decrease mosquito population and control malaria transmission (Hammond *et al.*, 2016).

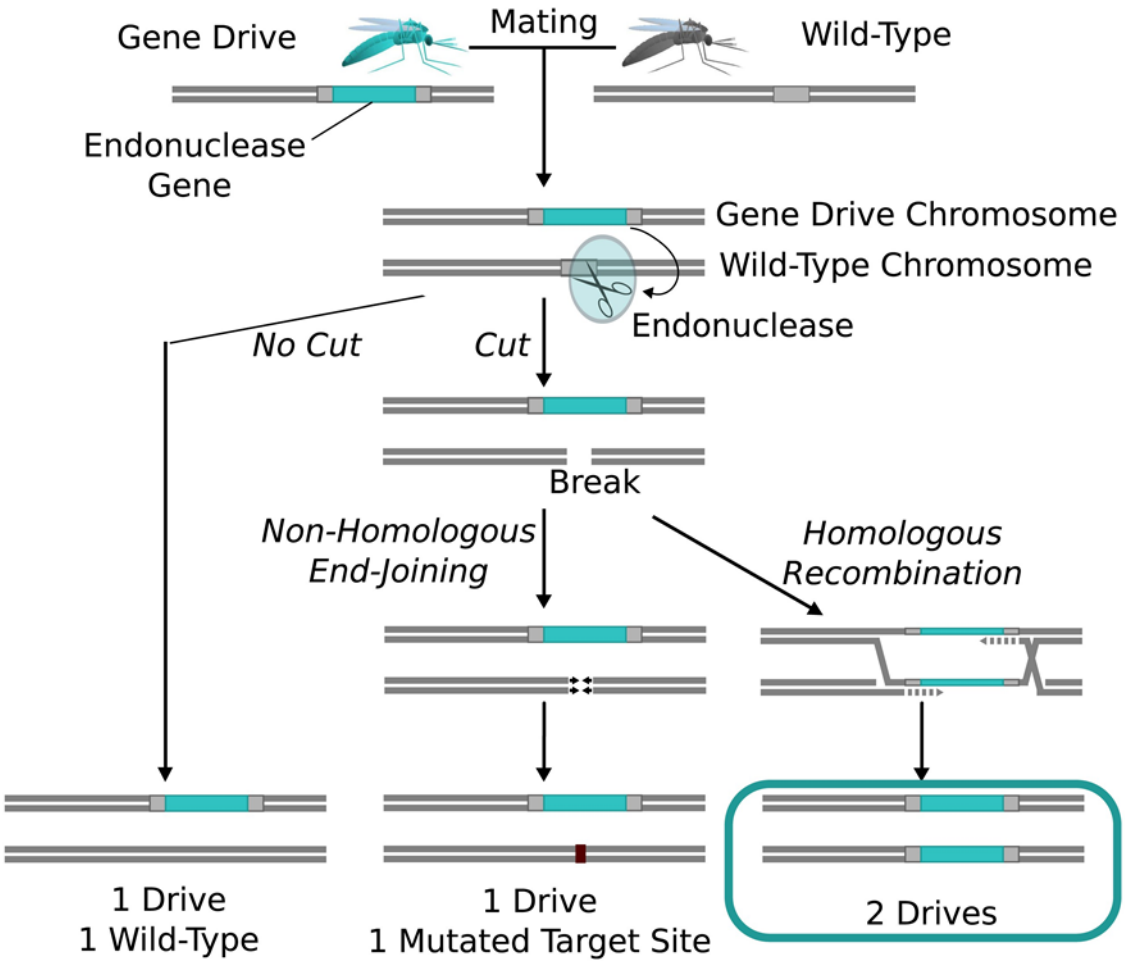

Figure 4: **Gene drives:** Endonuclease gene drives are preferentially inherited because the endonuclease cuts the homologous wild-type chromosome. When the cell repairs the break using homologous recombination, it must use the gene drive chromosome as a repair template, thereby copying the drive onto the wild-type chromosome. If the endonuclease fails to cut or the cell uses the competing non-homologous end-joining repair pathway, the drive is not copied. (Figure adapted from Esvelt et al., (2014)) (Esvelt *et al.*, 2014)

2.4 DISEASE RESISTANT PIGS

Before genome editors became available, the vision of disease resistant pigs and livestock existed within the scientific community. In 1991, Weidle et. al., genetically modified pigs, and rabbits to resist viral infections by randomly integrating mouse Ig heavy and light chain genes. The transgenes transcribing monoclonal mouse antibodies were meant to act as a general *in vivo* immunisation against influenza (Weidle, Lenz and Brem, 1991). Similarly, Lo et. al., (1991) integrated mouse IgA chains into pig and sheep genomes (Lo *et al.*, 1991). Another early study transferred the murine myxovirus-resistant system into the porcine genome, to facilitate resistance to influenza (Müller *et al.*, 1992). However, these studies all reported difficulties with insufficient protein translation of the transgenes. Since then, several studies have emerged investigating disease resistance by inducing genomic modifications in pigs (Table 1).

Table 1: Studies investigating viral resistance/resilience in pigs (adapted from Söllner et.al., (2021)).

Virus	Gene		SCNT/Micromanipulation	Method	Reference
	Host	Virus			
Pigs					
Influenza viruses	Mouse Mx1		PNI	DNA construct	(Müller *et al.*, 1992)
FMDV		Nonstructural protein 2B, Polymerase 3D	SCNT	RNA interference	(Hu *et al.*, 2012)
		Viral Protein 1	SCNT	RNA interference	(Hu *et al.*, 2015)
PRRSV	CD163		SCNT	CRISPR-Cas9	(Whitworth *et al.*, 2016)
	CD163 SRCR5		CMI	CRISPR-Cas9	(Burkard *et al.*, 2017)
	CD163-like homolog		SCNT	CRISPR-Cas9	(Wells *et al.*, 2017)
	CD163		SCNT	CRISPR-Cas9	(Yang *et al.*, 2018)
	CD163 SRCR5		SCNT	CRISPR-Cas9	(Guo *et al.*, 2019)
CSFV		NS4B	SCNT	CRISPR-Cas9 and RNAi	(Xie *et al.*, 2018)
PRRSV, TGEV, PDCoV	CD163 SRCR5 and ANPEP		SCNT	CRISPR-Cas9	(Xu *et al.*, 2020)
TGEV, PEDV	ANPEP		CMI	CRISPR-Cas9	(Whitworth *et al.*, 2018)
	ANPEP		SCNT	CRISPR-Cas9	(Luo *et al.*, 2019)
PEDV	CMAH		CMI	CRISPR-Cas9	(Tu *et al.*, 2019)
ASFV	RELA		CMI	Zinc-finger nucleases	(Lillico *et al.*, 2013; McCleary *et al.*, 2020)
	CD163		SCNT	CRISPR-Cas9	(Popescu *et al.*, 2017)
PERVs		pol	SCNT	CRISPR-Cas9	(Niu *et al.*, 2017)

PNI: Pronuclear microinjection, CMI: Cytoplasmic microinjection, SCNT: Somatic cell nuclear transfer, RNAi: RNA interference, FMDV: Foot-and-mouth disease virus, PRRSV: Porcine reproductive and respiratory syndrome virus, CSFV: Classical swine fever virus, TGEV: Transmissible gastroenteritis virus, PDCoV: Porcine deltacoronavirus, PEDV: Porcine epidemic diarrhoea virus, ASFV: African swine fever virus, PERV: Porcine endogenous retroviruses.

2.4.1 Foot-and-mouth-disease virus

With the development of engineered RNAi to target RNA viruses, the generation of resilient pigs became feasible. The viral protein 1 of the Foot-and-mouth-disease virus (FMDV), a destructive RNA virus causing fever and lesions in cloven-hoofed animals, was targeted by siRNAs to interfere with protein 1 synthesis (Hu *et al.*, 2015). The expression of the siRNAs varied between the animals and among different tissues. Therefore, for the infection study, the animals were grouped into high and low expressing groups. Differences between the low and high siRNA expressing groups were observed. Pigs with a high expression of siRNAs developed only a minor vesicle 9 dpi (days post-infection), while maintaining normal body temperatures. The low expressing group showed more severe signs, developing several lesions 7 dpi and mild fevers (39.5-40 °C). However, wild-type pigs displayed lesions already 3 dpi and were found to have higher viral loads in serum and lesions. Though, the transgenic pigs were not resistant, resilience to FMD was achieved (Hu *et al.*, 2015).

2.4.2 Porcine respiratory and reproductive syndrome virus

Fully resistant pigs against the viral disease Porcine respiratory and reproductive syndrome (PRRS) were first reported in 2016. In piglets, PRRSV causes respiratory symptoms while inducing abortions and stillbirth in gestating sows (Lunney *et al.*, 2016). Economic losses of PRRSV are estimated to be as high as 664 million dollars in the United States (Holtkamp *et al.*, 2013) and 1.5 billion in the European Union (EU) (de Paz, 2015). Three CRISPR-Cas9 edited pigs, lacking the porcine CD163 receptor, the receptor necessary for PRRSV infection, were infected with the PRRSV. The pigs did not develop clinical signs, viremia, or PRRSV specific antibodies (Abs) (Whitworth *et al.*, 2016). CD163, in particular the fifth scavenger receptor cysteine-rich (SRCR5) domain, serves as a fusion receptor for PRSSV infection (Van Gorp *et al.*, 2010) and is expressed by mature macrophages (Pulford *et al.*, 1992). Nonetheless, crucial biological functions such as preventing oxidative toxicity by removing haemoglobin from blood plasma are associated with the CD163 receptor (Kristiansen *et al.*, 2001; Van Gorp, Delputte and Nauwynck, 2010). Therefore, to maintain the physiological function of CD163, several studies have concentrated their Cas9 KO strategy to the SRCR 5 domain (Burkard *et al.*, 2017; Guo *et al.*, 2019; Xu *et al.*, 2020) or replaced it with a CD163-like homolog (Wells *et al.*, 2017). These improved strategies to minimise KO trade-offs have also proven effective against PRRSV (Burkard *et al.*, 2017; Wells *et al.*, 2017; Yang *et al.*, 2018; Guo *et al.*, 2019; Xu *et al.*, 2020).

In vivo pathogen genome targeting of PRRSV RNA was successful in MARC-145 cells. The infected cells showed a reduction of > 90 % mRNA expression, when Cas13b integrated into the genome and programmed to knockdown RNA expression in ORF5 and 7 of PRRSV (Cui *et al.*, 2020).

2.4.3 Coronaviruses

Alphacoronaviruses such as the Transmissible gastroenteritis virus (TGEV) and Porcine epidemic diarrhoea virus (PEDV) cause diarrhoea and dehydration in piglets with mortality rates reaching up to 100 % (Saif *et al.*, 2019). Through a targeted KO strategy of aminopeptidase N (ANPEP) with CRISPR-Cas9, the receptor was identified as crucial for TEGV infection but not for PEDV (Whitworth *et al.*, 2018; Luo *et al.*, 2019).

Identifying single receptors for viral entry renders the possibility to generate pigs with multiple receptor knock-outs to resist multiple infections. The double KO of the receptors CD163 and ANPEP induced resistance to PRRSV and TEGV in pigs. In addition, production and reproduction performances were evaluated and no differences were observed compared to wild-type pigs (Xu *et al.*, 2020). Furthermore, the study evaluated the susceptibility of these pigs to Porcine deltacoronavirus (PDCoV) which was discovered in 2012 (Woo *et al.*, 2012) and causes watery diarrhoea and dehydration (Saif *et al.*, 2019). Until now, its entry and replication mechanisms have not yet been fully understood (Koonpaew *et al.*, 2019). The involvement of ANPEP has been suggested by Wang et al. (2018) through a line of evidence such as, ANPEP functions as an entry receptor for PDCoV (Wang *et al.*, 2018). The challenged CD163 and ANPEP KO pigs developed delayed antibody formation compared to the control group, but at 14 dpi there was no difference in Ab levels between KO and control pigs. Disease symptoms were less severe in KO pigs. KO animals did not display thinning of the small intestinal wall or mesenteric hyperaemia, but still showed lesions in the small intestines to the same extent as the control animals. Infection of porcine alveolar macrophages of KO and WT pigs resulted in a significant decrease of susceptibility. Therefore, the study revealed that ANPEP plays a role in viral recognition, but is not the sole mechanism to facilitate PDCoV infection (Xu *et al.*, 2020).

2.4.4 African swine fever virus

While there are examples of resistant animals modified by a single genomic edit, virus-host interactions are in many cases more complex, as it has been shown for ASF. The disease causes mortality rates of up to 100 % in domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa ferus)* (Dixon *et al.*, 2004), whereas only subclinical infections occur in bushpigs (*Potamochoerus porcus*) and warthogs (*Phacochoerus africanus)* (Anderson *et al.*, 1998; Oura *et al.*, 1998), suggesting that genetic variation between the species may be responsible for the severity of the disease. One candidate locus which was identified as potential variation for disease development between the species; was the *RELA* (v-rel reticuloendotheliosis viral oncogene homolog A) locus. Variation in three amino acids (aa) between domestic pigs and warthogs were identified in the *RELA* locus (Palgrave *et al.*, 2011). The ASFV gene *A238L* and its translated protein A238L display partial homology to the porcine IκBα (Yáñez *et al.*, 1995) which bind to RELA (p65) thereby inhibiting binding of porcine IκBα.

(Tait *et al.*, 2000). A238L inhibits transcription factors which are crucial for certain regulations of proinflammatory cytokine expression, T- and B-cell functionality, and maintenance of immune homeostasis (Caamaño and Hunter, 2002). Therefore, ASFV infection blocks the NF-κB pathway and inhibits the immune response (Tait *et al.*, 2000). Three *RELA* modified pigs were generated via ZFNs, of which one only carried modifications at two aa (2aa), the other two carried the correct three aa (3aa) modifications (Lillico *et al.*, 2016). Homozygous offspring of these founder animals were challenged with ASFV, but no resistance was observed. However, a delayed onset of clinical symptoms, and decreased viral loads were observed in the 3aa pigs (McCleary *et al.*, 2020).

While the ASFV entry mechanism is not yet fully understood, CD163 has been suggested as an ASFV receptor (Sanchez-Torres *et al.*, 2003). The PRRSV resistant CD163 KO pigs were therefore infected with ASFV. However, no differences *in vitro* nor *in vivo* after infection were observed, ruling out CD163 as a crucial entry factor for ASFV (Popescu *et al.*, 2017).

In an approach of *in vivo* pathogen genome targeting, the viral gene CP204L translating p30 was targeted by Cas9 and gRNA expressing wild boar lung cells (WSL). The protein p30 is a phosphoprotein with involvement in internalisation and viral entry (Dixon *et al.*, 2004). The gRNA was designed to target the ORF (open reading frame) of CP204L, an important immunogenic protein (Dixon *et al.*, 2004). Upon integration of the transgenes the cells were infected with two ASFV isolates; ASFV Armenia 07 and Kenya1033. CP204L of ASFV Armenia 07 displayed complete homology to the gRNA sequence, whereas Kenya1033 revealed four mismatches within the target sequence. Plaque assay confirmed that the replication of ASFV Armenia 07 was inhibited, but replication of Kenya1033 was not affected. In addition, titration of virus progenies post infection revealed significant differences in growth kinetics. While Kenya1033 replicated in control and transgenic cell lines in a similar manner ($2x10^6$ plaque forming units (PFU)/ml 96 hours post infection), viral titers of ASFV Armenia 07 only reached 2 $x10^2$ PFU/ml in gRNA expressing cells (Hübner *et al.*, 2018). The results showed that the disruption of CP204L in the Armenia strain was causative for ASFV resilience in the WSL cells.

2.4.5 Porcine endogenous retroviruses

Porcine endogenous retroviruses (PERVs) are of concern for potential porcine xenografts to be employed in organ transplantation, i.e., xenotransplantation. PERVs which are integrated in the porcine genome do not cause clinical symptoms, but it has been shown *in vitro* that PERVs can translocate into the human genome (Moalic *et al.*, 2006) The observed integration sites in the human genome suggest that PERVs may facilitate tumorigenesis (Moalic *et al.*, 2006; Wilson, 2008). RNAi was employed to reduce expression of PERVs (Dieckhoff *et al.*, 2008; Ramsoondar *et al.*, 2009) and a reduction of 94 % of PERVs expression was reported (Dieckhoff *et al.*, 2008).

Recently, CRISPR-Cas9 genome editing generated pigs in which all copies of PERVs were inactivated to prevent viral transmission between pigs and humans. Karyotyping of the edited pigs revealed no abnormalities and the pigs remained healthy (Niu *et al.*, 2017).

3 MATERIAL AND METHODS

3.1 PROJECT OVERVIEW

In figure 5 the general workflow of the project is depicted. Transgenic pigs were generated for an *in vivo* pathogen genome targeting strategy to target the essential CP204L gene of the African swine fever virus (ASFV). The pigs were characterised, the Cas9 transgene activity investigated, and finally the pigs were submitted to an African swine fever infection study.

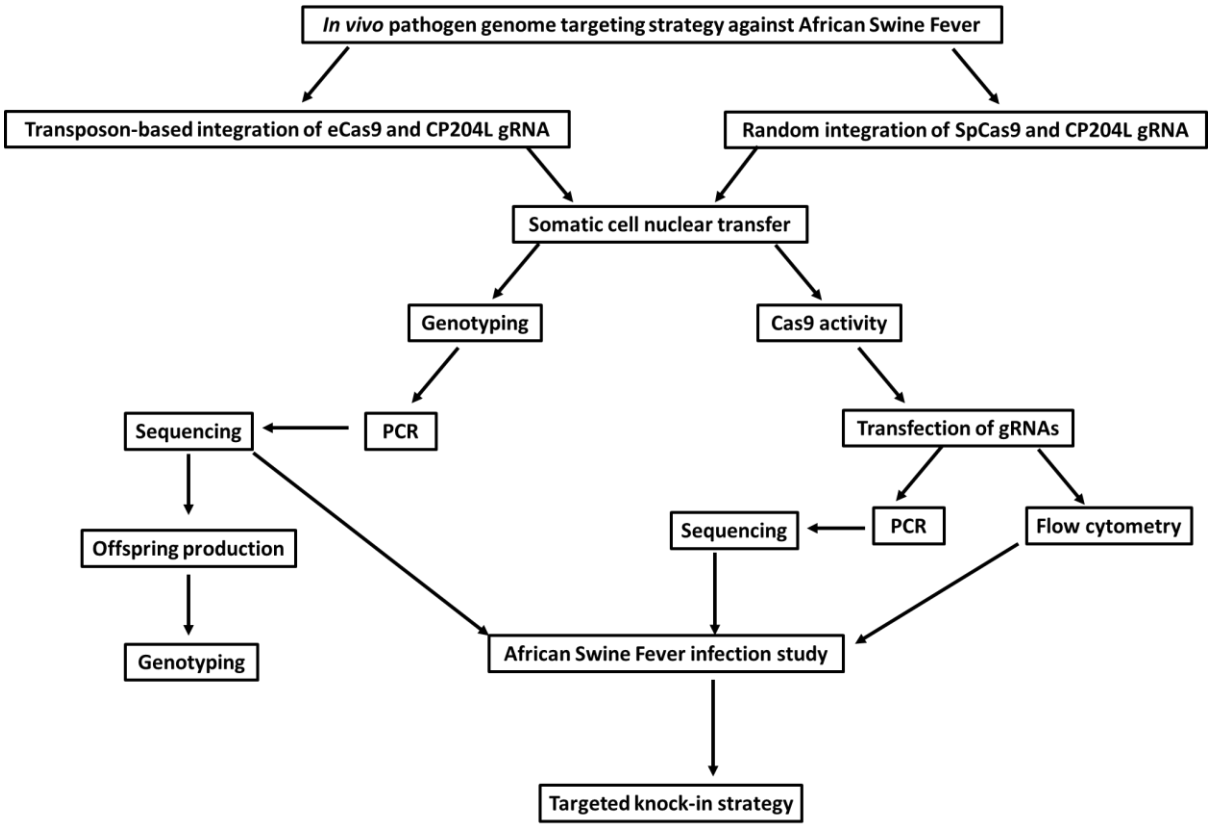

Figure 5: Project overview: To facilitate an *in vivo* pathogen genome targeting strategy against African swine fever (ASF), somatic cells were modified to express Cas9 and a gRNA targeting CP204L of ASFV. The cells were modified based on a transposon-based integration or random integration. After generating transgenic pigs by somatic cell nuclear transfer, the pigs were genotyped by PCR and Sanger sequencing. When the desired genotypes were confirmed a selection of boars were used to generate offspring. Isolated fibroblasts of transgenic pigs were transfected with CRISPR gRNAs (guide RNAs) to evaluate genome editing efficiency of the *in vitro* expressed Cas9. The gRNA and Cas9 expressing pigs were then admitted to an ASF infections study. Finally, a targeted knock-in strategy for Cas9 in the porcine Rosa26 locus was developed.

3.2 ETHICAL STATEMENT

All experiments in this thesis have been approved by the 'Landesamt für Verbraucherschutz und Lebensmittelsicherheit' (LAVES) in Lower Saxony, Germany (Animal Experiment No.: TVA 33.8-42502-04-18/2862, TVA 33.8-42502-04-16/2343). In addition, the *in vivo* infection study was approved by the 'Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern' (Animal Experiment No.: TVA 7221.3-1-017/20). The pigs were housed in line with the German Animal Welfare regulations and during the infection study according to biosecurity level 4 standards.

3.3 CONSTRUCTION OF TRANSFECTION VECTORS

Genetic modification of somatic cells was accomplished by designing and cloning plasmids to induce the desired modifications when transfected into fibroblasts. All vectors used in this project were amplified in NEB® 5-alpha Competent *E.coli* (High Efficiency) according to the High Efficiency protocol (C2987H) if not stated otherwise. In short, 3 µl digested plasmid were added to NEB® 5-alpha Competent *E. coli* cells, placed on ice for 30 minutes, heat shocked at 42 °C, transferred back on ice for 5 minutes before being incubated for 60 minutes with outgrowth media. Afterwards cells were plated on LB (Lysogeny broth) agar dishes (Annex 8.7 Table 20) with ampicillin (unless otherwise stated) and incubated over night at 37 °C. Colonies were picked the next day and transferred into 3 ml LB media (Annex 8.7 Table 19) containing 3 µl ampicillin. After another overnight incubation at 37 °C under constant agitation, plasmid DNA was purified with the GeneJET Plasmid-Miniprep-Kit (ThermoScientific™) according to the manufacturers' protocol. About 100 ng of purified DNA was sent for Sanger sequencing to confirm the desired genetic modification of the vector. When modification was confirmed, 100 µl of the cells were transferred into 100 ml LB media containing 100 µl ampicillin to amplify plasmid DNA (Maxi preparation). For Maxi preparation, the cell solution was centrifuged at 14,000 g at room temperature for 10 minutes. The pellet was then processed with the PureYield™ Plasmid Maxiprep System (Promega) as stated in the accompanied Promega protocol. After lysing, the solution was poured into the PureYield™ Clearing column which was stacked onto a PureYield™ Maxi Binding Column and a vacuum manifold (Vac-Man®, Promega). After filtration, the DNA on the binding column was washed with 5 ml Endotoxin Removal Wash and 20 ml Column Wash. The membrane of the binding column was dried, DNA was diluted with nuclease-free water and incubated for 5 minutes before the DNA concentration was determined.

3.4 CONSTRUCTION OF TRANSFECTION VECTORS FOR AN *IN VIVO* PATHOGEN GENOME TARGETING STRATEGY

Two vectors for transgene integration were prepared by the collaboration partners, encoding for Cas9 and gRNAs targeting CP204L of ASFV. The gRNAs targeting CP204L of strains isolated from Armenia and Kenya (Table 2) were previously published by Hübner et. al., (2018).

Table 2: Guide RNAs targeting CP204L of the African Swine Fever Virus.

African Swine Fever strain	gRNA name	gRNA sequence 5'-3'
Armenia 2007	ACP204L	GCAAGGGTATACTGAACATC
Kenya1033	KCP204L	GACAGGGATATACTGAACAGC

3.4.1 Sleeping Beauty transposon vector

The Sleeping Beauty (SB) transposon vector system is composed of two plasmids, the transposon containing the gene to be inserted and the vector encoding the transposase. As gene of interest (Figure 6) TurboGFP (green fluorescence protein), eCas9, gRNA, and a neomycin cassette as selection marker had been previously cloned into the vector. The collaboration laboratory (Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut) inserted a specific gRNA designed to target CP204L of the virulent Armenia 2007 ASF strain (Hübner *et al.*, 2018) and removed the NLS (Nuclear Localisation Sequence) of eCas9. ASFV replicates in the cytoplasm (Dixon *et al.*, 2013). By removing the NLS of Cas9, Cas9 could target the viral ASFV genome in the cytoplasm. The transgenes were flanked by inverted terminal repeats (ITRs), the binding site within the ITRs will be recognised by the SB transposase which was delivered by co-transfection. The transposase vector pCMV (CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid # 34879; http://n2t.net/addgene:34879 ; RRID:Addgene_34879) (Mátés *et al.*, 2009). When the transposase locates the ITRs, it excises the sequence within the ITRs and the insert is integrated into genomic DNA in a 'cut-and-paste' manner (Ivics *et al.*, 1997; Ivics and Izsvák, 2015).

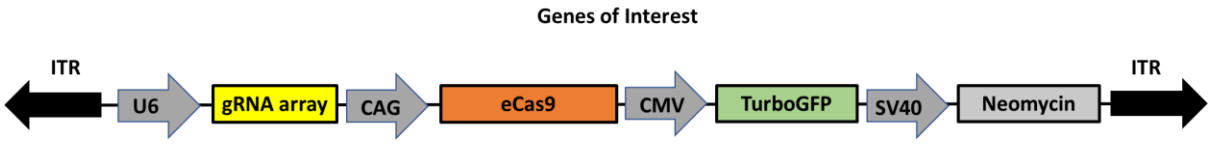

Figure 6: Transgenes within the Sleeping Beauty vector system: The gRNA (guide RNA), eCas9, TurboGFP, and the selection marker neomycin were flanked by inverted terminal repeats (ITRs) to be recognised by the Sleeping Beauty transposase.

3.4.2 pX330 vector

The pX330-U6- Chimeric_BB-CBh-hSpCas9 a gift from Feng Zhang (Addgene plasmid # 42230 ; http://n2t.net/addgene:42230; RRID:Addgene_42230) (Cong *et al.*, 2013) was the second vector which was transfected to generate transgenic pigs. The vector expresses SpCas9 and the gRNA array. In addition, the vector was previously adapted to express neomycin as selection marker and a gRNA adjusted to target CP204L of the Kenya1033 strain (Figure 7). Previously, random integration of the vector led to stable expression of the CP204L gRNA in modified wild boar lung (WSL) cells, rendering resilience to ASFV (Hübner *et al.*, 2018).

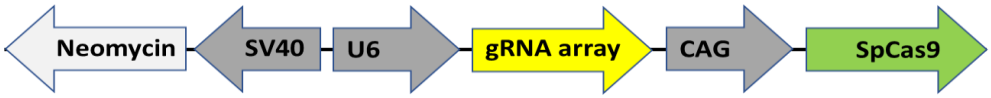

Figure 7: **Transgenes within the pX330 vector**: The gRNA (guide RNA), SpCas9, and the selection marker neomycin.

3.5 WORKFLOW OF GENERATING TRANSGENIC PIGS

Somatic cell nuclear transfer (SCNT) was employed to generate Cas9 expressing founder animals for an *in vivo* pathogen genome targeting strategy. After establishing transgenic fibroblast cell lines with transfection vectors, the cells were used as donor cells and fused into enucleated metaphase II oocytes. The general workflow is depicted in figure 8.

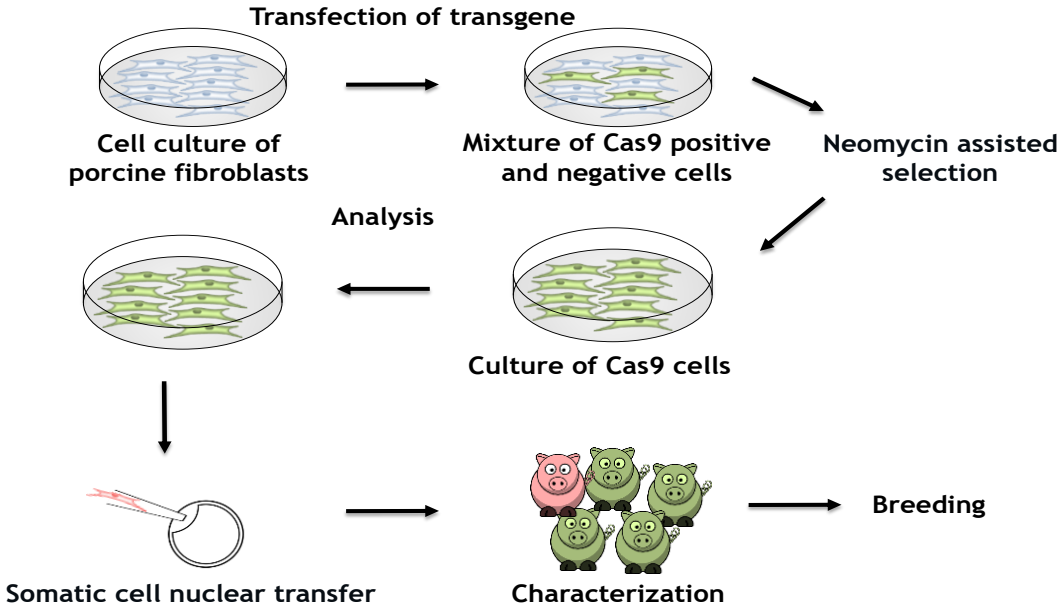

Figure 8: General workflow to generate transgenic pigs: Wild-type fibroblasts were transfected with the transgene carrying vector and vector integrated cells were selected with neomycin. The cells were then analysed and utilised for somatic cell nuclear transfer. After characterisation of the offspring, boars were used to produce progeny.

3.5.1 *In vitro* cell culture

Foetal fibroblasts were cultured, transfected, and used as donor cells for SCNT. Cells were either cultured in DMEM (Dulbecco's Modified Eagle's Medium) based D10, D20, or T3 media, containing 10 %, 20 %, or 30 % foetal calf serum (FCS), respectively. All manufactures and ingredients used for media or solutions can be found in Annex 8.5.

3.5.2 Isolation of foetal fibroblasts

Foetal fibroblasts were cultured after terminating gestation at day 25. The foetuses were retrieved from the uterus and washed in PBS (phosphate-buffered saline) containing 2 % of Pen/Strep (Penicillin/Streptomycin). After removal of excess organs and the cephalic parts, the remaining tissue was added to 500 µl EDTA/trypsin (0.02 % Ethylenediaminetetraacetic acid/0.05 % trypsin) and incubated at 37 °C under constant shaking. After 20 minutes the digested tissue was transferred into a T25 (25 cm^2) culture dish and 5 ml of T3 with 2 % Pen\Strep was added. The tissue was then cultured at 37 °C and 5 % CO_2 in air to ensure proliferation of the fibroblasts.

3.5.3 Cultivation of fibroblasts

Fibroblasts were cultured in humidified air at 37 °C and 5 % CO_2 and media were changed every 2-3 days. FCS concentration was adjusted depending on cell passage. Cells were trypsinized with EDTA/trypsin for splitting, freezing or further processing. The cells were stored in T3 media containing 10 % DMSO (dimethyl sulfoxide) for freezing.

3.5.4 Transfection of fibroblasts

For transfection of expression vectors, fibroblasts were electroporated with the Neon® Transfection System (ThermoScientific). When cells reached 70-90 % confluency on a T75 (75 cm^2) flask, fibroblasts were trypsinized, washed with PBS, and centrifuged at 1000 rpm for 4 minutes. After aspiration of PBS, 200 µl resuspension buffer R (ThermoScientific) were added to resuspend the cell pellet. The cells were transferred into a 1.5 ml tube and the vector(s) was added. For SB transposon integration, the transposon vector (625 ng/µl) and the transposase vector (595 ng/µl) were co-transfected. The concentration of the pX330 vector was 655 ng/µl. A total amount of 5 µg was transfected. The Neon® tube was inserted into the Neon® pipette station and filled up with 3 ml electroporation puffer E2 (ThermoScientifc). Before the suspension was slowly aspirated with a 100 µl Neon® pipette and tip, the solution was carefully mixed. The tip was then inserted into the Neon® tube. Electroporation was set to 2 pulses for 20 ms at 1350 V, afterwards the cells were transferred back into pre-warmed antibiotic free T3 media in T25 flasks or 6-well plates. The remaining 100 µl were also aspirated and electroporated before being added to the media. Approximately 12-24 h later the media was changed to T3 containing Pen/Strep.

Twenty-four hours after transfection the cells were selected (neomycin marker) for 10 days with G418 (800 µg/ml) (Roth).

3.5.5 Genotyping of transfected cells

To characterise the transfected cells, fibroblasts were lysed with cell lysis buffer containing proteinase K (10mg/ml) (Thermo Fisher Scientific) (Annex 8.6 Table 17). First, the cells were washed with PBS and then lysis buffer was added. The cells were either incubated for 2 h at 55 °C or overnight at 37 °C. Proteinase K was inactivated at 96 °C for 12 min. For end point PCR, the lysate was used without further DNA purification. PCR was used to amplify Cas9 integration (Annex 8.8 Table 22). The concentration of the Master Mix for end-point PCR can be found in table 3. Depending on whether the PCR product would be further processed, a 25 µl or 50 µl mix was prepared. PCR amplicons were visualised with gel electrophoresis on 1 % gels at 80 V for 30-60 min.

Table 3: Master Mix components for end-point PCR*.

Component	Supplier	End concentration
Nuclease-free water		28/14 µl
5x Colourless GoTaq Flexi Buffer	Promega	1x
Forward Primer	Eurofins Genomics	0.6 µM
Reverse Primer	Eurofins Genomics	0.6 µM
$MgCl_2$	Promega	1.5 mM
dNTPs	Life technologies	0.2 mM
GoTaq G2 Hot Start Polymerase	Promega	1.2 U
DNA		5/2.5 µl

* The described Master Mix is applicable for all end-point PCR protocols described here.

3.5.6 Somatic cell nuclear transfer

To generate transgenic pigs, SCNT was employed. SCNT provides the opportunity to generate transgenic offspring by replacing the nuclear DNA of an oocyte with a modified nuclear DNA from a somatic cell. Pig ovaries were collected from a local slaughterhouse and oocytes were retrieved by vacuum aspiration with 18 Gauge needles. The oocytes were washed in PXM + 1 % NBCS (Porcine X media and new born calf serum) (Annex 8.4 Table 2) and cumulus-oocyte-complexes (COCs) were selected for maturation. The COCs were matured for 40-44 h at 38 °C in humidified air containing 5 % CO_2 in FLI maturation media (Annex 8.4 Table 3). The FLI maturation media contained FGF2 (Peprotech), LIF (ESGRO Mouse LIF), and IGF1(R&D Systems) and has been described previously (Yuan *et al.*, 2017). In the meantime, the previously modified fibroblasts were incubated for 48 h in DMEM media containing 0.001 % FCS (Annex 8.5 Table 15) to synchronise cells at the G0/G1 cell cycle stage. After maturation of the oocytes the cumulus cells were removed by incubating cells for 2 minutes in TL (Tyrode's lactate)-HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 296 Ca^{2+}

+0.1 % hyaluronidase media (Annex 8.4 Table 4 and 5). After careful up and down pipetting to remove cumulus cells, oocytes with polar bodies were chosen for SCNT. First, the oocytes were enucleated by removing the metaphase-II-plate and polar body with a glass pipette while being kept in TL-HEPES-296 Ca^{2+} media. At the same time, the donor cells were trypsinized with EDTA/trypsin and the pellet resuspended in Calcium-free TL-HEPES 296 media. Next, the enucleated oocytes and the modified donor fibroblasts (chapter 3.5.4) were fused in Calcium free Sor2 media (Annex 8.4 Table 8) by inducing a 16 V pulse for 100 µs. After fusion, the complexes were transferred into Ca^{+} Sor2 media (Annex 8.4 Table 9) for electrical activation at 24 V for 45 µs, followed by a 3 h chemical activation in PZM-3 (Porcine-zygote media) (Annex 8.4 Table 13) and 6-DMAP (6-Di-methylaminopurine) (Annex 8.4 Table 10). Finally, activated complexes were cultured in PZM-3 for 24 h at 39 °C and 5 % CO_2 and O_2 and one-two cell embryos were transferred into hormonally synchronised 7-9 months old German Landrace gilts. The gilts were synchronized with 20mg/day Altrenogest (Regumate®, MSD) for 12 days. On day 13, the gilts received 1500 I.U. PMSG (pregnant mare gonadotropin, Pregmagon®, IDT Biologika), followed by 500 I.U. hCG (human chronic gonadotropin, Ovogest®300, MSD Germany) 78 hours later.

3.6 GENOTYPING OF TRANSGENIC OFFSPRING

3.6.1 Tissue DNA extraction

Tail tips were retrieved from transgenic animals. About 50 mg of the tissue was added to 600 µl tail lysis buffer (Annex 8.6 Table 18) and 43 µl Proteinase K (10mg/ml). The solution was incubated overnight at 50 °C at constant agitation. The following day the lysate was centrifuged for 15 min at 14.000 rpm. 560 µl of the supernatant was transferred into saturated NaCl solution and thoroughly mixed. After another centrifugation step (15 minutes, 14.000 rpm), 700 µl of the supernatant was mixed with 100 % ethanol and centrifuged again. The supernatant was discarded, and DNA pellet was washed 2-3 times with 70 % ethanol. After drying, the pellet was eluted in nuclease-free water.

3.6.2 Isolation of fibroblasts

Fibroblasts from offspring generated by SCNT were prepared for re-cloning, genotyping, and *in vitro* experiments. The same protocol as explained in chapter 3.5.2 to isolate foetal fibroblasts was followed, except fibroblasts were isolated from ear biopsies. The subcutaneous tissue of the biopsy was washed and shredded int 1-2mm pieces in 2 % Pen/Strep PBS, then incubated in EDTA/Trypsin and transferred into T25 flask. Cultivation of fibroblasts has been described in chapter 3.5.3.

3.6.3 Transgene integration

To confirm transgene integration in the piglets the isolated tail DNA was diluted to 20ng/µl and amplified by PCR (Chapter 3.5.5.). PCR was used to confirm Cas9, gRNA, and neomycin integration, primers and temperature protocols can be found in annex 8.8. For Sanger sequencing PCR products were cleaned up with Invisorb® Fragment CleanUp from Stratec diluted to 20 ng/µl and 5 µM of the corresponding primer was added.

3.6.4 Reverse-Transcription quantitative PCR

A SYBER™ Green reverse-transcription qPCR (RT-qPCR) was established to detect Cas9 RNA expression. Fibroblasts RNA was isolated with a Direct-zol Miniprep Kit (Zymo Research). Two boars were sacrificed at the age of 12 months (transposon integrations) and 10 months (random integration), respectively. RNA was isolated from muscle, tonsil, spleen, kidney, lymph nodes, oral mucosa, and liver with TRIzol™ Reagent (Invitrogen) from homogenized organs (100mg) according to manufactures protocol. For analysing fibroblast expression, two biological and two technical replicates were prepared, for organ expression two technical replicates. Prior to cDNA synthesis, 1 µg purified RNA was digested with 2 U DNAse for 30 min at 37 °C. The cDNA synthesis was performed with GoScript™ Reverse Transcriptase (Promega) as described in the protocol provided by Promega. For qPCR 20 ng of cDNA was added to the SYBER™ Green master mix (Power SYBER™ Green PCR Master Mix, Applied Biosystems™) and amplified by qPCR Cas9 primers (Annex 8.8 Table 22). The Relative Standard Curve Method was used to calculate differential mRNA expression. Cas9 expression was normalised to the reference gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). A cDNA dilution from pooled muscle RNA was included on every plate to give standard curves for the calculation of relative expression values for Cas9 and GAPDH.

3.7 GERMLINE TRANSMISSION

While the SCNT technology was employed to generate founder animals, reproductive technologies such as *in vitro* fertilisation (IVF) and artificial insemination (AI) are required to establish a desired pig line. Hence, two founder boars were kept to investigate if they could produce offspring and to see whether the transgenes were passed down to the next generation. Transgenic boar semen was collected and frozen according to standard practice. The quality of the sperm was assessed by computer-assisted sperm analysis (CASA).

3.7.1 *In vitro* fertilisation

For IVF with transgenic sperm, oocytes were collected, matured, and selected as described above in chapter 3.5.6. The frozen semen was thawed at 37 °C for 30 sec and motility was visually evaluated.

After thawing, the semen was washed twice with 3 ml Androhep®Plus + Gentamicin (Minitube) and centrifuged at 2200 rpm for 3 minutes. Motility was again assessed after centrifugation. Different sperm cell/oocyte ratios and two different batches of frozen semen were tested. Sperm cell/oocyte ratio ranged from 100 sperm cells to 7500 cells per oocyte. The sperm cells and oocytes were co-incubated in Fert-Talp media (Annex 8.4 Table 12) for 4 hours. After fertilisation the zygotes were cultured in PZM-3 for 6 days and blastocyst rates were determined. Blastocysts were lysed individually with 15 µl cell lysis buffer (Annex 8.6 Table 17) for 2 h at 55 °C as well as inactivated at 96 °C for 12 minutes. Cas9 and gRNA integration were confirmed by PCR.

3.7.2 Artificial insemination

Semen was collected from boar 762-7 with random integration and diluted 1:1 with Androhep®Plus. Superovulated sows were inseminated twice within 24 hours. The sows were synchronized as described before (Chapter 3.5.6.). One pregnancy was established and terminated at day 25. Foetal fibroblasts were isolated as described above (Chapter 3.5.2). DNA was extracted from the cephalic parts of foetuses and genotyped (Chapter 3.6.3.). Similarly, four sows were inseminated with transgenic semen from boar 765-6 and three established pregnancies were carried to term.

3.8 FUNCTIONALITY OF THE CAS9 TRANSGENE

Part of the *in vivo* pathogen genome targeting strategy is that the integrated Cas9 transgene remains functional to induce DSBs in DNA. To test whether the transcribed Cas9 maintained its cleaving potential, previously validated gRNAs were transfected into isolated Cas9 expressing fibroblasts. The transfected gRNAs must form a complex with the *in vivo* translated Cas9 to induce indels or deletions (Figure 9) and thereby validates the Cas9 activity.

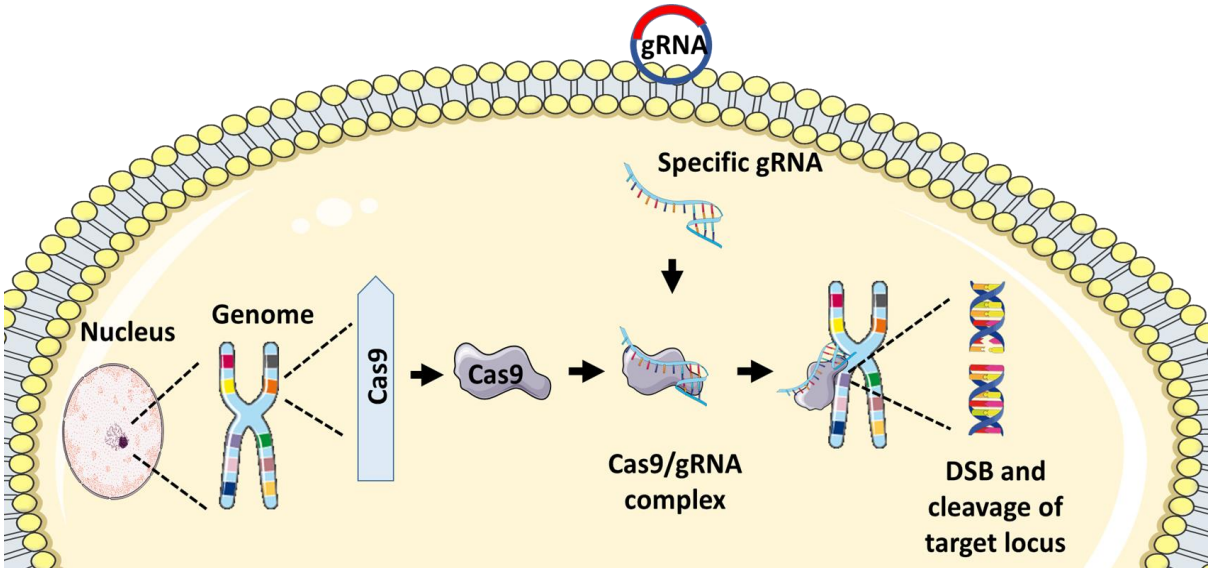

Figure 9: Strategy to validate Cas9 activity: Fibroblasts were isolated from Cas9 expressing pigs and transfected with plasmids encoding gRNAs or synthetic gRNAs. Indel formations and deletions at desired loci confirmed the functionality of the transgenic Cas9.

3.8.1 Guide RNA encoding vector BPK1520

Several gRNAs were cloned into the BPK1520 vector, a gift from Keith Joung (Addgene plasmid # 65777 ; http://n2t.net/addgene:65777 ; RRID:Addgene_65777) (Kleinstiver *et al.*, 2015). The gRNAs were designed to target the following porcine loci: beta-2-microglobulin (B2M), Beta-1,4 N-acetylgalactosaminyltransferase 2 (B4GALNT2), and alpha-1,3-galactosyltransferase (GGTA1) and were well characterised from previous studies on xenotransplantation (Hein *et al.*, 2020; Sake, 2021). The loci encode three different epitopes that are critical for acceptance of a porcine xenografts after transplantation into human patients (Byrne *et al.*, 2018). The encoded gRNAs can be found in table 4. Two gRNAs each for B2M and B4GALNT2 were transfected to induce deletions within the target loci. GGTA1 was targeted with a single gRNA to generated indels (insertions and deletions). BPK1520 was digested with BsmBI (NEB #R0739) according to the provided protocol (Joung Lab gRNA Cloning Protocol). After linearisation control by electrophoresis, the digested plasmid was purified with Invisorb® Fragment CleanUp from Stratec. Before ligation, crRNA oligos with corresponding overhangs (5’ CACC 3’ upper strand and 5’ AAAC 3’ lower strand) were annealed with 2 µl 10x T4 DNA Ligase Buffer (NEB) as stated in the Joung Lab gRNA Cloning Protocol. For ligation, 0.5 µM of oligo duplex was added to 20 ng digested vector and ligated with 0.5 µl T4 DNA ligase (NEB) and 1 µl 10x T4 DNA ligase buffer in a total volume of 10 µl and incubated for 2-12 hours at room temperature or 4 °C. Fibroblasts isolated from pig 731-1, 732-3, 733-1, 759-5, and 762-7 were transfected. In addition, foetal fibroblasts sired by 762-7 were also transfected with BPK1520 encoding gRNAs for GGTA1 and B2M.

Table 4: Guide RNA sequences to target B2M, GGTA1, and B4GALNT2NT.

Gene	Sequence 5'→3'
B2M	GAGTAAACCTGAACCTTCGG
	TGAGTTCACTCCTAACGCTG
B4GALNT2	ATTGTCTGGGACGTCAGCAA
	AGAGTACCACCTCCACAGAG
GGTA1	CTGACGAGTTCACCTACGAG

3.8.2 Synthetic gRNAs

To further validate Cas9 activity, synthetic Alt-R® CRISPR-Cas9 crRNAs targeting B2M, B4GALNT2, and GGTA1 (Table 4) and Alt-R® CRISPR-Cas9 tracrRNA were purchased from Integrated DNA Technologies (IDT). The RNAs were resuspended to 100 µM and assembled at equimolar concentrations at 95 °C for 5 minutes and cooled at 5 °C/min to from crRNA:tracrRNA complexes. In total 300 pmol of ice chilled gRNA complexes were added for the transfection.

3.8.3 Validation of genome edits

The transfected cells isolated from pig 731-1, 732-3, 733-1, 759-5, and 762-7 were lysed (Chapter 3.5.5) and the gRNA target regions of GGTA1, B2M, and B4GALNT2 were amplified. PCR protocols and primers for target regions can be found in (Annex 8.8 Table 23). After generation of Cas9 expressing foetal fibroblasts, cells isolated from foetuses 102-12 and 102-14 were also transfected and edits validated by PCR. To validate edits in foetal fibroblasts further by Sanger sequencing, foetal GGTA1 knock-out cells were counter-selected with magnetic beads (Dynabeads™, Invitrogen) based on α-galactose expression (Fujimura *et al.*, 2008; Hauschild *et al.*, 2011) (Annex 8.10). Transfected B2M foetal fibroblasts were diluted on a 96-plate, harbouring five cells per well. Confluent wells were amplified, and deletion positive wells sent for Sanger sequencing.

3.8.4 Next generation sequencing

To determine the genome editing efficiency of the Cas9 expressing fibroblasts, NGS was performed. The target regions B2M, B4GALNT2, and GGTA1 were amplified in cell lysates of the gRNA transfected cells from pigs 731-1, 732-3, 733-1, 759-5, and 762-7. PCR products were purified, and DNA concentrations were determined by the Invitrogen Qubit 4 Fluorometer. The amplicons were pooled by fragment size to a total concentration for 5 nM and sent for Illumina MiSeq sequencing. Genome editing efficiencies were calculated with Geneiouse Prime Version 2021.0.1. First, the reads were paired and merged before being mapped to reference gene. Second, pairwise identity was

calculated by the software. Foetal Cas9 expressing fibroblasts were retrieved after boar 762-7 reached sexual maturity. At that time point NGS of the cells isolated from founder animals was already performed.

3.8.5 Flow cytometry GGTA1

To assess Cas9 efficiencies at the phenotypic level, gRNA transfected Cas9 expressing cells were stained for flow cytometry. Cas9 expressing cells which were transfected with a GGTA1 gRNA were stained with isolectin to detect expression of α-galactose. Edited cells would not express α-galactose which is synthesised by GGTA1 (Sharma *et al.*, 2003). A negative control was obtained from a GGTA1 knock-out fibroblast cell line (Hein *et al.*, 2020) and the corresponding untreated Cas9 expressing cell lines served as positive control. Fibroblasts ($1x10^6$) were trypsinized and washed in PBS. The cells were equally divided to provide $0.5x10^6$ cells for staining and $0.5x10^6$ for an unstained control. The samples to be stained were incubated with 3 µl GSL I-B_4 isolectin conjugated with DyLight 649 (Vector laboratories) for 5 minutes at 37 °C. After incubation and a centrifugation step, the supernatant was discarded, and the cell pellet was resuspended in 500 µl FACS-Buffer (Miltenyi Biotec). Expression of α-galactose was measured in a MACSQUANT® YB Flow Cytometer with the corresponding software.

3.8.6 Flow cytometry B2M

Guide RNA transfection targeting B2M was assessed by measuring the expression of MHC-I (major histocompatibility complex-I). As for GGTA1 flow cytometry a B2M knock-out cells line (Hein *et al.*, 2020) served as negative control and corresponding untreated Cas9 expression cells as positive control. The monoclonal antibody anti-swine MHC I (AB1) (Kingfisher-Biotech Inc #WS0550S-100) was diluted in PBS + 5 % FCS 1:50, the secondary PE-Vio labelled IgG2ab anti-mouse antibody (AB2) (Miltenyi Biotec #130-123-498) 1:11, and the mouse Ig2b kappa isotype 1:25 (Invitrogen #14-4732-85). Each cell line was analysed with three controls: no staining, isotype and AB2 staining, and AB2 staining. First, $0.5x10^6$ cells were stained with 20 µl of AB1/isotype for 20 minutes on ice and washed twice. Second, the samples were incubated with AB2 for another 20 minutes on ice and washed twice again. Finally, the supernatant was discarded, cells resuspended in 500 µl FACS-Buffer, and MHC-I expression was measured by MACSQUANT® YB Flow Cytometer.

3.8.7 Anti-CRISPR protein

To confirm that genome edits were really induced by the Cas9 transgene, an anti-CRISPR (acr) encoding vector was transfected in combination with BPK1520 encoding either for a GGTA1 gRNA or B2M gRNAs. Anti-CRISPR proteins are able to inhibit Cas9 activity (Harrington *et al.*, 2017; Pawluk, Davidson and Maxwell, 2018). The co-transfection of gRNA and the anti-CRISPR AcrIIA4 was

expected to result in lower genome editing efficiencies, in comparison to transfections with only gRNAs. AcrIIA4 inhibits the binding of the Cas9:gRNA complex to target DNA (Bubeck *et al.*, 2018). The vector CMV-NLS-AcrIIA4 translating the Ca9 inhibitor AcrIIA4 was a gift from Dominik Niopek (Addgene plasmid # 113037; http://n2t.net/addgene:113037 ; RRID:Addgene_113037) (Bubeck *et al.*, 2018). CMV-NLS-AcrIIA4 was co-transfected in foetal Cas9 expressing fibroblasts at a concentration of 2.5 µg. The transfected cells were evaluated by flow cytometry for B2M and GGTA1 (described above) to assess genome editing inhibition.

3.8.8 Off-target analysis

Potential off-target sites of transfected gRNAs were determined by the online tool CRISPOR (*http://crispor.tefor.net/*) (Concordet and Haeussler, 2018). Off-targets are associated with alterations in genomic regions similar to the target sequence of the gRNA. Since the integrated Cas9 of the isolated fibroblasts is most likely permanently expressed, Cas9:gRNA formation may have been prolonged causing unwanted off-target effects. Three of the most likely assessed off-target sites for each guide were selected and corresponding primers designed. PCR amplicons were purified and sent for Sanger sequencing. The sequences were aligned to the WT locus to investigate potential off-targets.

3.9 INFECTION STUDIES WITH AFRICAN SWINE FEVER VIRUS

Transgenic (TG) and wild type (WT) control pigs were infected with ASFV Armenia to investigate whether the *in vivo* pathogen genome targeting strategy would facilitate resistance or resilience against ASF. *In vivo* and *in vitro* studies were designed and conducted in close collaboration with the Institute of Virus Diagnostic and Institute of Molecular Virology and Cell Biology – Friedrich-Loeffler-Institut.

3.9.1 *In vitro* infection of peripheral blood mononuclear cells

EDTA blood samples were taken from a 4-months-old transgenic and control boar. To isolate PBMCs (peripheral blood mononuclear cells) the blood was diluted 1:2 with PBS and slowly added on top of 12 ml Pancoll (1,077 density) (PAN™ Biotech) for a total volume of 45 ml. The samples were then centrifuged for 40 minutes at 720g at room temperature. During centrifugation PBMCs accumulate on top of the Pancoll and were collected with a serological pipette. PBMCs were washed with PBS twice and centrifuged at 310g for 12 minutes. Erythrocytes were retrieved from the bottom of the falcon. $7x10^7$ PBMCs were seeded per 96-well plate. The cells were incubated over night at 37 °C and 5 % CO_2 in IMDM (Iscove's Modified Dulbecco's Medium, Gibco™) supplemented with 5.32 g/l Ham's F-12 (Gibco™), 2.45 g/l $NaHCO_3$ (Roth), 10 % FCS, 1 % Pen/Strep, and Mercaptoethanol 160 µl/l. The

following day medium was replaced with new medium containing 2 ng/ml GM-CSF (Granulocyte-macrophage colony-stimulating factor) and incubated overnight. A haemadsorption assay was performed to determine viral titers associated with an infection in TG animals. In this assay infected cells bind red blood cells on their surface and form rosette-like clots which can then visually observed to determine viral titers. The previously collected erythrocytes were diluted in PBS (1 % erythrocytes). PBMCs were infected with ASFV Armenia ($2x10^6$/ml) in a serial dilution (Figure 10) and the erythrocyte solution was added. Twenty-four to 48 h post infection the 96-well plates were inspected under a microscope for rosettes and 50 % endpoint was determined. Also, viral replication in TG and WT PBMCs was assessed by infecting five 6-well plated seeded with $2x10^7$ PBMCs with a MOI (multiplicity of infection) of 0.1. Viral titers determining 50 % haemadsorbing dose (HAD_{50}) were analysed at 0h, 12h, 24h, and 48h post infection, respectively.

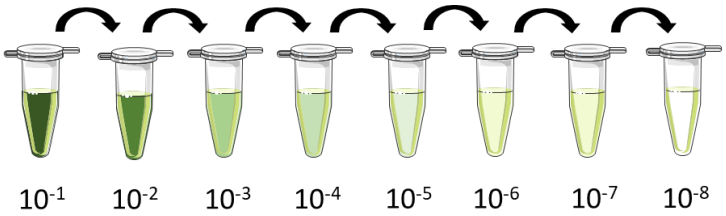

Figure 10: Serial dilution of African swine fever virus: Serial dilution of African swine fever virus for hemadsorption assay.

3.9.2 *In vivo* infection

The *in vivo* infection study was conducted in a biosecurity level 4 animal facility. WT-control and TG animals were transported from the biosecurity level 1 facility 3-7 days before the infection study was initiated. The TG pigs were immunocastrated (Improvac®, Zoetis) at 10 months and were 12 months at the time of infection. The control animals were 6 months at the time of infection and had been previously surgically castrated. It was not expected that age or castration method would influence disease development. The TG boars were kept individually to avoid any aggressive behaviour. The control pigs were kept as a group (Figure 11). Seven WT-control and seven TG animals were oronasally infected with approximately 2 ml 10^5 $TCID_{50}$/ml (50 % Tissue Culture Infectious Dose) ASFV Armenia. Blood samples, nasal, and rectal swaps were taken on days 0, 4, and 7 dpi, respectively, temperature was measured once a day, and the animals were checked twice daily and monitored by cameras. Two TG pigs had to be reinfected at 13 dpi with a larger volume of 10 ml 10^5 $TCID_{50}$/ml.

Figure 11: Logistic set-up for African Swine Fever infection study: The transgenic pigs were housed individually in three different stables. The control pigs were housed together (upper left corner).

DNA was extracted from blood samples at 4 and 7 dpi with the NucleoMag® Vet kit for viral DNA isolation (Macherey-Nagel). The samples were prepared for automatic DNA purification with the Thermo Scientific™ KingFisher™ Flex Purification System, KingFisher with 96 Deep-well Head. For cell lysis, 100 µl blood was added to 20 µl proteinase K and 100 µl lysis buffer VL, mixed, and centrifuged. For binding 350 µl binding buffer VEB and 20 µl NucleoMag® B-Beads were added, followed by three washes with wash buffer VEW 1, VEW 2, and 80 % ethanol. Finally, the samples were eluted with elution buffer VEL. Multiplex qPCR of ASFV p72 and beta-actin was performed with a QuantiTect Multiplex PCR Kit (Qiagen). Protein 72 is the major capsid protein of ASFV and is involved in virus entry (Dixon *et al.*, 2013). Probes and primers were based on Tignon et. al., (2011) and Toussiant et al., (2007) (Toussaint *et al.*, 2007; Tignon *et al.*, 2011) (Annex 8.8 Table 28) and the qPCR protocol was optimized by the German ASF reference laboratory of the Institute for Virus Diagnostics (Table 5-7).

Table 5: Composition of primer/probe composition for the detection of p72 of African swine fever virus.

ASF Primer/Probe	End concentration
ASF-P72-F	10 pmol/µl
ASF-P72-R	10 pmol/µl
ASF-Probe-Tignon-FAM	1.25 pmol/µl

Table 6: Composition of primer/probe composition for the detection of Beta-Actin.

ACTB Primer/Probe	End concentration
ACT2-F	2.5 pmol/µl
ACT2-R	2.5 pmol/µl
ACT-Probe-HEX	2.1 pmol/µl

Table 7: qPCR Master Mix composition for the detection of African Swine Fever Virus.

Master Mix component	End concentration
RNAase free Water	3.5 µl
2x QuantiTect Multiplex	12.5 µl
ASF-Mix	2 µl
ACT-Mix	2 µl
DNA	5 µl

Clinical scores were performed once a day. Several indicators (Table 8) were assessed and scored into one of four categories (0, 1, 2, and 3), indicating no symptoms (0) to severe clinical signs (3). At the end of the scoring, the points of the different parameters were summed up. When the total score reached 10, and/or the animal displayed fever above 40.5 °C on three executive days in addition to clinical signs, and/or isolated itself in combination with delayed reactions, and/or vomited for three executive days, and/or increased respiratory frequency, and/or display severe lameness for more than 48 hours, the pig was euthanised.

Table 8: Clinical scores to assess animal health.

Parameters	0	1	2	3
Liveliness				
Posture				
Respiratory frequency				
Motion				
Body temperature				
Skin				
Eyes				
Faeces				
Appetite				
Energy level				

3.10 Targeted Cas9 integration into the Rosa26 locus

SB transposition of Cas9 and the CP204L gRNA did not result in ASF resistant or resilient pigs (see results section), therefore a targeted knock-in strategy to integrate Cas9 and multiple gRNAs was designed. A targeted knock-in has the advantage that the integration site of the transgene is known. In addition, a 'safe harbour' locus (Rosa26) in the porcine genome has been identified. Integrating transgenes into a safe harbour locus results in stable transgene expression without unwanted site effects (Kong *et al.*, 2014; S. Li *et al.*, 2014; X. Li *et al.*, 2014). Recently, Cas9 integration in the Rosa26 locus has already been successful and resulted in stable expression of Cas9 (Wang *et al.*, 2017; Rieblinger *et al.*, 2021). Integrating multiple gRNAs targeting the viral genome of MDV have shown to induce resistance to MD in chickens (Challagulla *et al.*, 2021). A targeted knock-in strategy of Cas9 and multiple gRNA transgenes may lead to resistance against ASF in modified pigs.

3.10.1 Template design

A plasmid pUC57-Brick-Rosa26-HA-Cas9-HiFi-Neo-dTK (pUC-Rosa26) was designed to generate fibroblasts with a targeted Cas9 integration into the Rosa26 locus (Figure 12). The vector was purchased and synthesised by Genscript. The template contained homologous arms to the DSB and a

neomycin and ΔThymidine kinase sequence for selection. The template was designed to facilitate diverse *in vivo* pathogen genome targeting. Therefore, LoxP and Lox2272 binding sites were added, flanking a neomycin and ΔThymidine kinase cassette for later excision and insertion of gRNAs targeting ASFV genes. Based on Recombinase Mediated Cassette Exchange (RMCE) with the Cre-Lox system, gRNA arrays can be introduced into the flanking binding sites by exchanging the neomycin and ΔThymidine kinase cassette with the gRNA array (Figure 12). Cells with integrated gRNA array can then be selected in the absence of ΔThymidine kinase. The vector was amplified in NEB® Stable Competent *E. coli* (High Efficiency) cells in accordance with the provided protocol (C3040H).

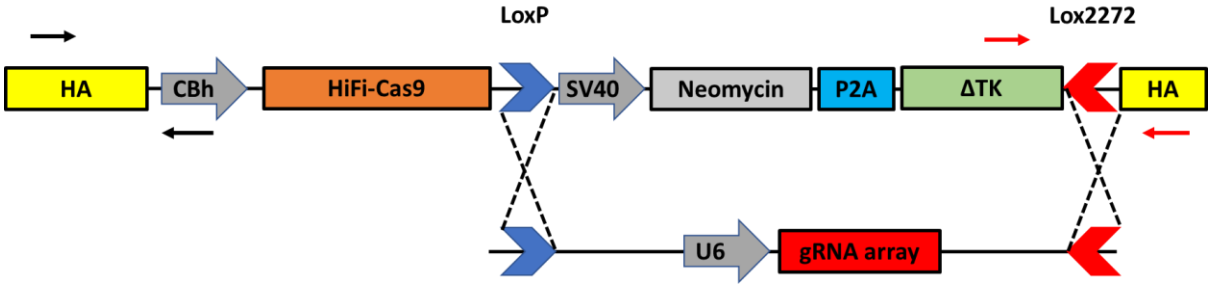

Figure 12: Template design for targeted Cas9 integration in to the Rosa26 locus: The transgenes were flanked by homologous arms (HA), within the flanked region a HIFI-Cas9 cassette driven by a CBh promotor, LoxP (Lox2272), and the selection markers neomycin and ΔThymidine kinase (ΔTK) linked by a 2A peptide (porcine teschovirus-1 2A) sequence. The Lox flanked markers facilitate Recombinase-mediated cassette exchange (RMCE) based on a Cre-Lox system. Cre recognises the Lox sites, excises the selection markers, and integrates a provided gRNA array also flanked with Lox sites. Primers for left integration site (black arrows) and primers for right integration site (red arrows).

3.10.1.1 Rosa26 gRNAs

Three gRNAs targeting Rosa26 were cloned into the BsbI restriction site of a pX330 vector. Guide RNAs 1 and 3 were designed with CRISPOR (*http://crispor.tefor.net/*) (Concordet and Haeussler, 2018) based on Sscrofa 11.1 (NCBI Sus scrofa isolate TJ Tabasco breed Duroc chromosome 13) and gRNA 2 was previously published by Xie et. al., (2017) (Xie *et al.*, 2017) see table 9.

Table 9: Different gRNA sequences to target the porcine Rosa26 locus

gRNA	Sequence 5'→3'
gRNA 1	GAGGCGATGACGAGATCGCG
gRNA 2*	GTGAGAGTTATCTGACCGTA
gRNA 3	AATCCCGCCCATAATCGAGA

* gRNA published by Xie et.al., (2017)

One µg of the vector was digested with 1 µl BsbI-HF® (NEB R3539) and 2 µl rCutSmart Buffer™ (NEB) in a volume of 20 µl for approximately 2 hours. Afterwards digestion was confirmed by

electrophoresis control and the plasmid purified. Ten µl of 100 mM gRNA oligos with BsbI overhangs were annealed with 80 µl annealing buffer (Annex 8.7 Table 21) at 1. 37 °C for 30 min, 2. 95 °C for 5 min, and cooled to 25 °C at 5°C/min. Before ligation the oligos were diluted 1:200. The vector and oligos were ligated for 2 hours at room temperature or overnight at 4 °C using components listed in table 10.

Table 10: Ligation components for guide RNA insertion into the pX330 vector.

Amount	Component
x µl	50 ng of linearised pX330
1 µl	Oligo duplex
2 µl	10x T4 ligation buffer (NEB)
1 µl	T4 ligase (NEB)
x µl	Nuclease-free water
Total 20 µl	

3.10.2 T7 endonuclease-I assay

A T7 endonuclease-I assay (T7 assay) (NEB M0302) was performed to visualise mismatches in the DNA to confirm Indel formation after NHEJ (Figure 13). The target site was amplified by PCR before the T7 assay (Annex 8.8 Table 24). For the T7 assay, 12 µl of WT and genome edited un-purified amplicons were hybridized after initial denaturation at 95 °C for 10 minutes, followed by two cooling steps to 85 °C at 2 °C/sec and to 25 °C at 0.1 °C/sec. Second, the 1 µl of T7 endonuclease-I cleaves the annealed products at mismatch site during an incubation of 15 min at 37 °C. Before running the product for 60 min on a 1.5 % electrophoresis gel, digestion was stopped with 1.5 µl 0.25 M EDTA.

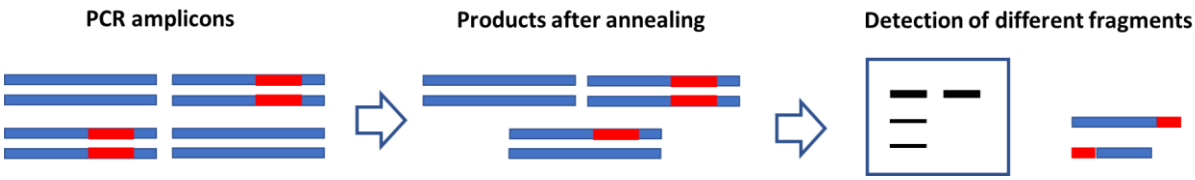

Figure 13: Schematic overview of the T7 endonuclease-I assay: Through denaturation of the PCR amplicon homoduplexes or heteroduplexes with mismatches from. These mismatches are detected and cleaved by the T7 endonuclease which will result in shorter DNA fragments.

3.10.3 ICE Analysis

When indel editing efficiencies had to be determined after Sanger sequencing, a reference sequence and an edited sequence were submitted to the online tool ICE ,provided by Synthego (https://ice.synthego.com/, Synthego Performance Analysis, ICE Analysis. 2019. v3.0. Synthego). The

tool calculates, based on the provided sequences and gRNAs the indel formation and to what percentage these indels lead to gene knock-outs (KO-Score).

3.10.4 Transfection and validation of target Cas9 integration

Foetal wild-type fibroblasts were co-transfected with 15 µl (243 ng/µl) pUC-Rosa26 template and 5 µl (678 ng/µl) Rosa26 gRNA 2 vectors as described in chapter 3.5.3. After G418 selection (Chapter 3.5.4) for 10 days, the cells were lysed to confirm template integration. Template integration was validated by amplification of the Cas9 and neomycin sequence, followed by amplification of the left and right integration site at the Rosa26 locus see figure 12 (Annex 8.8 Table 24).

4 RESULTS

4.1 QUANTITATIVE RESULTS OF GENERATING TRANSGENIC PIGS

Transgenic pigs for an *in vivo* pathogen genome targeting strategy were generated through somatic cell nuclear transfer (SCNT). The pigs expressed Cas9 and a gRNA targeting p30 of the ASFV. Two genetic approaches were applied to modify somatic cells: one by Sleeping Beauty (SB) transposon-based integration and the other one by random integration (RI).

4.1.1 Somatic cell nuclear transfer with Sleeping Beauty transposon integration

SB transposon-based transgenic pigs were generated by SCNT (from here on out SB pigs) (Figure 14). The modified donor cells expressed eCas9 and a gRNA targeting p30 of Armenia ASFV. Six embryo transfers resulted in four successful pregnancies. A total of 59-85 one-two cell stages embryos were transferred per recipient (Table 11). In total 22 piglets were born. Nine piglets were euthanised within the first week due to low birth weight and leg deformities most likely related to the SCNT process. Fibroblasts isolated from one of the SB transgenic piglets were reused for SCNT and the constructed embryos were transferred into two recipient sows. One sow established pregnancy and delivered eleven piglets with a cloning efficiency of 13.6 %. Within the first week, four piglets were euthanised (leg deformities and low birth weight) and two were stillborn. One piglet only displayed one testicle.

Figure 14: Transgenic piglets: Healthy piglets with transposon eCas9 integration generated by somatic cell nuclear transfer.

Table 11: Quantitative results of somatic cell nuclear transfer for Sleeping Beauty transposon integration.

Number of transfers	6
Number of pregnancies	4
Number of zygotes/sows	59-85
Number of piglets (Cloning efficiency %)	22 (4.7-10 %)
Number of piglets born alive	20
Number of piglets euthanised within first week	9

4.1.2 Somatic cell nuclear transfer with random integration

Embryos cloned from fibroblasts with random pX330 integration of SpCas9 and a gRNA targeting p30 of an ASFV Kenya strain (from here on out RI pigs) were transferred into two sows. The two sows gave birth to 15 piglets (Table 12). Also, for the RI pigs, fibroblasts were isolated and used for another round of SCNT. Two sows carried to term and delivered six piglets of which three were stillborn. The other three piglets remained healthy.

Table 12: Quantitative results of somatic cell nuclear transfer for random integration.

Number of transfers	2
Number of pregnancies	2
Number of zygotes/sows	80
Number of piglets (Cloning efficiency %)	15 (8.8-10 %)
Number of piglets born alive	14
Number of piglets euthanised within first week	2

4.2 GENOTYPING OF TRANSGENIC PIGLETS

4.2.1 Transposon-based integration

Of the 22 SB piglets that were generated, 15 carried the Cas9 transgene (Figure 15A). Sanger sequencing confirmed integration of Cas9 and the Armenia ASFV targeting gRNA sequence (Figure 16A and B). Also, integration in re-cloned piglets was confirmed by PCR (Figure 15 B) and Sanger sequencing (Figure 16B) to carry the gRNA. In addition, GFP expression was visually detectable (Figure 17).

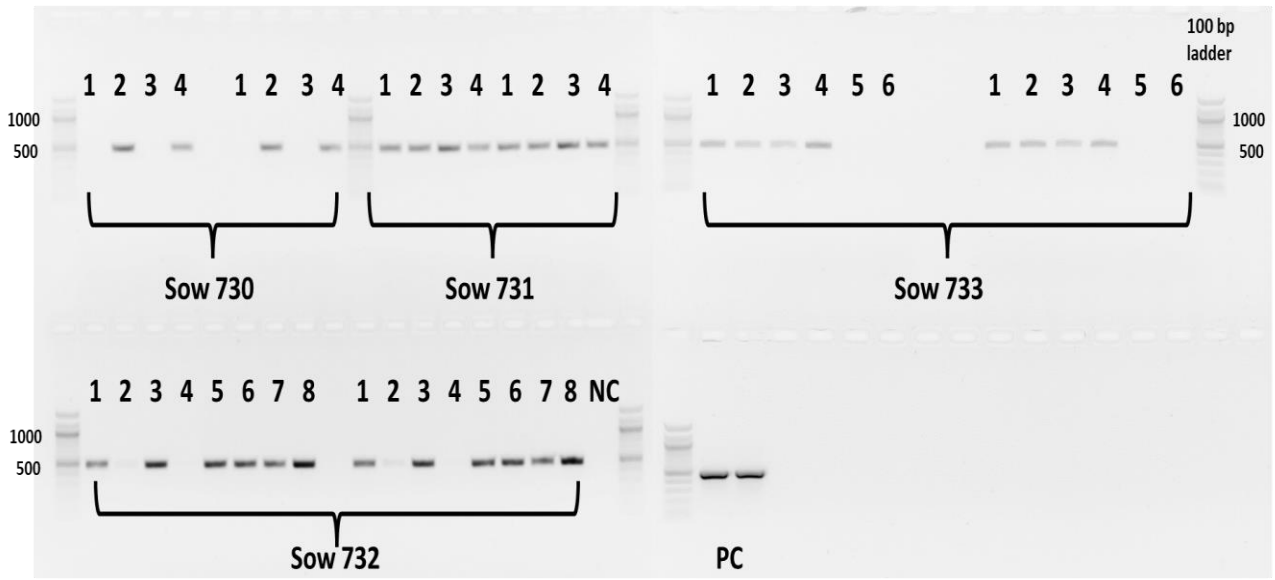

Figure 15: Gel electrophoresis of Cas9 PCR amplicon (500 bp): A: Tail DNA extracted from founder piglets and amplified for Cas9. **B**: Cas9 amplified from tail DNA extracted from re-cloned piglets (right).

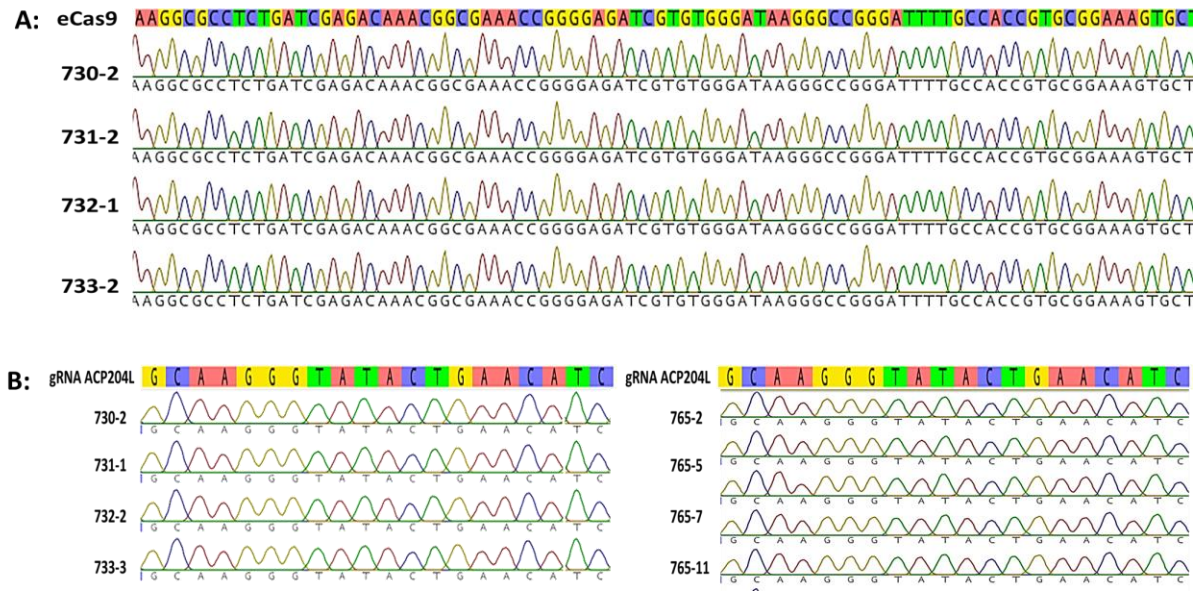

Figure 16: Sanger sequence of eCas9 and Armenia gRNA: A: Amplified Cas9 product of four founder piglets sequenced and aligned to eCas9 reference. B: Sanger sequence of founder animal and re-cloned piglets aligned to the Armenian CP204L guide RNA (gRNA) sequence.

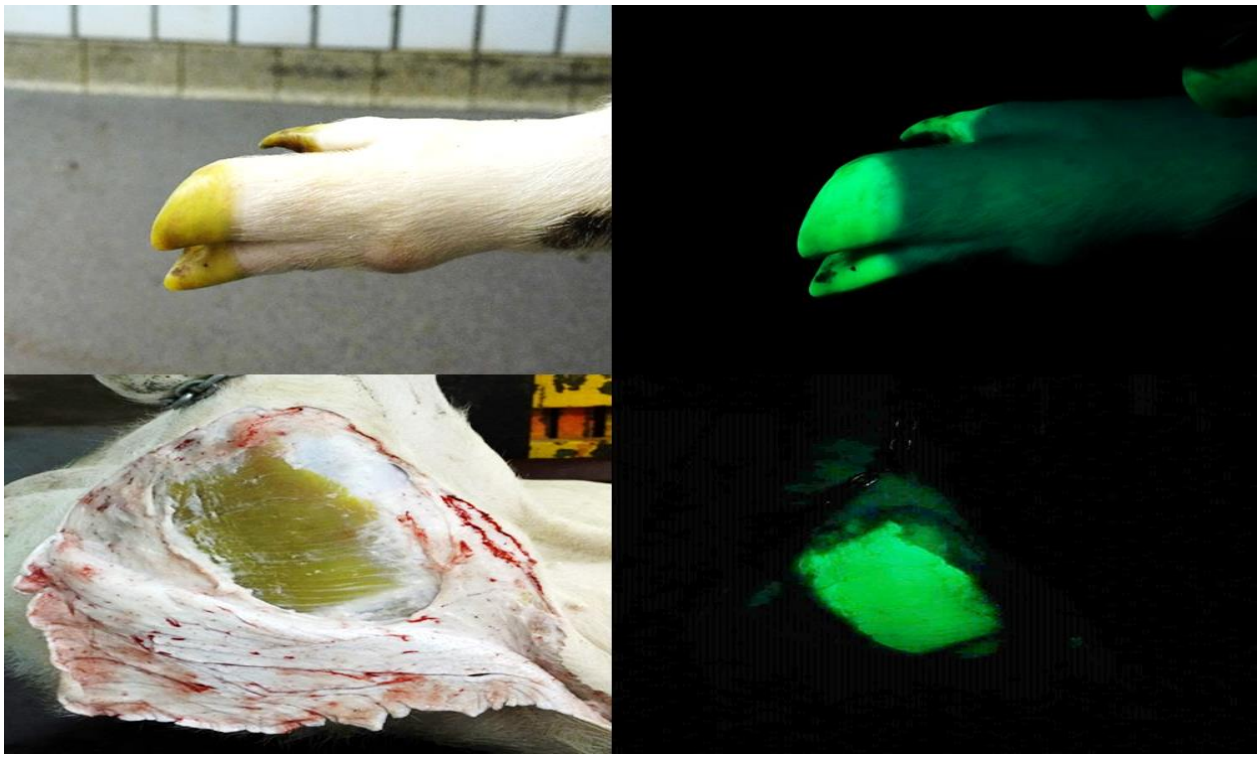

Figure 17: Green fluorescent protein integration (GFP): Sacrificed boar with integrated GFP transgene in muscle and horn tissue.

4.2.2 Random integration

Two out of 15 piglets showed the SpCas9 and Kenya ASFV gRNA transgene integration. Integration was confirmed by Sanger sequencing shown in figure 18A and B. Re-cloning of 759-5 was successful and the offspring was positive for gRNA integration (Figure 18B).

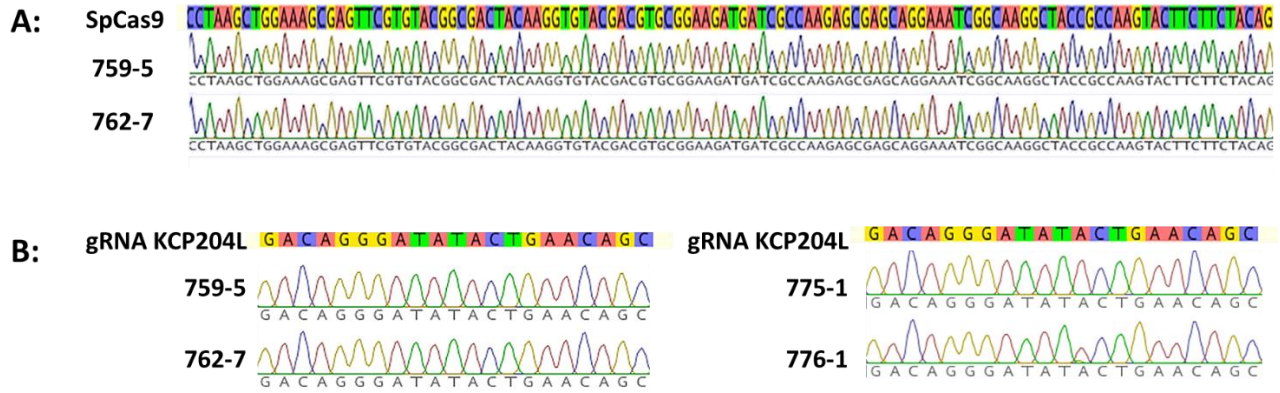

Figure 18: Sanger sequence of SpCas9 and Kenya gRNA: A: Amplified Cas9 product of the two founder piglets sequenced and aligned to SpCas9. **B**: Sanger sequence of founder and re-cloned piglets aligned to the Kenyan CP204L guide RNA (gRNA) sequence.

4.3 Reverse-Transcription quantitative PCR

4.3.1 RT-qPCR of fibroblasts

RT-qPCR was performed with extracted RNA from isolated fibroblasts to confirm transcription of the integrated Cas9 gene. Cas9 expression in foetal fibroblasts of foetus 102-5 (sired by 762-7) was set to 1 as baseline to calculate fold changes in the other cell lines (next chapter results from germline transmission). Overall, fold changes were higher in isolated transposon-integrated cell lines, 26.88 (731-1), 13.56 (732-3), and 13.87 (733-1). Isolated cells with random integration (pigs 759-5, 762-7, and foetuses 102-5,102-12,102-14) the fold changes were lower (0.53,0.67,1.05,0.87) but expression levels did not decrease between foetal offspring (102-5, 102-12, 102-14) and sire 762-7 (Figure 19).

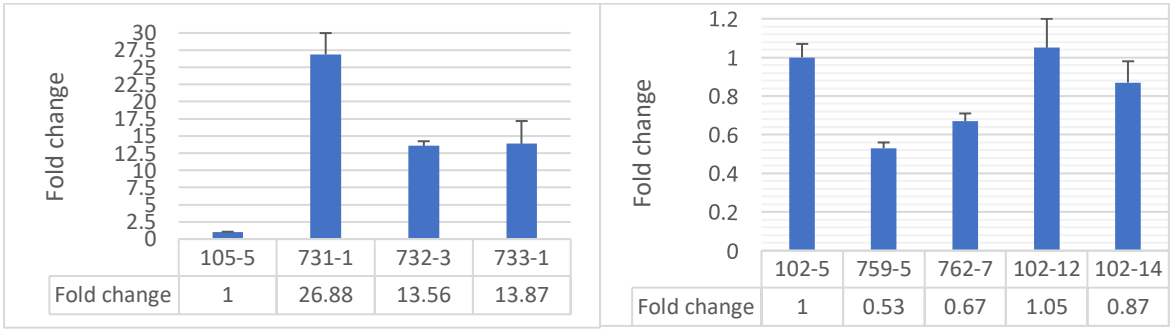

Figure 19: Fold changes of Cas9 transcription of isolated fibroblasts: Fold changes of transposon and random Cas9 integration normalised against GAPDH and compared to foetal Cas9 expressed in cells isolated from 102-5.

4.3.2 RT-qPCR of organ tissue

To confirm Cas9 transcription in several organs, one SB and one RI pig were sacrificed. RNA was extracted from muscle, tonsil, spleen, kidney, lymph nodes, oral mucosa, and liver. Only tissue from the same organs were compared. Overall, tissue extracted from 776-3 (RI pig) showed lower Cas9 expression (Table 13), except in tonsils and liver.

Table 13: Cas9 transcription of isolated organ tissue.

Animal ID	Organ	Normalised Cas9 expression[1]	Fold change* (RI:SB)
732-6	Muscle	1.23	0.60
776-3		0.74	
732-6	Tonsil	0.36	1.18
776-3		0.42	
732-6	Spleen	0.28	0.12
776-3		0.03	
732-6	Kidney	1.53	0.22
776-3		0.34	
732-6	Lymph nodes	0.33	0.22
776-3		0.07	
732-6	Oral mucosa	2.14	0.36
776-3		0.78	
732-6	Liver	0.89	3.87
776-3		3.43	

[1] $\frac{\text{relative expression value Cas9}}{\text{relative expression value GAPDH}}$

*Fold changes were compared between tissues isolated from the same organs e.g., liver tissue from 776-3 with random integration (RI) against 732-6 with Sleeping beauty (SB) transposon integration.

4.4 GERMLINE TRANSMISSION

The ability to generate offspring from transgenic boars which harbour Cas9 and an ASFV specific gRNA would be crucial to establish resistant offspring. Therefore, boars derived from both integration approaches which were confirmed to express the transgenes were kept for breeding (Figure 20A). Computer-assisted sperm analysis (CASA) was performed after collection of semen to assess the motility (Annex 8.9 Table 29). Semen from boar 765-6 (SB pig) was not considered for *in vitro* fertilisation due to severe deformation (Annex 8.9 Figure 9).

4.4.1 *In vitro* fertilisation

In vitro fertilisation (IVF) was performed with sperm from the RI pig (762-7). Different concentrations were used to compensate for the low motility of the first batch. Blastocysts developed and a second batch of semen was frozen and used for IVF. Blastocyst rates are shown in table 14 below. Analysis of 12 blastocysts revealed integration of Cas9 in 10 samples (Figure 20B).

Table 14: Results of *in vitro* fertilisation of 762-7, a boar carrying a random Cas9 integration.

762-7 Batch	Sperm cell/ oocyte ratio	Blastocyst rate (%)
1	7500	17/60 (28.3 %)
	1500	3/67 (4.48 %)
	1000	14/14 (28.3 %)
	750	4/74 (5.4 %)
2	1000	31/76 (40.8 %)
	75	19/81 (23.5 %)
	100	10/68 (14.7 %)
	150	12/73 (16.4 %)

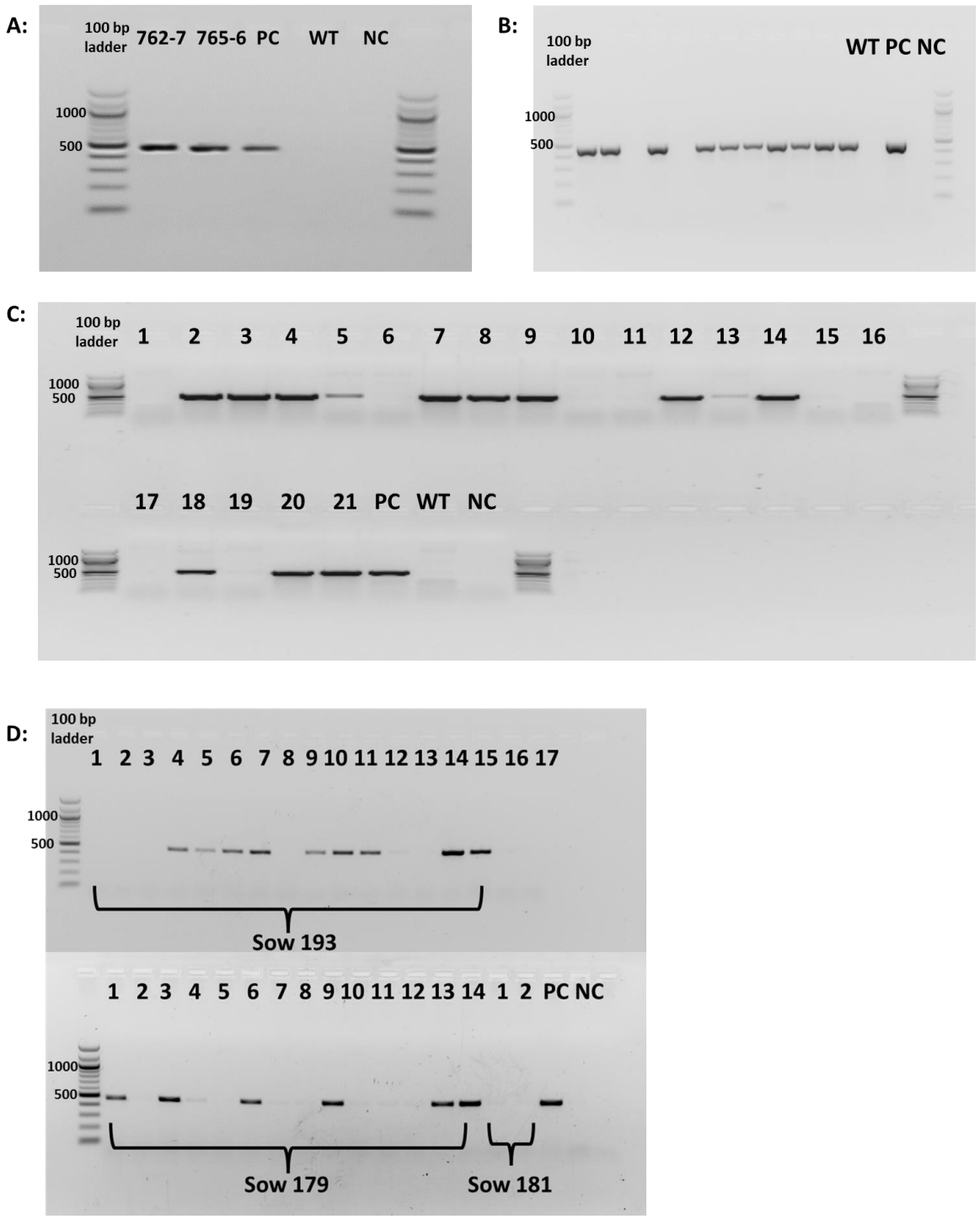

Figure 20: Gel electrophoretic image of the Cas9 PCR amplicon (500 bp) to detect germline transmission: **A**: Amplified SpCas9 (762-7) and eCas9 (765-6) integration of sperm DNA. **B**: SpCas9 integration of blastocysts fertilised with 762-7. **C**: DNA extracted from foetuses sired by 762-7 for Cas9 amplification. **D:** DNA extracted from live piglets sired by 765-7 (eCas9).

4.4.2 Artificial insemination

Semen from both boars 762-7 and 765-6 was used for AI. The pregnancy from the RI boar was terminated at day 25 post insemination. Twenty-one foetuses were retrieved from the sow. Of the 21 foetuses 13 were positive for Cas9 (Figure 20C). Four sows were inseminated with semen from boar 765-6 (SB pig) and three sows carried to term. In total 38 piglets were born of which five were stillborn (Table 15). Of the 33 piglets, 15 animals were confirmed to carry a Cas9 integration (Figure 20D).

Table 15: Quantitative results of artificial insemination with semen from boar 765-6.

Number of inseminations	4
Number of pregnancies	3
Number of piglets	38
Number of piglets born alive	33
Number of piglets euthanised within first week	3
Number of transgenic piglets	15

4.5 FUNCTIONALITY OF CAS9

After confirmation of a random and transposon-based Cas9 integration, the genome editing efficiency was evaluated. Crucial for the *in vivo* pathogen genome targeting strategy is that the Cas9 element is translated and can induce DSBs in genomes as it does for genome editing purposes. To validate the cleavage potential of Cas9, isolated fibroblasts were transfected with previously established gRNAs. To generate DSBs at the desired loci the transfected gRNAs must form a duplex with the expressed Cas9. Indel or deletion induction in the target locus would then confirm the functionality of the integrated Cas9. The isolated fibroblasts from pigs 731-1, 732-3, 733-1 (SB pigs), 759-5, 762-7 (RI pigs) were transfected with BPK1520 expressing gRNAs targeting GGTA1, B4GALNT2 and B2M. In addition, cells from pigs 731-1, 732-3, 733-1 were transfected with synthetic gRNAs also targeting GGTA1, B4GALNT2 and B2M. Synthetic gRNAs have the advantage that they reduce off-target editing due to rapid degradation of the gRNAs, compared to prolonged plasmid expressed gRNAs (Liu *et al.*, 2017). Next generation sequencing (NGS) and flow cytometry was performed for transfected cells isolated from 731-1, 732-3, 733-1, 759-5, and 762-7. After boar 762-7 reach sexual maturity, the boar was mated with a wild-type sow and fibroblasts of the foetuses were retrieved. Isolated fibroblasts from foetuses 102-12 and 102-14 were also transfected with plasmid expressed gRNAs and successful edits were confirmed by Sanger sequencing and flow cytometry.

4.5.1 Detection of genome edits: GGTA1

The GGTA1 target site of Cas9 expressing cell lines was amplified by PCR for next generation sequencing. NGS reads revealed genomic editing efficiency of 0.1-4.2 % between the different cell lines and transfections (Table 16). No reads were generated for synthetic gRNA transfection of cells from SB pig 732-3 and plasmid transfection of fibroblasts from pig 733-1 (SB pig). In addition, the plasmid transfection of cells from pig 731-1 produced only a coverage mean of 209 reads. The transfected cell lines were subjected to flow cytometry to detect expression of α-galactose (Figure 21). **Median** fluorescence intensity (MFI) was determined from a negative control (GGTA1 knock-out cell-line), the transfected cell lines, and the respective untreated cell line as positive control (Table 17). The synthetic gRNA transfected cells from pig 731-1 showed only a minor MFI decrease of 1.79 % from untreated to treated cells. Synthetic gRNA transfection of cells from pig 732-2 did not result in a MFI reduction, median fluorescence intensity increased by 30.48 % compared to untreated cells. Foetal fibroblasts were Sanger sequenced after transfection and counter-selection for α-galactose expression was performed. Selected cells showed modifications within the target region as expected by the flow cytometry data (before counter-selection) (Figure 22).

Table 16: Editing efficiencies of GGTA1 of Cas9 expressing cells generated by next generation sequencing.

Transfection	Coverage mean	Editing efficiency (%)
731-1 Plasmid	209	0.1
731-1 RNA	6268	0.1
732-3 Plasmid	947	0.3
733-1 RNA	824	4.1
759-5 Plasmid	1855	2.7
762-7 Plasmid	3280	2.9

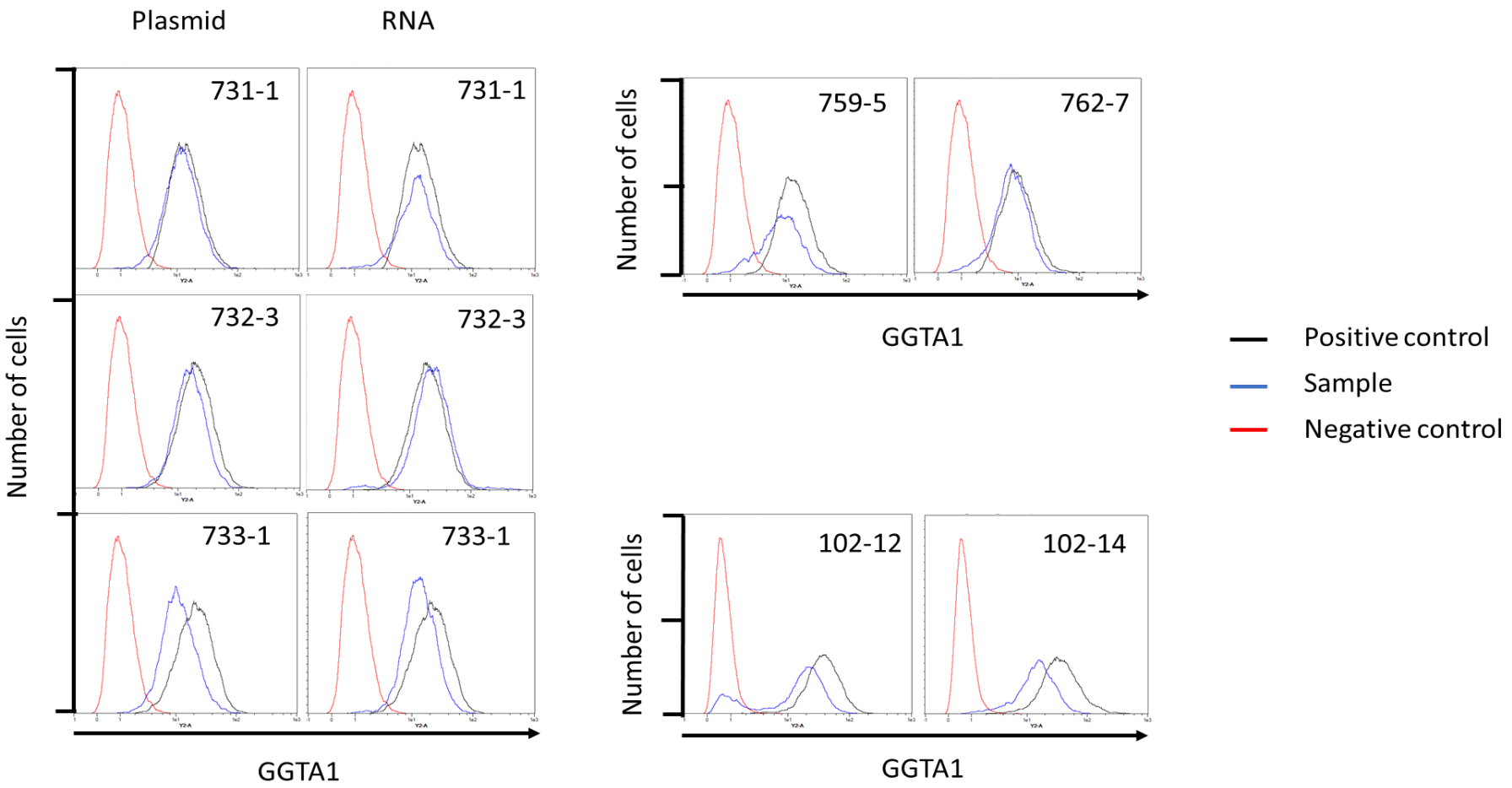

Figure 21: Lectin based flow cytometry for the detection of α-galactose: Isolated Cas9 expressing fibroblast were transfected with a GGTA1 targeting gRNA to induce indel formation. The transfected cells were compared to a negative control (GGTA1 knock-out cell line) untreated cell of the same isolate served as positive control. Measurements of foetal cells from 102-12 and 102-14 before counter-selection against α-galactose expression.

Table 17: Median fluorescence intensity (MFI) of lectin-based flow cytometry to detect α-galactose.

Transfection	MFI negative control*	MFI positive control[1]	MFI gRNA treated cell line	MFI reduction (%)±
731-1 Plasmid	4.17	13.42	12.69	0.73 (5.44)
731-1 RNA	4.17	13.42	13.18	0.24 (1.79)
732-3 Plasmid	4.17	19.37	15.77	3.6 (18.59)
732-3 RNA	4.17	19.37	23.46	na
733-1 Plasmid	4.17	20.64	11.23	9.41 (45.59)
733-1 RNA	4.17	20.64	13.55	7.09 (34.35)
759-5 Plasmid	4.17	13.23	10.01	1.25 (9.45)
762-7 Plasmid	4.17	9.42	8.54	0.88 (9.43)
102-12 Plasmid	3.98	37.18	20.87	16.31 (43.87)
102-14 Plasmid	3.98	35.02	15.29	19.73 (56.34)

*GGTA1 knock-out cell line

[1]Untreated corresponding Cas9 expressing cell line

± Reduction between positive control and gRNA transfection

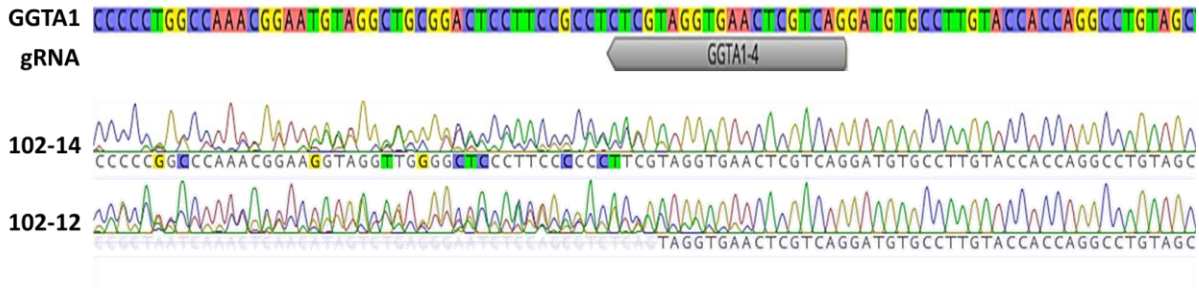

Figure 22: Sanger sequencing of GGTA1 gRNA targeted region. Cas9 expressing foetal fibroblasts (sired by 762-7) transfected with a GGTA1 targeting gRNA after counter-selection for α-galactose expression.

4.5.2 Detection of genome edits: B2M

Genomic deletions in the B2M target sequence of transfected cell lines were detected by PCR (Figure 23), followed by NGS. Cas9 editing efficiency of B2M deletions was confirmed and varied greatly from 0.5- 32.4 % between transfections and cell lines (Table 18). B2M knock-out cell lines were also analysed by flow cytometry (Figure 24 and Table 19). MFI of transfected cells decreased in all treated cell lines, indicating efficient editing of the B2M loci. However, isolated cells from pig 732-3 transfected with synthetic gRNAs were observed to have a minor MFI decrease of 2.48 % compared with untreated cells. Transfected foetal fibroblasts were diluted and individual wells were Sanger sequenced. Foetal fibroblasts of foetuses 102-12 and 102-14 were Sanger sequenced showing modifications within the B2M loci (Figure 25).

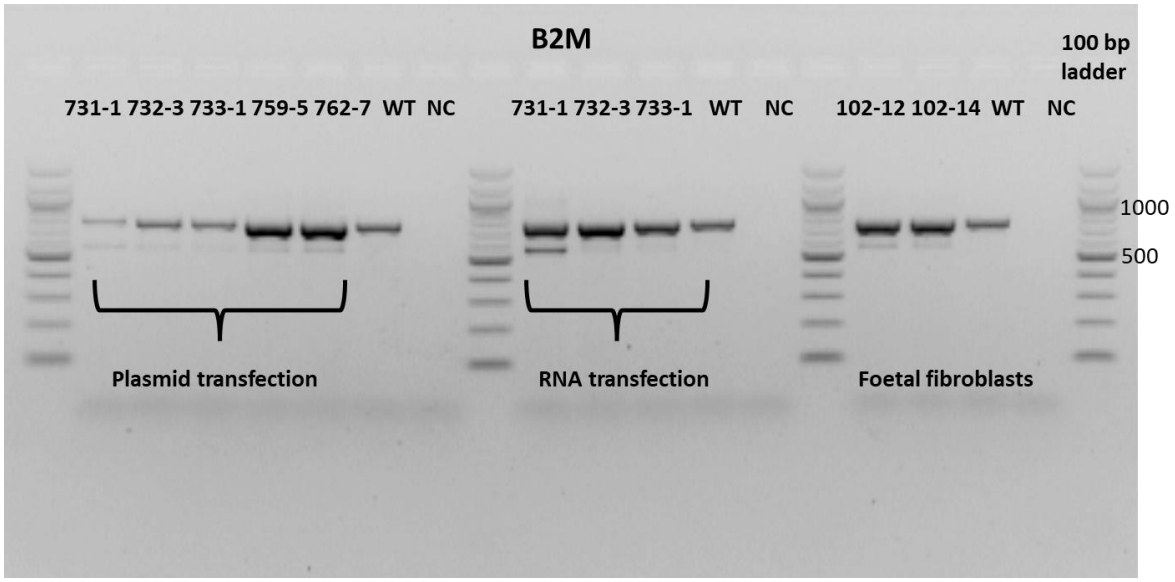

Figure 23: End-point PCR of B2M deletion (wild-type fragment 763 bp): Isolated Cas9 expressing fibroblast transfected with B2M gRNAs to induce a deletion. Amplification of foetal fibroblast prior to dilution.

Table 18: Editing efficiencies of B2M of Cas9 expressing cells generated by next generation sequencing.

Transfection	Coverage mean	Editing efficiency (%)
731-1 Plasmid	7352	27.6
731-1 RNA	33769	32.4
732-3 Plasmid	13039	2.7
732-3 RNA	25911	4.9
733-1 Plasmid	10869	3.4
733-1 RNA	18097	0.5
759-5 Plasmid	17322	7.2
762-7 Plasmid	25469	3.2

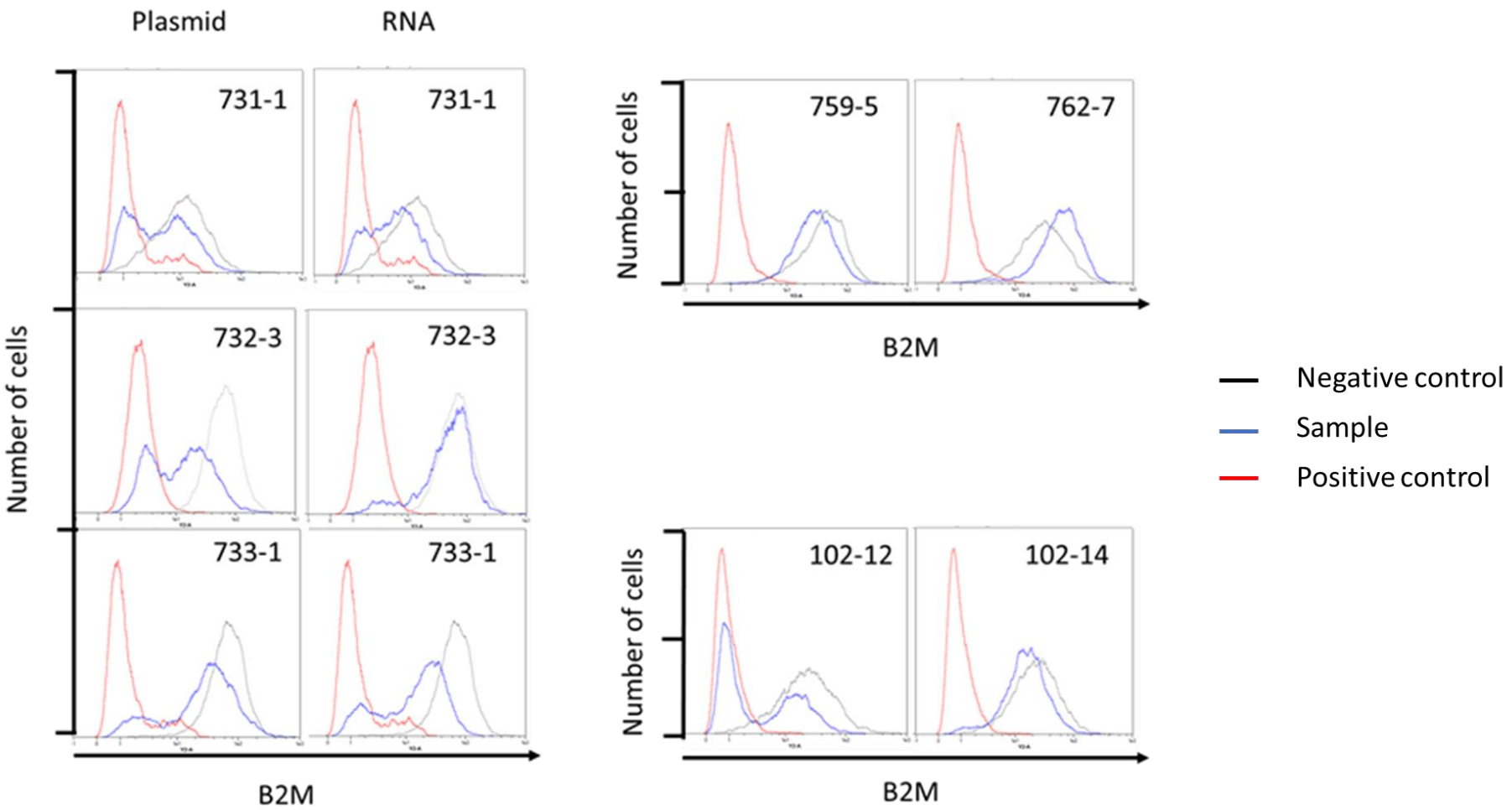

Figure 24: Flow cytometry for the detection of MHC-I expression. Isolated Cas9 expressing fibroblast were transfected with a B2M targeting gRNAs to induce a deletion. The transfected cells were compared to a negative control (B2M knock-out cell line) as positive control served untreated cells of the same isolate. Measurements of foetal fibroblasts 10-12 and 102-14 before dilution.

Table 19: Median fluorescence intensity (MFI) of MHC-I expression.

Transfection	MFI negative control*	MFI positive control[1]	MFI gRNA treated cell line	MFI reduction (%)±
731-1 Plasmid	11.32	15.68	13.00	2.68 (17.09)
731-1 RNA	11.32	15.68	11.63	4.05 (25.83)
732-3 Plasmid	16.23	63.90	28.25	35.65 (55.79)
732-3 RNA	16.23	63.90	62.25	1.65 (2.58)
733-1 Plasmid	11.32	67.86	40.14	27.72 (40.85)
733-1 RNA	11.32	67.86	26.11	41.75 (61.52)
759-5 Plasmid	10.74	65.66	31.23	34.43 (52.44)
762-7 Plasmid	10.74	64.48	34.30	30.18 (46.81)
102-12 Plasmid	16.72	32.74	22.22	10.52 (47.43)
102-14 Plasmid	16.72	30.87	23.23	7.5 (24.30)

*B2M knock-out cell line

[1]Untreated corresponding Cas9 expressing cell line

± Reduction between positive control and gRNA transfection

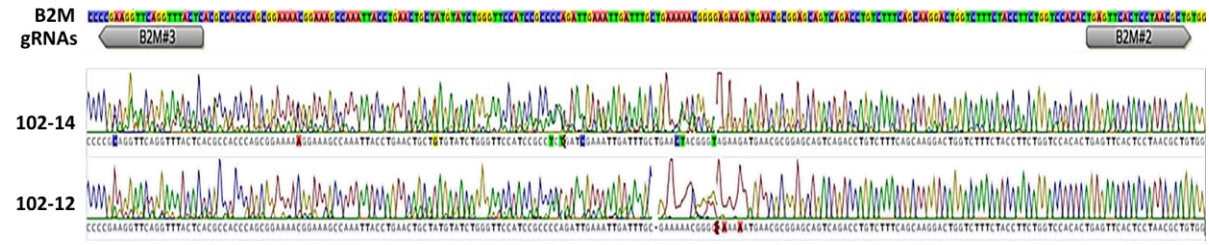

Figure 25: Sanger sequencing of B2M gRNA targeted region: Cas9 expressing foetal fibroblasts (sired by 762-7) transfected with two B2M targeting gRNAs. Sequences obtained from diluted cell population.

4.5.3 Detection of genome edits: B4GALNT2

Deletions in the B4GALNT2 loci were also detected by PCR (Figure 26) and NGS. Unfortunately, pooling of the library resulted in a low coverage of B4GALNT2 amplicons. Coverage results are listed in table 20, but not all results can be considered reliable (731-1 and 732-3 RNA transfection). Nonetheless, deletions of B4GALNT2 showed greater editing efficiencies compared to B2M and GGTA, ranging from 19.4 -66.3 %.

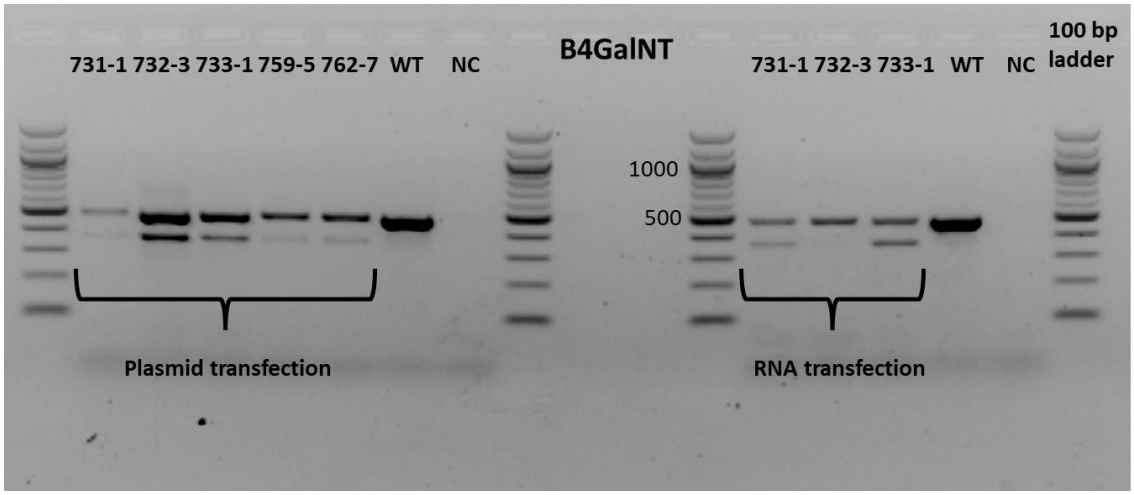

Figure 26: End-point PCR of B4GALNT2 deletion (wild-type fragment 419 bp): Isolated Cas9 expressing fibroblast transfected with B4GALNT2 gRNAs to induce a deletion.

Table 20: Editing efficiency of B4GALNT2 of Cas9 expressing cells generated by next generation sequencing.

Transfection	Coverage mean	Editing efficiency (%)
731-1 Plasmid	1390	36.9
731-1 RNA	122*	52.2
732-3 Plasmid	6298	60.2
732-3 RNA	80*	13.5
733-1 Plasmid	3209	51.2
733-1 RNA	312	66.3
759-5 Plasmid	499	30.7
762-7 Plasmid	719	19.4

* low coverage mean

4.5.4 Inhibition of Cas9 activity

SpCas9 expressing foetal fibroblasts (102-12) were transfected with gRNAs and AcrIIA4, an anti-CRISPR protein inhibiting Cas9 activity. Inhibition of Cas9 was measured by flow cytometry for GGTA1 and B2M (Figure 27 and Table 21). The inhibition of Cas9 would result in lower editing efficiencies with a greater proportion of unedited cells. The greater proportion of unedited cells would lead to a lower MFI reduction compared to gRNA transfected cells. MFI of GGTA1 gRNA transfection was reduced by 39.55 % compared to the untreated cells. When the cells were transfected with AcrIIA4 and GGTA1 gRNA, MFI decreased by only 21.78 % compared to the untreated sample. Similarly, B2M gRNAs transfection resulted in 51.06 % MFI reduction, whereas the combination with AcrIIA4 and gRNAs lead to only 22.00 % MFI reduction. The results show that Cas9 activity was inhibited by the anti-CRISPR protein AcrIIA4 to a certain extent.

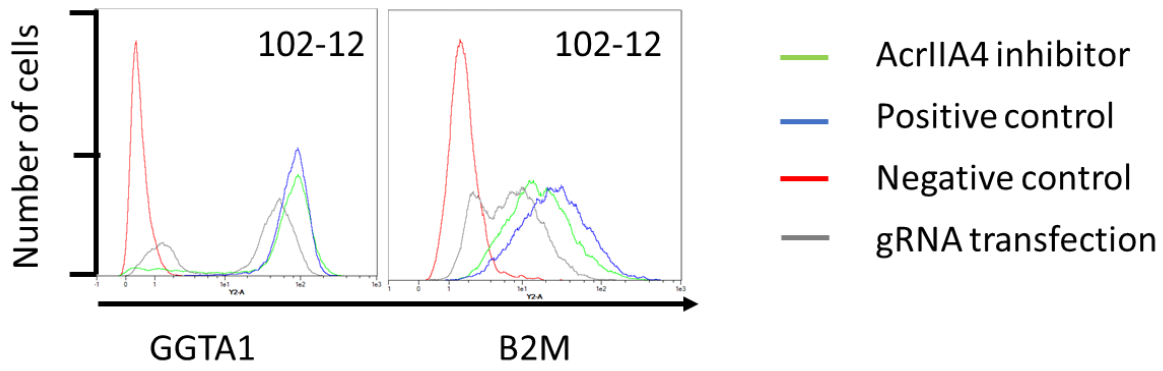

Figure 27: Flow cytometry after Cas9 inhibition: Isolated foetal Cas9 expressing fibroblasts were transfected with a Cas9 inhibitor (AcrIIA4) and compared to gRNA only transfected cells and corresponding controls.

Table 21: Median fluorescent intensity (MFI) after Cas9 inhibition.

Transfection	MFI negative control*	MFI positive control[1]	MFI gRNA transfection	MFI reduction (%)[±]	MFI AcrIIA4 inhibitor	MFI reduction after inhibition (%)[+]
102-12 GGTA1	21.86	82.25	49.66	32.59 (39.55)	64.34	17.91 (21.78)
102-12 B2M	15.74	33.59	16.44	17.15 (51.06)	26.20	7.39 (22.00)

*Knock-out cell lines

[1]Untreated corresponding Cas9 expressing cell line

[±] Reduction between positive control and gRNA transfection

[+]Reduction between positive control and after inhibition

4.5.5 Off-target analysis

Off-target GGTA1

Off-target analysis was performed for transfected cells isolated from pigs 731-1, 732-3, 733-1, 759-5, 762-7 (731-1, 732-3, and 733-1 also RNA transfections). Three off-targets for the GGTA1 gRNA 5' CTGACGAGTTCACCTACGAG 3' were identified. Off-targets were located on chromosome 13 (NC_010455.5) position 188,635,819-188,635,841 5' CT**AT**C**A**A**C**TTCACCTACGAG 3' (mismatches in bold), chromosome 3 (NC_010445.4) position 83,562,713-83,562,735 5' **G**T**T**A**GA**AGTTCACCTACGAG 3', and chromosome 16 (NC_010458.4) position 79,888,603-79,888,625 5' CTGACG**T**GT**A**CACCTAC**A**AG 3'. The targets were amplified (Annex 8.8 Table 25) and aligned to (NCBI) reference sequence. None of the three off-targets showed any variation compared to the reference sequence or wild-type DNA (Annex 8.3 Figure 1).

Off-targets B2M

Similarly, three of the most likely off-targets for the two gRNAs targeting B2M were analysed in greater detail. Off-targets for gRNA 5' TGAGTTCACTCCTAACGCTG 3' were located on chromosome 13 (NC_010455.5) position 106,638,730-106,638,752 5' T**T**AGTT**T**ACTC**A**TAA**A**GCTG 3', chromosome 12 (NC_010454.4) position 40,369,128-40,369,150 5' T**T**A**A**TTC**G**CTCC**C**AACGCTG 3', and chromosome 7 (NC_010449.5) position 39,698,969-39,698,990 5' TGAGTTCA**A**TCCT**TGA**GCTG 3'. Potential off-targets for the second guide 5' GAGTAAACCTGAACCTTCGG 3' were found on chromosome 9 (NC_010451.4) position 134,729,454-134,729,476 5' G**G**GTAAA**TA**TGAACCTTC**A**G 3', chromosome 5 (NC_010447.5) position 59,310,453-59,310,475 5' GAGTAAACC**A**GAA**T**C**A**TC**T**G 3', and chromosome 1 (NC_010443.5) position 189,493,129-189,493,151 5' GAGTAA**G**CCTGAA**T**CTTC**A**G 3'. Also, in these off-targets no mismatches were found (Annex 8.8 Table 26 and Annex 8.3 Figure 2 and 3), except in one; chromosome 9. However, the WT sequence showed the same variation (Figure 29).

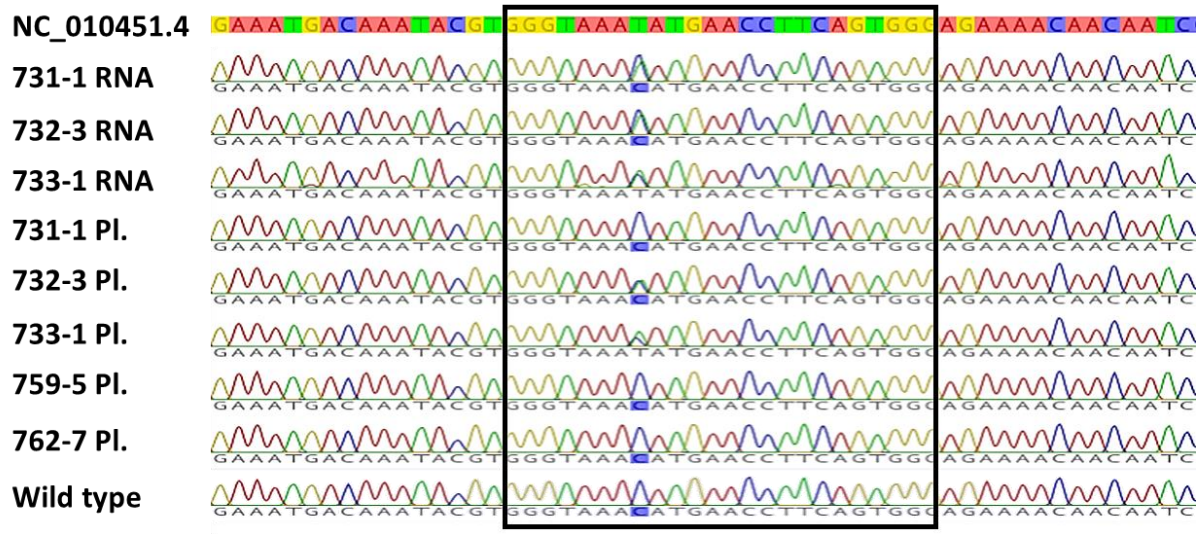

Figure 29: B2M Chromosome 9 off-target alignment. Off-target sequence of chromosome 9 targeted by gRNA 5' GAGTAAACCTGAACCTTCGG 3'. One mismatch to the reference sequence was shown in transfected cells but also in wild-type sequence.

Off-targets B4GALNT2

B4GALNT2 off-targets for gRNA 5' ATTGTCTGGGACGTCAGCAA 3' were analysed for chromosome 7 (NC_010449.5) position 49,347,420-49,347,442 5' **T**TTG**G**CTGGGAC**A**TCAGCA**G** 3', chromosome 9 (NC_010451.4) position 8,914,295-8,914,317 5' AT**CAG**CTGGGACGTCAGCAA 3', and chromosome 11 (NC_010453.5) position 52,308,178-52,308,199 5' ATTGTCTGG**A**AA**G**A**A**CAA 3'. For gRNA 5' AGAGTACCACCTCCACAGAG 3' following off-targets were amplified; chromosome 2 (NC_010444.4) position 3,798,436-3,798,458 5' AGAG**C**ACCACCTCCAAAGAG 3', chromosome 9 position 36,004,607-36,004,629 5' **T**GA**AC**A**T**CACCTCCACAGAG 3', and chromosome 17 position 24,525,562-24,525,584 5' AGAG**C**ACCA**AA**TCCA**A**AGAG 3'. Also, for the B4GALNT2 targets, no variation compared to the reference sequence was found (Annex 8.8 Table 27 and Annex 8.3 Figure 4 and 5).

4.6 AFRICAN SWINE FEVER VIRUS INFECTION STUDIES

4.6.1 *In vitro* infection study

Haemadsorption assay of TG and WT macrophages was performed to see whether TG macrophages would be more resilient to ASFV infection. One animal of each group was slaughtered, and isolated macrophages were infected with ASFV Armenia. No difference in haemadsorption between the TG and WT animals was observed, for both plates a HAD_{50} (50 % haemadsorbing dose) was calculated to be $10^{-4.7.}$. Also, viral replication at different time points was assessed. The macrophages of the TG animal indicated a lower trend of viral replication (Figure 30).

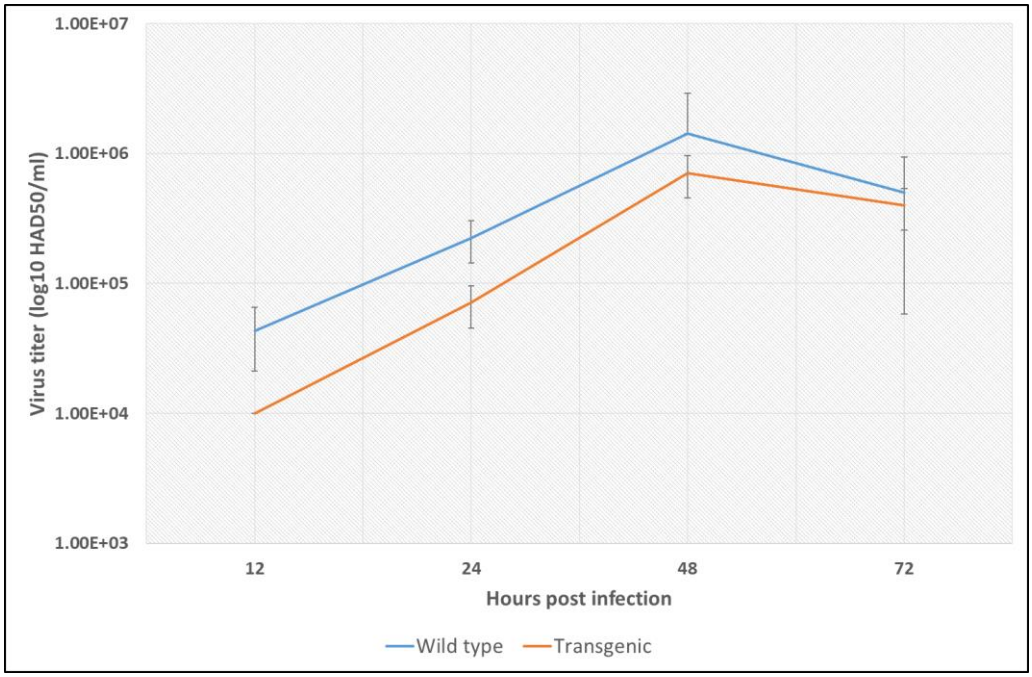

Figure 30: Viral replication of African swine fever in infected macrophages: Isolated macrophages from a transgenic and control pig were infected with ASFV. A tendency of lower viral replication was observed in transgenic macrophages upon 48 hours post-infection.

4.6.2 *In vivo* infection study

To investigate whether the transgenic SB pigs (T1- T7) would be resistant or resilient against ASF, seven transgenic and seven WT-control pigs (C1-C7) were infected with 2 x $10^{4.35}$ $TCID_{50}$ ASFV (Figure 31). First clinical signs such as elevated temperatures, differences in motion, and decreased liveliness were seen 5 dpi (days post infection) in control animals and in transgenic pigs simultaneously. In animals which showed first clinical signs 5 dpi the disease further progressed, and five WT (C1, C3, C4, C5, C6) and two TG animals (T3 and T4) were euthanised 7 dpi, and T2 8 dpi. The remaining two WT-control animals (C2 and C7) developed delayed symptoms, C7 10 dpi and C2 on 12 dpi. C7 was euthanised 12 dpi and C2 14 dpi. Two of the remaining TG pigs (T1 and T5) which were housed together with TG pigs that were infected, started to show clinical signs 11 dpi and were euthanised 14 dpi. Animals T6 and T7 remained healthy until 14 dpi. ASFV p72 was detected in blood samples at 4 dpi. No viremia was detected by qPCR in animals C1, C2, C4, C5, C7, T1, T2, T5, T6, and T7. Blood samples were again evaluated at 7 dpi, viremia was now detected in most control animals except C2 and C7. No viremia was detected in T1, T5, T6, and T7. A new inoculation was prepared. However, in the meantime animal C2, C7, T1, and T5 started to display ASF symptoms and were euthanised, while T6 and T7 were inoculated a second time. Before reinfection, blood was taken and checked for antibodies against ASFV to ensure that the animals were not infected during the first round of inoculation. Animals T6 and T7 were then inoculated with 10 x $10^{5.75}$ $TCID_{50}$. After four days, viremia

was confirmed in the reinfected animals, and they were euthanised 7 dpi due to severe symptoms. Viral loads were not compared between the animals due to different infection routes, time points, and infectious dosages.

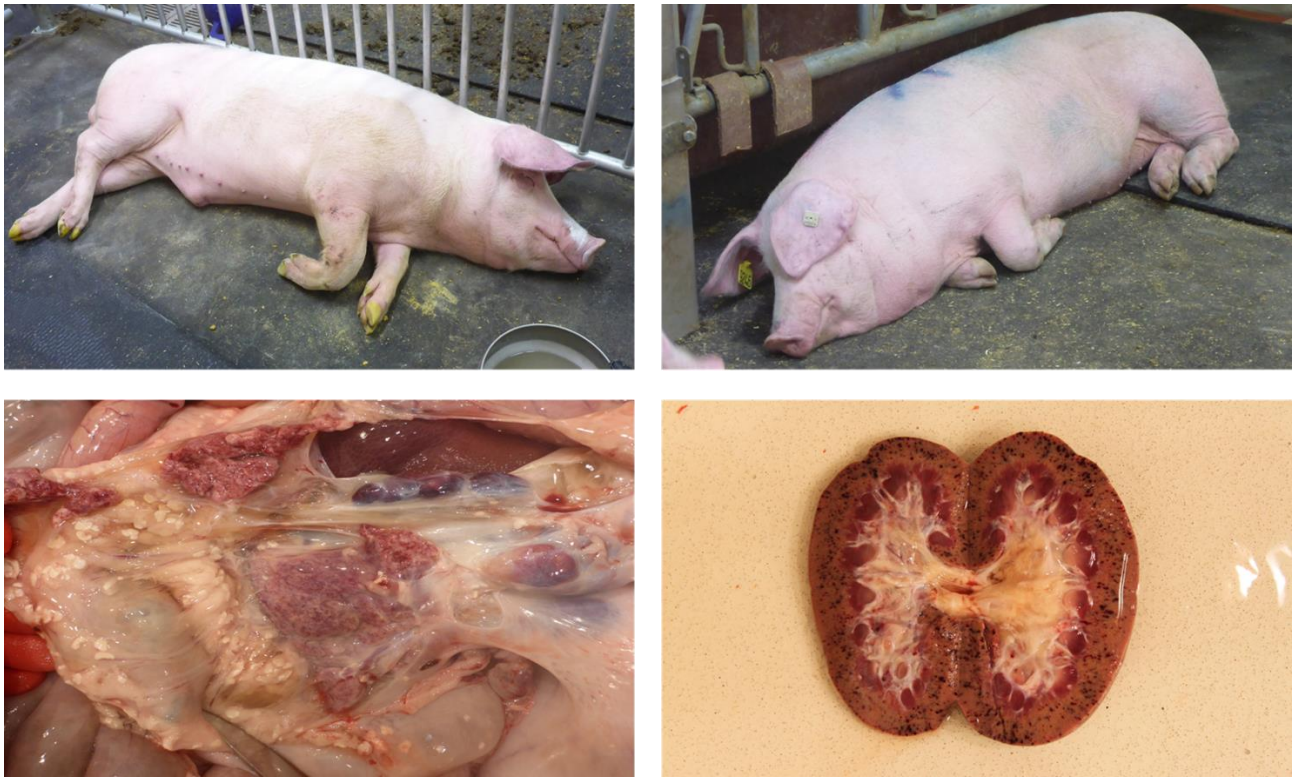

Figure 31: *In vivo* African swine fever infection study: Transgenic pig (left) and control pig (right) at end-point. Necrotic pancreas of a transgenic pig and a haemorrhagic kidney of a control pig.

4.7 TARGETED CAS9 INTEGRATION

Since the SB pigs were not resistant to ASFV infection a different approach was designed to further explore an *in vivo* pathogen genome targeting strategy against ASF. A template was designed to integrate a Cas9 expression cassette into the porcine 'safe harbour' locus (Rosa26). The template was designed to facilitate a Recombinase Mediated Cassette Exchange (RMCE) with the Cre-Lox system. The system can be used to integrate different and multiple gRNA arrays.

4.7.1 Guide RNAs targeting porcine Rosa26

Three gRNAs were evaluated for genome editing efficiency in the Rosa26 locus. A T7 assay confirmed editing of all three gRNAs (Figure 32). ICE analysis of Sanger sequences further supported editing of all three gRNAs, however efficiency varied. The first gRNA established 25 % indels, the second 29 %, and the third 11 % (Figure 33). For the HDR experiment gRNA 2 was selected which also proofed to be efficient in the study from Xie et. al., (2017).

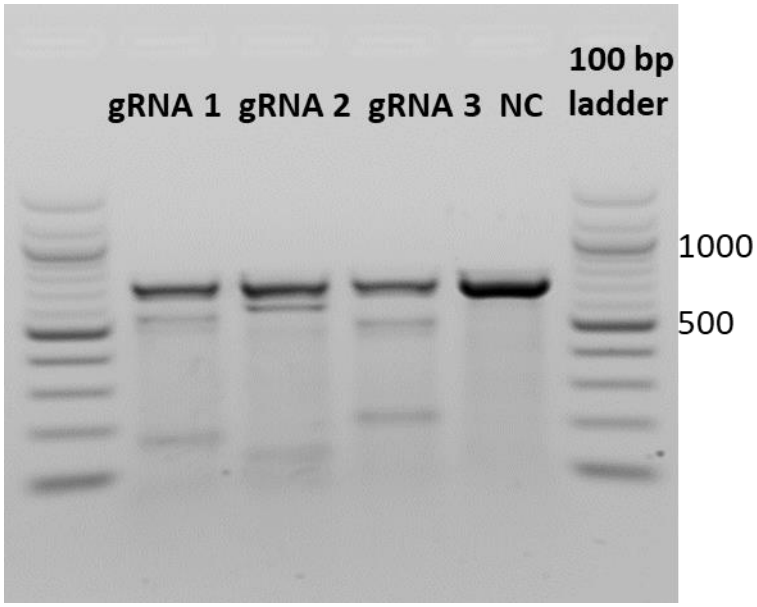

Figure 32: T7 endonuclease-I assay of Rosa26 targeting gRNAs. Guide RNA 1-3 and a negative control (NC). PCR amplicon (699 bp) upper band shows the wild-type sequence, bands below the wild-type bands indicate mismatches detected by the T7 assay.

Figure 33: Synthego ICE analysis of Rosa26 gRNAs editing efficiency. The different sequences found in the Sanger sequence after PCR amplification of Rosa26 in the edited samples. Indel formations in the edited samples are indicated by e.g. +1 or -1. The contribution (%) of each sequence in the Sanger sequence. The contribution of indel sequences in gRNA 1 sample was 25 % in total. For gRNA 2 29 % and for gRNA 3 11 %. Black dotted line indicates the gRNA cut site and the sequnce with '0' refers to the wild-type sequence. Synthego Performance Analysis, ICE Analysis. 2019. v3.0. Synthego; [29.10.2020].

4.7.2 Targeted integration

Foetal fibroblasts were transfected with pUC-Rosa26 to integrate a Cas9 transgene cassette into the porcine Rosa26 locus. Two transfections were carried out and cells were selected with G418. First, Cas9 integration was confirmed by amplifying the Cas9 gene (Figure 34). Second, primers were designed which were positioned in the genomic region of the HDR template and the transgenic region of the insert. Integration sites were Sanger sequenced to confirm the insertion of the transgene in the Rosa26 locus (Figure 35). Cells of the first transfection were used for SCNT, but no pregnancies were established.

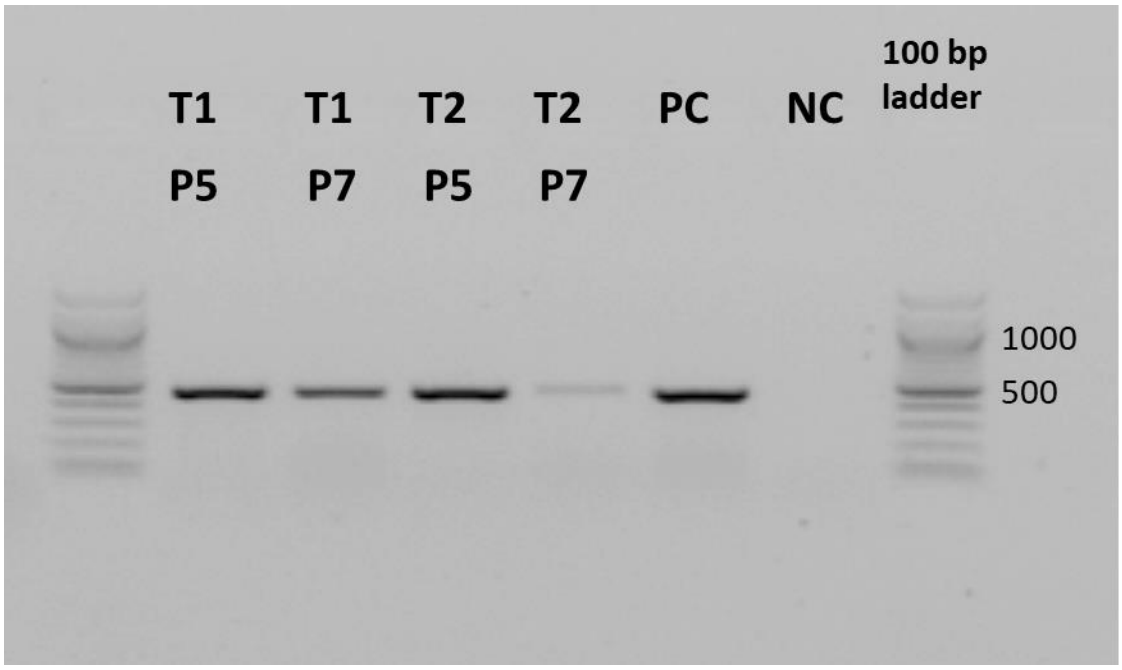

Figure 34: Cas9 amplification of Cas9 integration (500 bp) in Rosa26 locus: Cas9 was amplified from two transfections (T1 and T2) with the Cas9 integration template. Integration was confirmed in cell lysate at passage 5 and 7 (P5 and P7).

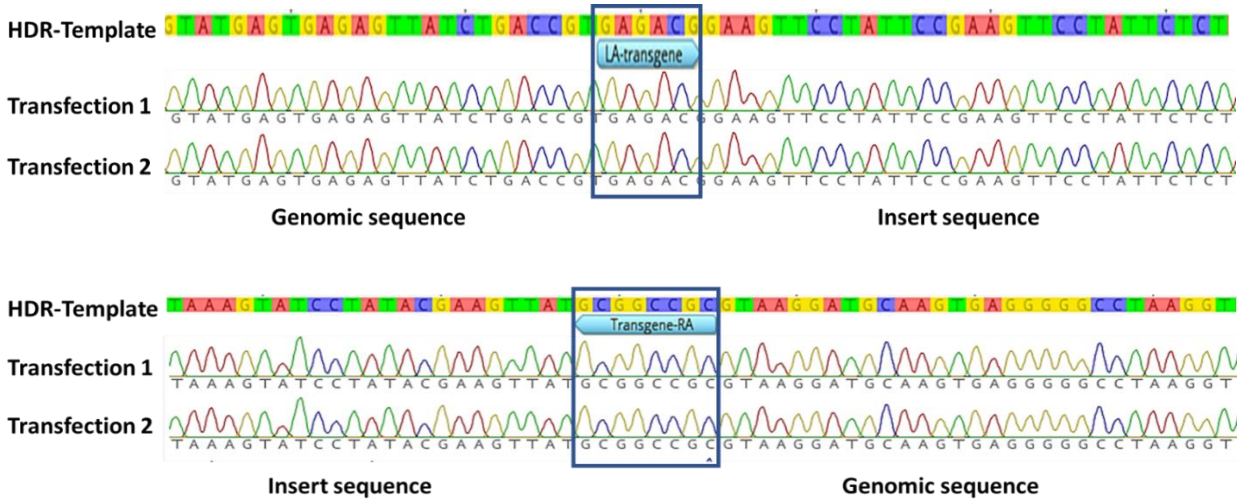

Figure 35: Templated integration of pUC57-Rosa26. End-point PCR covering genomic DNA and transgene integration was Sanger sequenced to confirm integration sites of the left and right homologous arms. Square indicates transition between genomic and insert sequence.

5 DISCUSSION

Generating disease resistant animals with the use of genome editors such as CRISPR-Cas may help to understand host-pathogen interactions and these results can lead to vaccine and drug development in the long term.

This study was based on the findings of Hübner et al., (2018), which showed that modified wild boar lung (WSL) cells expressing Cas9 and an African swine fever virus (ASFV) specific gRNA were resilient to infection (Hübner *et al.*, 2018). Thus, an *in vivo* pathogen genome targeting strategy was developed to test whether the strategy could induce ASFV resistance in domestic pigs. The resultant pigs were characterised in detail, bred and the functionality of the integrated Cas9 element was investigated.

5.1 SOMATIC CELL NUCLEAR TRANSFER-BASED PIG PRODUCTION

In this study the founder pigs were produced by SCNT. Nine out of 20 piglets had to euthanised within the first week after birth, from those that were generated based on a Sleeping Beauty (SB) transposon transposition. SCNT is accompanied by nuclear reprogramming by which the somatic donor cell is epigenetically reprogrammed to become totipotent (Gurdon and Melton, 2008). Reprogramming events include DNA demethylation, histone modification changes, histone replacement, chromatin remodelling, and changes in the transcriptome (Niemann, 2016; Matoba and Zhang, 2018). Incomplete or false reprogramming is known to affect development of the offspring (Hill, 2014; Niemann, 2016). Incomplete reprogramming may negatively affect the development of the inner cell mass, trophectoderm, and the extraembryonic membrane which will give rise to the placenta (Chae *et al.*, 2009). An inadequate intrauterine environment and placentation may result in abnormalities such as lower birth weight and malformation (Estrada *et al.*, 2007; Yang *et al.*, 2007). The nine euthanised piglets and the two stillborn piglets displayed SCNT related health problems such as low birth weight, oversized tongue, and leg abnormalities. The remaining pigs grew up healthy.

5.2 TRANSGENE INTEGRATION APPROACH

Transgenic pigs carrying Cas9, and gRNA transgenes were generated either via transposon-based integration or by random vector integration. A total of 22 piglets were born of which the donor cells were transfected with the SB vector system containing an eCas9, gRNA, GFP, and neomycin expression cassette. The integrated gRNA had been designed to target the CP204L gene of the Armenian ASFV (Hübner *et al.*, 2018). The SB vector system generates highly efficient and stable

transgenesis (Ivics *et al.*, 2009; Garrels *et al.*, 2011; Jakobsen *et al.*, 2011). In addition, it is compatible with several kilobase transposition of transgenes (Zayed *et al.*, 2004), which was required to integrate the multiple transgenes (Cas9, gRNA array, neomycin, GFP) in this study. In combination with selections markers, transgene expressing cells can be selected and sufficient numbers of founder animals can be generated. Here, we generated 15 transgenic founder pigs out of a total of 22 pigs. Despite the unregulated integration of SB directed transgenes, it is known that SB integrates preferably in palindromic AT repeats (Vigdal *et al.*, 2002) and not in sites of active transcription (Yant *et al.*, 2005). If integration occurs within active transcription units, then the preferred site are introns (Yant *et al.*, 2005; Ivics *et al.*, 2009). Several studies have successfully generated transgenic pigs by means of SB transgenesis (Carlson *et al.*, 2011; Garrels *et al.*, 2011; Jakobsen *et al.*, 2011).

Somatic cells for SCNT were selected based on a neomycin selection marker. As indicated by seven WT piglets born among the 15 transgenic piglets, the G418 selection did not result in a completely homogenous cell population. During the selection process a vast majority of wild-type cells will be eliminated, but a fraction of wild-type cells will remain (Lanza, Kim and Alper, 2013). The wild-type piglets must have resulted from wild-type fibroblasts which remained after selection.

5.2.1 Random transgenesis

Surprisingly, only two transgenic pigs out of 15 were generated after random integration of transgenes. Unstable integration of the transgenes may have resulted in loss of transgenes during *in vitro* culture of the somatic cells (Migliaccio *et al.*, 2000; Kong *et al.*, 2009) or embryo development (Kong *et al.*, 2009). Unstable integration of transgenes allows for transcription of the transgenes, but will not result in long-term integration (Migliaccio *et al.*, 2000). Most likely, short-term transcription of the selection marker neomycin resulted in successful selection of Cas9 and gRNA expressing cells, but due to unstable integration of the transgenes, the genes were ultimately lost. Since the vector pX330 was designed as expression vector for Cas9 and gRNAs CRISPR-Cas9 experiment (Cong *et al.*, 2013; Ran *et al.*, 2013), genomic locations on where or whether stable/unstable vector integration occurs is mostly unknown. The previously ASFV resistant WSL cells were modified to express Cas9 and gRNA with the pX330 vector. In WSL cells the gRNA targeting CP204L of ASFV was stably expressed for over more than 50 passages (Hübner *et al.*, 2018).

5.2.2 Integration of Cas9 and gRNAs for *in vivo* pathogen genome targeting

For the purpose of *in vivo* pathogen genome targeting, Cas9 and gRNA transgenes must be firmly incorporated into the host genome. We integrated eCas9, SpCas9 and gRNAs into the porcine genome. The pigs grew up normally and two boars were raised for breeding. In line with other

studies which generated Cas9 expressing pigs, no abnormalities were observed (Wang *et al.*, 2017; Rieblinger *et al.*, 2021). The integrated gRNA targeting CP204L of ASFV, showed no homology to the porcine genome, and therefore has no target site in the porcine genome to induce DSBs. Chickens which expressed gRNAs targeting MDV also experienced no health impairments (Challagulla *et al.*, 2021). Constant Cas9 expression within animals may be of concern and calls for control of Cas9 to avoid unwanted cleavage. A proposed 'on-switch' may be able to control Cas9 activity *in vivo* (Oakes *et al.*, 2019). Cas9 was inactivated by circular permutation and conjugated AA linkers between the N and C-terminal and called ProCas9. Only after exposure to a sequence-specific protease, Cas9 activity is reinstated by proteolytic cleavage of the linker sequence. The 'on-switch' was engineered for viruses such as West Nile Virus and Zika Virus (Oakes *et al.*, 2019).

5.3 GENE EDITING EFFICIENCIES OF CAS9

As proof of principal to confirm the functionality of the transgenic Cas9, fibroblasts of the generated pigs were isolated and subjected to transfections with specific gRNAs. The chosen gRNAs targeted three different epitopes which are crucial for the development of porcine xenografts (Byrne *et al.*, 2018). For the purpose of studying genetically modified pigs for xenotransplantation, the gRNAs targeting B2M, GGTA1, and B4GALNT2 were previously validated and resulted in successful genome edits (Hein *et al.*, 2020; Sake, 2021). Therefore, the induction of genome edits with validated gRNAs would prove the functionality of the integrated Cas9 cassette in the transgenic pigs.

5.3.1 Next generation sequencing

Three genes, GGTA1, B2M, and B4GALNT2 were targeted with gRNAs in the Cas9 expressing cells. GGTA1 was targeted with a single gRNA to induce indel formation, B2M and B4GALNT2 were each targeted with two gRNAs to induce a fragment deletion. Overall, the NGS results showed greater editing efficiency in the B2M (0.5 – 32.4 %) and B4GALNT2 (19.4 – 66.3 %) targets compared to GGTA1 (0.1 – 4.1 %). Single gRNA targeting generates only one DSB in the DNA, while two DSBs were produced when two gRNAs had been transfected. The repair of the single DSB may not lead to indel formation, instead it will be perfectly repaired and thereby reducing editing efficiency (He *et al.*, 2015), explaining why B2M and B4GALNT2 may had greater efficiencies. It is known that editing varies between target genes (Van Campenhout *et al.*, 2019). For example, Cas9 expressing pigs which were submitted to *in vivo* genome editing also showed great differences between target genes. The targeted genes APC, BRCA1, and BRCA2 displayed different editing efficiencies of 8.1 %, 20.2 %, and 78.8 %, respectively (Wang *et al.*, 2017).

5.3.2 Flow cytometry

Phenotypic modification of GGTA1 and B2M transfected Cas9 expressing cells, was analysed by flow cytometry. Analysis of MHC-I expressions indicated greater genome editing efficiency than it was predicted from NGS results. The most prominent sample was the synthetic gRNA transfected sample of pig 733-1, NGS calculated an efficiency of 0.5 % but MFI was reduced by 61.51 % between gRNA treated and untreated cells. *In vitro* culture and passaging may have reduced expression of MHC-I (Martini *et al.*, 2010). If MHC-I expression in the fibroblasts decreased, antibody binding would have been influenced, skewing the measurements. To avoid such misrepresentation in the future MHC-I, expression could be stimulated with interferon-γ (Martini *et al.*, 2010; Wei and Kryczek, 2011).

5.4 AFRICAN SWINE FEVER VIRUS INFECTION STUDIES

Previously, transgenic WSL were generated to test an *in vivo* pathogen genome strategy to induce resistance to ASF. The cells expressing Cas9 and a CP204L targeting gRNA inhibited viral replication when infected with ASFV (Hübner *et al.*, 2018). The question was whether the combination of transgenes would be able to inhibit ASFV infection *in vivo*. Therefore, pigs were generated expressing Cas9 and the gRNA targeting CP204L of ASFV. Based on their earlier results and the fact that the integrated Cas9 proved to be functional (see above), infection studies with ASFV were conducted.

Both the *in vitro* nor the *in vivo* infection study with the Cas9 expressing pigs resulted in lower viral replication levels. The *in vitro* experiment with isolated macrophages from the Cas9 expressing pig indicated a trend for lower replication at the beginning of the infection, when p30 is expressed. The p30 protein is involved in viral attachment and entry (Gómez-Puertas *et al.*, 1998; Dixon *et al.*, 2013), Cas9 may have disrupted p30 translation at the onset of the infection. However, no delayed disease onset was observed in in the *in vivo* study and individual differences may have been responsible for a lower replication in macrophages *in vitro*. It must be kept in mind that the *in vitro* study only reflected the immune response of one transgenic animal. If there was a delayed viral replication, the disruption of CP204L may have led to viral escape mutants. Viral escape mutants have been previously reported for *in vivo* pathogen genome targeting of the human immunodeficiency virus I (HIV-I) (G. Wang *et al.*, 2016; Yoder and Bundschuh, 2016; Z. Wang *et al.*, 2016). Though, frequency of infectious escape mutants in Cas9 expressing WSL cells was low, viruses carrying one or two nucleotide exchanges were found. These indels led to a different amino acid composition (Hübner *et al.*, 2018). Another possibility why the pigs were not resistant to ASFV infection, is that Cas9 and gRNA expression levels in macrophages were not sufficient. ASFV replicates mainly in macrophages (Alcamí, Carrascosa and Viñuela, 1990; Galindo *et al.*, 2015), but

Cas9 and gRNA expressions in macrophages were not quantified in this study. With higher expression levels of Cas9 and gRNAs in macrophages viral replication might have been inhibited efficiently.

During the *in vivo* infection study the oronasal inoculations with 2 ml suspension was not sufficient to induce infection in all control and transgenic animals. Since the transgenic pigs were 12-months-old and weighed about 120 kg it was difficult to handle and infect them with a 2 ml suspension. The animals that got infected through the inoculations developed clinical ASF symptoms at 4 dpi. Two of the remaining controls and two of the transgenic pigs must have gotten infected through contact with infected animals, since a delayed onset of disease was observed before re-inoculation. However, these findings indicate that the transgenic pigs would also not have resisted natural infection.

5.5 Conclusions and future perspectives

In conclusion, the present study generated transgenic pigs based on an *in vivo* pathogen genome strategy. The Cas9 and gRNA integration was confirmed and the Cas9 expressed from isolated fibroblasts of the transgenic pigs proved to be able to generate DSBs when transfected with gRNAs. However, expression of Cas9 and the gRNA targeting CP204L of ASFV in the pigs was not sufficient to induce ASF resistance. Several improvements must be made to optimise an *in vivo* pathogen genome editing strategy for ASF resistance. Achieving resistance to ASF will provide further understanding of ASFV-host interaction, which may contribute to the development of anti-viral drugs or vaccines in the long term. Treatment and prevention of ASF is urgently needed as the virus travels around the globe threatening pork production.

5.5.1 Targeting essential viral genes

While the designed gRNA targeting p30 inhibited viral replication in WSL cells, *in vivo* experiments did not result in ASFV resistant pigs. In chickens, expression of Cas9 and six gRNAs targeting ICP4 of MDV, resulted in animals resistant to Marek's disease (Challagulla *et al.*, 2021). The integrated gRNAs generated deletions (Figure 1) within ICP4, and thereby the virus was not able to replicate anymore. Generation of deletions was also suggested for targeting HIV-1 to prevent the virus from creating infectious escape mutants (G. Wang *et al.*, 2016). In addition, several genomic regions can be targeted simultaneously (G. Wang *et al.*, 2016). When targeting multiple genes of a virus, DSBs in essential viral genes should be introduced. Indels through DSBs in non-essential genes may also lead to escape mutants, since indel formation will prevent recognition of the target sequence by the gRNA and viral replication will not be inhibited (G. Wang *et al.*, 2016; Z. Wang *et al.*, 2016). Essential

viral genes for ASFV have been identified and may be subject for targeting. B646L encoding p72 the capsid protein of ASFV or G1211R the polymerase of ASFV may be potential targets, though previously DSBs in the open reading frame did not lead to a significant reduction of viral replication (Hübner *et al.*, 2018). However, optimising targeting strategies by targeting several ASFV genes in combination with inducing deletions might efficiently inhibit viral replication.

5.5.2 Targeted integration of transgenes

Genome editors can insert transgenes at desired loci. The porcine Rosa26 locus has been identified as so-called 'safe harbour' for integration and expression of transgenes (X. Li *et al.*, 2014). The locus expresses a non-coding RNA which can be found in several tissues such as spleen, liver, brain, and tongue. Several studies have targeted the Rosa26 locus to integrate transgenes and found stable integration and expression of transgenes (Kong *et al.*, 2014; X. Li *et al.*, 2014; Xie *et al.*, 2017; Rieblinger *et al.*, 2021). By integrating the Cas9 and gRNA expression cassettes into a well characterised locus, the transgenes should be stably expressed in most tissues. In combination with multiple gRNAs inducing deletions in the ASFV genome, resistance to ASFV may be achieved. Therefore, a vector was designed to integrate Cas9 and a neomycin selection marker into the Rosa26 locus, and cells which were positive for Cas9 integration were used for SCNT, but unfortunately no pregnancies could be established. In case pigs will be generated with a targeted Cas9 integration, a long-range PCR covering the genomic and integration site needs to be established. Thereby, correct integration of the template will be confirmed. The selection marker was flanked with Lox binding sites, so the marker could be excised, and a multiple gRNA array could be inserted. The interchangeable gRNA array gives the opportunity to experiment with different gRNAs also targeting other viruses e.g., Pseudorabies virus, another DNA virus infecting pigs.

Illegitimate integration of donor templates or vectors are of great concern when applying genome editors in livestock or biomedical therapeutics. Upon successful generation of polled cattle with TALEN (Carlson *et al.*, 2016), it was later reported that the template and the vector backbone were integrated at the target site (Norris *et al.*, 2020). Up to now, there are few reports on integration errors during targeted transgenesis that obviously can be easily be overlooked (Norris *et al.*, 2020). Inducing DSBs and providing any kind of template DNA increases the probability of unintended integration, as already shown for ZFNs, but also for CRISPR-Cas9 (Olsen *et al.*, 2010; Radecke *et al.*, 2010; Gutierrez-Triana *et al.*, 2018; Skryabin *et al.*, 2020). It highlights the necessity of careful genotyping in gene edited livestock. The generated cells with a targeted Cas9 integration have not yet been evaluated for any illegitimate integrations.

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7 ANNEX

7.1 LIST OF FIGURES

7.2 LIST OF TABLES

7.3 OFF-TARGET ALIGNMENTS

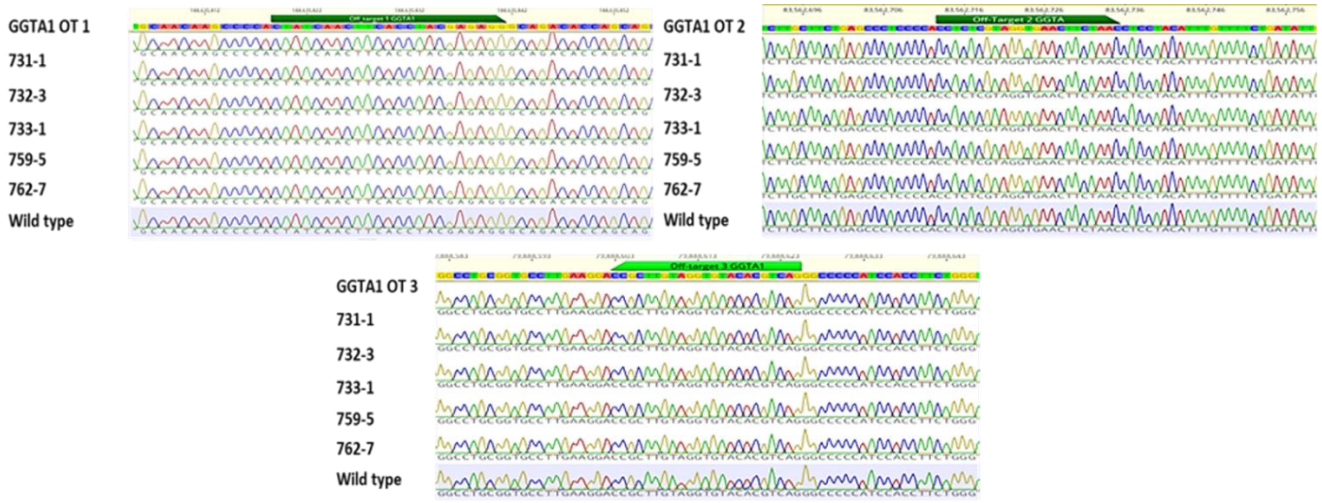

Figure 1: GGTA1 gRNA off-target alignments.

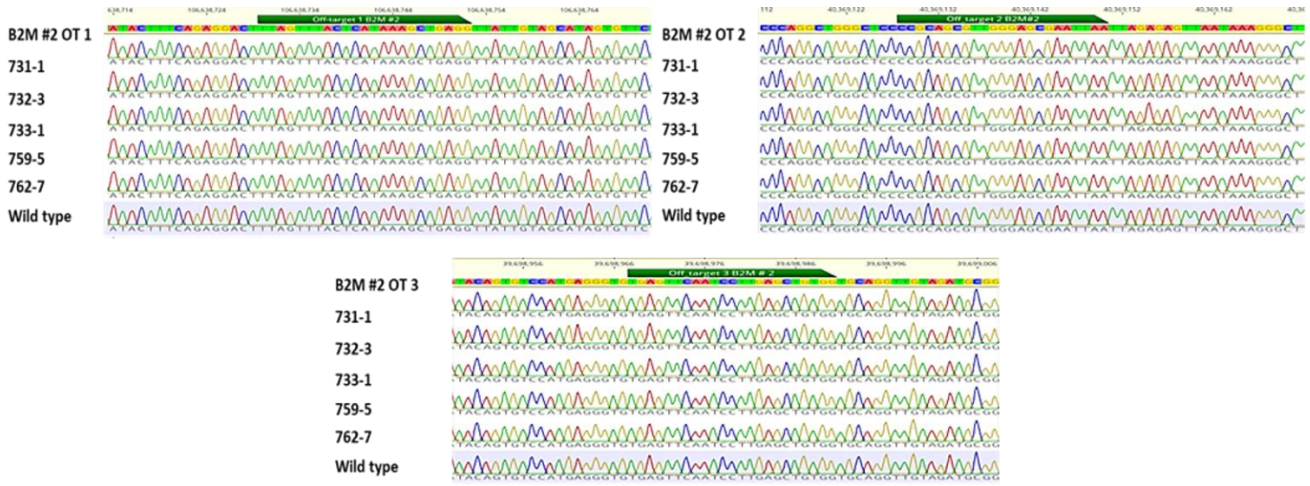

Figure 2: B2M #2 gRNA off-target alignment.

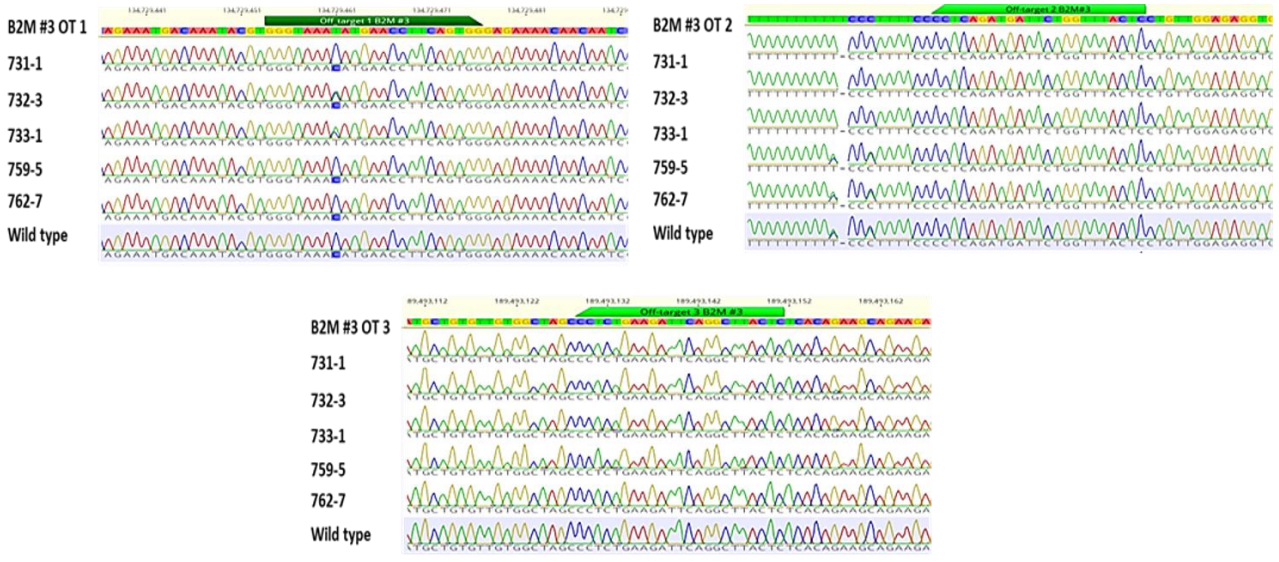

Figure 3: B2M #3 gRNA off-target alignment.

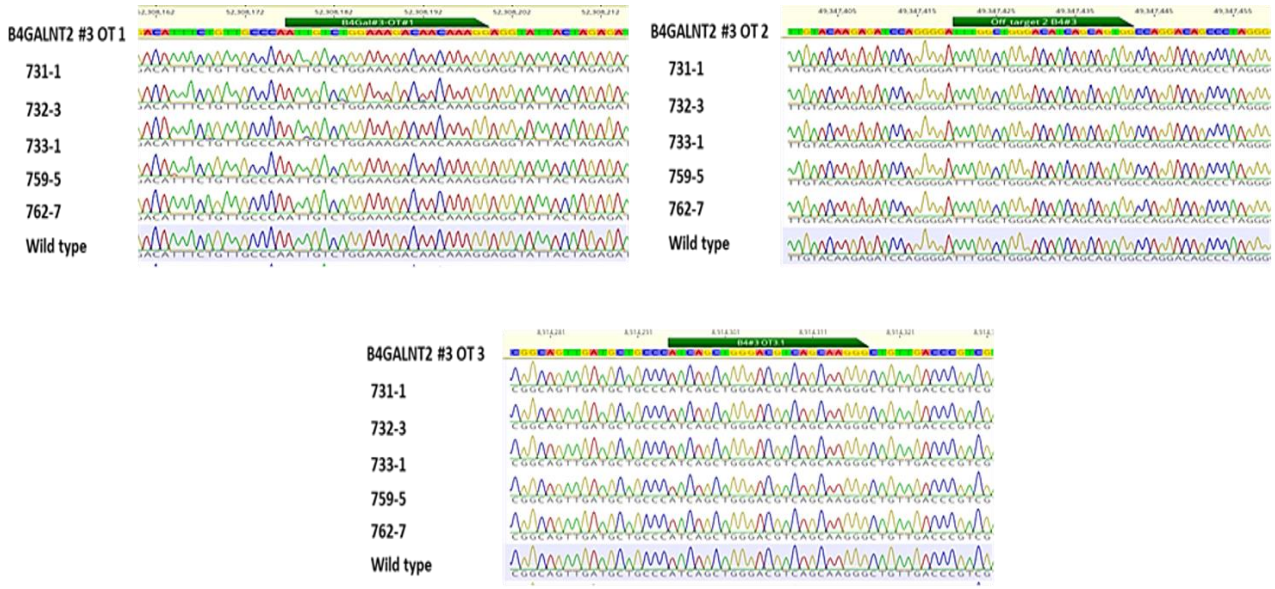

Figure 4: B4GALNT2 #3 off-target alignment.

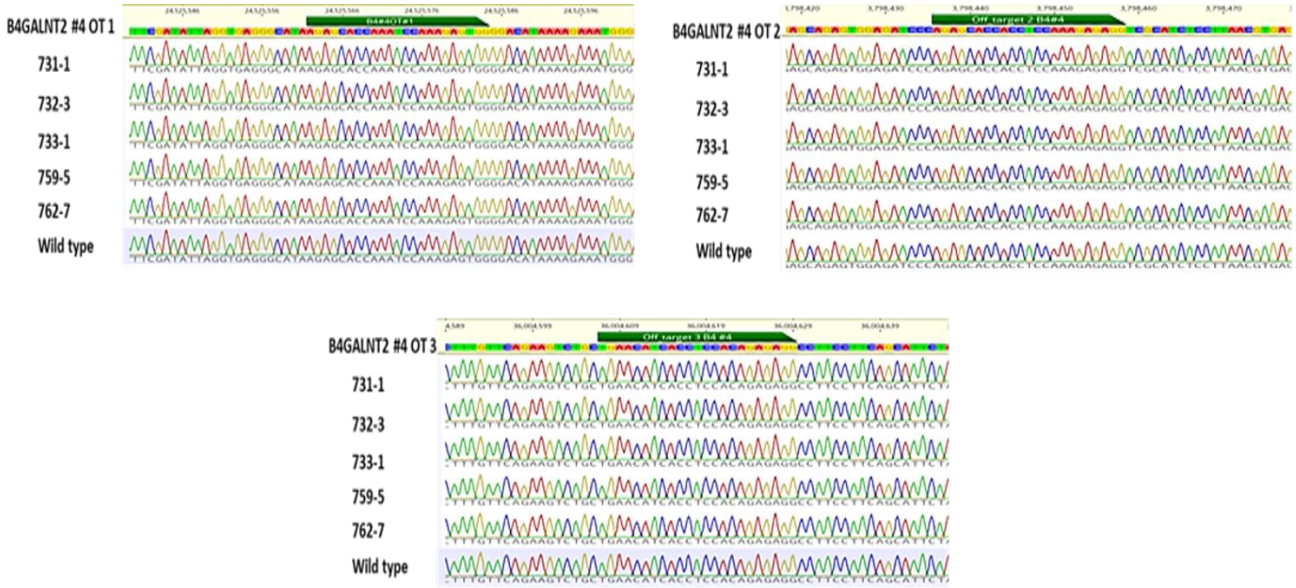

Figure 5: B4GALNT2 #4 off-target alignment.

7.4 OOCYTES AND ZYGOTES

Table 1: Ovarian wash buffer.

Substance	Concentration	Manufacturer
NaCl	0.9 %	Roth
Penicillin G	0.06 g/l	Sigma-Aldrich
Streptomycin	0.131 g/l	Sigma-Aldrich

Table 2*:* PXM buffer for selection of oocytes.

Substance	Concentration	Manufacturer
NaCl	1 M	Roth
KCL	0.1 M	Roth
KH_2PO_4	3.5 mM	Sigma-Aldrich
$MgSO_4$	4 mM	Sigma-Aldrich
$NaHCO_3$	0.25 M	Roth
HEPES	1.25 M	Roth
Na-Pyruvate	2 mM	AppliChem
Ca-Lactate	40 mM	Roth
Gentamycin	10 mg	Sigma-Aldrich
BSA	1 g	Sigma-Aldrich
Total	1l	

Table *3:* FLI maturation media Yuan et al., (2017).

Substance	Concentration	Manufacturer
TCM 199		Sigma-Aldrich
Gentamycin-Sulfate	0.05 g/l	Sigma
Na-Pyruvate	22 mg/l	AppliChem
$NaHCO_3$	22 g/l	Sigma
BSA	1 g/l	Sigma-Aldrich
EGF	10 ng/ml	Sigma-Aldrich
LIF	2,000 U/ml	ESGRO Mouse LIF
L-Cysteine	0.57 mM	Sigma-Aldrich
IGF-1	20 ng/ml	R & D Systems
FGF	40 ng/ml	Peprotech
Ovogest (hCG)	10 I.U./ml	MSD
Pregmagon (PMSG)	10 I.U./ml	IDT Biologika

Table 4: TL-HEPES Ca^{2+} stock solution.

Substance	Concentration	Manufacturer
NaCl	114 mM	Roth
KCL	3.2 mM	Roth
$CaCl_2$ x $2H_20$	2 mM	AppliChem
NaH_2PO_4 x H_20	0.4 mM	Merck
$MgCl_2$ x H_20	0.5 mM	Roth
$NaHCO_3$	2 mM	Roth
HEPES	10 mM	Roth
Na-Lactate (60%)	10 mM	Sigma-Aldrich
Penicillin G	100 U/l	Sigma-Aldrich
Streptomycin	50 mg/l	Sigma-Aldrich
Total	1l	

Table 5: TL-HEPES 296 + Ca^{2+}**.**

Substance	Concentration	Manufacturer
Na-Pyruvate	0.25 mM	Sigma
Sucrose	32 mM	Merck
BSA	0.4 %	Sigma
TL-HEPES Ca^{2+} Stock	Add 50 ml	

Table 6: Ca-free TL-HEPES stock solution.

Substance	Concentration	Manufacturer
NaCl	114 mM	Roth
KCL	3.2 mM	Roth
NaH_2PO_4 x H_20	0.4 mM	Merck
$MgCl_2$ x H_20	0.5 mM	Roth
HEPES	10 mM	Roth
Na-Lactate (60%)	10 mM	Sigma
Penicillin G	100 U/l	Sigma
Streptomycin	50 mg/l	Sigma

Table 7: TL-HEPES 296**.**

Substance	Concentration	Manufacturer
Na-Pyruvate	0.25 mM	Sigma
Sucrose	32 mM	Merck
BSA	0.4 %	Sigma-Aldrich
TL-HEPES Stock	Add 25 ml	

Table 8: Ca^{2+} -free Sor 2 (fusion media).

Substance	Concentration	Manufacturer
Sorbitol	0.25 mM	Sigma-Aldrich
Mg-Acetate	0.5 mM	Sigma-Aldrich
BSA	0.1 %	Sigma-Aldrich

Table 9: Ca^{2+} Sor 2 (electrical activation).

Substance	Concentration	Manufacturer
Sorbitol	0.25 mM	Sigma-Aldrich
Ca-Acetate	0.1 mM	Sigma-Aldrich
Mg-Acetate	0.5 mM	Sigma-Aldrich
BSA	0.1 %	Sigma-Aldrich

Table 10: 6-DMAP (chemical activation).

Substance	Concentration	Manufacturer
6-Di-methylaminopurine	2 mM/1.5 µl	Sigma-Aldrich

Table 11: Talp stock solution.

Substance	Concentration	Manufacturer
PVA (polyvinyl-alcohol)	1.0000 g/l	Sigma
Gentamycin	0.0500 g/l	Sigma-Aldrich
Phenol red	0.0010 g/l	Sigma
NaCl	6.6600 g/l	Sigma-Aldrich
KCl	0.2400 g/l	Sigma-Aldrich
$MgCl_2$ x 6 H_2O	0.1000 g/l	Roth
Na-Lactate	2.4000 g/l	Sigma
NaH_2PO_4 x H_2O	0.0480 g/l	Merck
Glucose	0.9000 g/l	Roth
$NaHCO_3$	2.1000 g/l	Sigma-Aldrich
Caffeine	0.3880 g/l	Riedel-de-Haen
Ca-Lactate x H_2O	2.4660 g/l	Roth

Table 12: Fert-Talp Media (IVF).

Substance	Concentration	Manufacturer
BSA	0.60 g	Sigma-Aldrich
Na-Pyruvate	200 µl (0.12 g/ml)	Sigma
Talp-Stock	Add 200 ml	

Table 13*:* PZM-3 culture media.

Substance	Concentration	Manufacturer
NaCl	108 mM	Sigma-Aldrich
KCl	10 mM	Sigma-Aldrich
KH_2PO_4	0.35 mM	Sigma-Aldrich
$MgSO_4$ x 7 H_2O	0.4 mM	Sigma-Aldrich
$NaHCO_3$	25.07 mM	Sigma-Aldrich
Na-Pyruvate	0.2 mM	Sigma
Ca-Lactate x H_2O	2 mM	Roth
L-Glutamine	1 mM	AppliChem
Hypo taurine	5 mM	Sigma-Aldrich
BME	20 ml/l	Sigma-Aldrich
MEM	10 ml/l	Sigma-Aldrich
Gentamicin-sulfate	0.05 mg/ml	Sigma-Aldrich
BSA	3 mg/ml	Sigma-Aldrich

7.5 CELL CULTURE

Table 14*:* DMEM Stock.

Substance	Concentration in 500 ml	Manufacturer
DMEM		Capricorn Scientific
L-Glutamine	0.2 mM	AppliChem
B-Mercaptoethanol	0.1 mM	Sigma

Table 15: Cell culture media.

Substance	D10 in 50 ml	D20 in 50 ml	T3 in 50 ml	Serum reduced media in 10 ml	Manufacturer
DMEM-Stock	43.5 ml	38.5 ml	33.5 ml	9.65 ml	
FCS	5 ml	10 ml	15 ml	50 µl	Capricorn Scientific
Pen/Strep (100x)	500 µl	500 µl	500 µl	100 µl	Sigma-Aldrich
Non-essential amino acids	500 µl	500 µl	500 µl	100 µl	Sigma-Aldrich
Sodium pyruvate (100x)	500 µl	500 µl	500 µl	100 µl	Sigma-Aldrich

Table 16: Cryopreservation media.

Substance	Concentration	Manufacturer
T3 media		
Dimethyl sulfoxide	10 %	Sigma

7.6 Lysis

Table 17: Cell lysis buffer.

Substance	Concentration	Manufacturer
10 % SDS	0.02 %	Roth
Proteinase K (20mg/ml)	50 µg/ml	Thermo Fisher Scientific
Tris HCL (1M, pH 8.4)	20 mM	Roth

Table 18: Tail lysis buffer.

Substance	Concentration	Manufacturer
SDS	1 %	Roth
NaCl	100 mM	Roth
EDTA	100 mM	AppliChem
Tris HCL (pH 8.0a)	100 mM	Roth

7.7 Media and buffers

Table 19: LB Media.

Substance	500 ml	Manufacturer
Bacto tryptone	5 g	Roth
NaCl	5 g	Roth
Yeast extract	2.5 g	Roth

Table 20: LB Agar.

Substance	200 ml	Manufacturer
LB Media	200 ml	
AgarAgar	5 g	Bioscience
Antibiotic	100 µg/ml	

Table 21: Annealing buffer.

Substance	200 ml	Manufacturer
Tris (7.5)	10 mM	Roth
EDTA	1 mM	AppliChem
NaCl	50 mM	Roth

7.8 PRIMER AND PCR PROTOCOLS

Table 22*:* Genotyping transgenic animals.

Target	Primer (5'-3')	Annealing °C	Cycles	Product (bp)
Cas9	CTAGAAATCCCAGAGGTTAC	62	30	500
	TCCTTGTCCTGGAGGATTCC			
ASF gRNA	ATGCTTACCGTAACTTGAAAG	58	30	250
	ATTTGTCTGCAGAATTGGCG			
Neomycin	CAGGATGATCTGGACGAAGA	59	35	300
	GATGCGCTGCGAATCGGGAG			
qPCR Cas9	CCCAAGAGGAACAGCGATAAG	60	45	106
	CTATTCTGTGCTGGTGGTGG			

Table 23*:* Detecting genome edits.

Target	Primer (5'-3')	Annealing °C	Cycles	Product
B2M	TGTGGGCAAGTCACTACGTC	62	32	763
	ATGCTCAGATTCGGTTGGCA			
B4GALNT2NT2	ACTCTGCATGCCAAGAGTTAAGA	62	35	419
	CCTGGAGACTTTGAGAGCCG			
GGTA1	CTAGAAATCCCAGAGGTTAC	59	35	553
	TCCTTGTCCTGGAGGATTCC			

Table 24*:* Targeted Cas9 integration.

Target	Primer (5'-3')	Annealing °C	Cycles	Product (bp)
Rosa26	CGAGCTGCAATCCTGAGGGA	61	32	699
	TTCATGACTTGCTGGCTACCT			
Left integration site	TCCAATCGCAGTGGTAGTCA	62	35	495
	GGTTCACCCGTCAAATGGCA			
Right integration site	CCGTCCCATGCACGTCTTTA	62	35	834
	TGGACTAAGAACCCGCAACA			

Table 25*:* Off-targets GGTA1.

Target	Primer (5'-3')	Annealing °C	Cycles	Product (bp)
Chromosome 13 (188,635,819-188,635,841)	CCTAGGCACACAACCTCCAC AGGTTGCTTACTTCCAGTTCACTT	62	35	311
Chromosome 3 (83,562,713-83,562,735)	ACCAGAAGGAGGGGAGACTG GATGGGCCAGAGCTGAAAGTG	62	35	452
Chromosome 16 (79,888,603-79,888,625)	AGATTCAGCCACAGAAGCCC CAGGATGAGCTCCACGTCTG	62	35	194

Table 26*:* Off-targets B2M.

Target	Primer (5'-3')	Annealing °C	Cycles	Product (bp)
chromosome 13 (106,638,730-106,638,752)	CTCAGCTTGGGAGCAAAACAC ACAACACAGGAAGTACAGCCAA	62	35	977
chromosome 12 (40,369,128-40,369,150)	GCCAGGGTGTTGCTTTAGGT TTCCGCTGCAAACACAAACA	62	35	858
chromosome 7 (39,698,969-39,698,990)	TAAGGCCACGGAAGTGTGAG GCCACAGCCCGTCAAATACA	62	35	965
chromosome 9 (134,729,454-134,729,476)	TCATTGTTGTGGGTCCGTTT AGGAATTTTGCAGGTGGTTTG	60	35	1080
chromosome 5 (59,310,453-59,310,475)	GGAATCGTGCTGAAACGTGG TGGGAACCAGCAAGGAAAGG	62	32	356
chromosome 1 (189,493,129-189,493,151)	TGCGGTTCAGATCCCTTGTT CCGGATCCTTAACCTGCTTCA	62	35	707

Table 27*:* Off-targets B4GALNT2.

Target	Primer (5'-3')	Annealing °C	Cycles	Product (bp)
chromosome 7 (49,347,420-49,347,442)	TGCCTTCTGGCTTTGTGGTA ATGGGAGAAGAGATGGAGGACA	59	32	1354
chromosome 9 (8,914,295-8,914,317)	CTAAGATCCCGGTGTTCGGG GGTCAAGCTCTGGGAACTGG	62	35	930
chromosome 11 (52,308,178-52,308,199)	GGGGGACATGTTTTCAGGTT GCCCTCAGTGTCAATGGTGATA	59	32	995
chromosome 2 (3,798,436-3,798,458)	GATGGTCTCTGCCTAAGCTCC GCAATGAAGCTCGGTTCCAG	62	35	1089
chromosome 9 (36,004,607-36,004,629)	CCTGGTTGTAGCACCCAATGA CCCCTGGATAGCACATCCTTC	62	35	671
chromosome 17 (24,525,562-24,525,584)	CCCAACGTGATCTGACTCCT TCCCCCACACAAGGAATTTGT	62	32	381

Table 28*:* Duplex ASF qPCR.

Target	Primer (5'-3')	Probe	Annealing °C	Cycles
ASF-P72	TGCTCATGGTATCAATCTTATCG	FAM-TTCCATCAAAGTTCTGCAGCTCTT-BHQ-1	60	45
	CCACTGGGTTGGTATTCCTC			
ACTB-1135	AGCGCAAGTACTCCGTGTG	HEX- TCGCTGTCCACCTTCCAGCAGATGT -BHQ-1		
	CGGACTCATCGTACTCCTGCTT			

7.9 SEMEN QUALITY OF TRANSGENIC BOARS TO GENERATE OFFSPRING

Table 29: Results of computer-assisted sperm analysis (CASA)

		Fresh Sperm		Frozen Sperm	
Batch	**Boar**	**Mean motility (%)**	**Mean progressive motility (%)**	**Mean motility (%)**	**Mean progressive motility (%)**
1	765-6	74.6	68.6	NA	NA
2		73	57.7	6.8	5.1
1	762-7	52.9	16.1	5.4	3.6
2		74.9	34.8	49.7	39.5

Figure 7: Sperm abnormalities: Head deformation (blue arrow) in the sperm samples from boar 765-6.

7.10 MAGNETIC BEAD COUNTER-SELECTION OF GGTA1 KNOCK-OUT CELLS

Foetal Cas9 expressing fibroblasts transfected with a GGTA1 gRNA were counter-selected for α-galactose expression (Fujimura *et al.*, 2008). For selection, 10^6 cells were incubated on ice for 15 minutes with 50 µl biotin-conjugated isolectin-B4 (Enzo Life Science) in TL-Hepes 296 + $Ca2^{=}$. Afterwards, the cells were washed twice with PBS. Dynabeads™ (Invitrogen) were washed three times with PBS. Beads and cells were resuspended and placed on magnet for one minute. Supernatant containing GGTA knock-out cells, was transferred and again placed on the magnet. Selected cells were then transferred into T3 media.

AFFIDAVIT

I herewith affirm that I am the sole author of the thesis titled 'An experimental approach of an *in vivo* genome pathogen targeting strategy to generate African Swine Fever resistant pigs', which was written according to the principles of good scientific practice. The following persons provided assistance:

Walter Fuchs (Institute of Molecular Virology and Cell Biology): Vector cloning for ASFV specific gRNAs and supported design of *in vivo* infection study

Sandra Blome (Institute for Diagnostic Virology): Guided the design and execution of *in vivo* and *in vitro* infection studies

Katrin Pannhorst (Institute of Molecular Virology and Cell Biology): Equal contribution to *in vivo* infection studies

Lutz Wiehlmann and Colin Davenport (Research Core Unit Genomics, Medizinische Hochschule Hannover): MiSeq and raw data generation

I did not make use of any paid dissertation services or other consultants. Nor did anyone receive unpaid services from me for work related to the contents of the submitted thesis.

The thesis was completed at the following institution: Institute for Farm Animal Genetics, Friedrich-Loeffler-Institut.

This thesis has not been previously submitted for evaluation for admission to an examination, doctoral graduation, or any such purpose.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

_____

[07.02.2022], signature

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