Efficiency and safety of gene editing in pigs: Evaluated in an experimental and in a disease relevant model

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der Tierärztlichen Fakultät der Ludwig-Maximilians-
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Efficiency and safety of gene editing in pigs: Evaluated in an experimental and in a disease relevant model

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Für meine Familie

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Abbreviations

Ala	Alanine
Ampho B	Amphotericin B
Bp	Base pair
BSA	Bovine serum albumin
BW	Body weight
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPR/CAS 9	Clustered regularly interspaced short palindromic repeats / Cas9
crRNA	CRISPR-RNA
D-PBS	Dulbecco's phosphate buffered saline
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DE	Deutsches Edelschwein
Digenome-Seq	Digested Genome Sequencing
DL	German Landrace
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ET	Embryo transfer
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FLI	FGF2 LIF IGF
GE	Gene editing
GGTA1	α1,3-galactosyltransferase
Gln	Glutamine
GOTI	Genome-wide off-target analysis by two-cell embryo injection
GSH	Glutathione

GUIDE-Seq	Genome-Wide, Unbiased Identification of DSBs enabled by Sequencing
HDR	Homology-directed repair
HR	Homologous recombination
IGF	Insulin-like growth factor
Indel	Insertion or deletion
IPTG	Isopropyl-β-D-thiogalactopyranoside
IVC	*In vitro* culture
IVF	In vitro fertilisation
IVP	*In vitro* production
LIF	Leukaemia inhibitory factor
MACS	Magnetic-activated cell sorting
mRNA	Messenger ribonucleic acid
NEAA	Non-essential amino acid
NHEJ	Non-homologous end joining
NLS	Nuclear localisation signal
OD	Oedema Disease
PAM	Protospacer adjacent motif
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/Streptomycin
PFF	Porcine foetal fibroblast
PFM	Porcine fertilisation medium
PI	Piétrain
PKF	Porcine kidney fibroblasts
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
PSS	Porcine Stress Syndrome
PZM5	Porcine zygote medium 5
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT	Room temperature
SDS	Sodium dodecyl sulphate
sgRNA	Single guide RNA
ssODN	Single stranded oligonucleotide
T7E1	T7 endonuclease 1
TAE	Tris-acetate-EDTA-buffer

TALEN	Transcription activator like effector nuclease
TBE	Tris-borate-EDTA-buffer
TCM 199	Tissue culture medium 199
TIDE	Tracking of indels by decomposition
tracrRNA	Trans-activating CRISPR RNA
VIVO	Verification of *in vivo* off-targets
WGS	Whole genome sequencing
ZFN	Zinc finger nuclease
αGal	Galactose-α1,3-galactose

1. INTRODUCTION

Pork is the most widely consumed type of meat worldwide. In 2019 the average German per capita pork consumption was 36kg which represents 60% of total meat consumption [1]. Large scale animal farming and climate change put increasing demands on animal health which is highly influenced by genetic factors [2, 3]. Gene editing (GE) could complement traditional livestock breeding to improve animal welfare. Due to similarities in size and physiology to humans, the pig has gained significance as a model organism in biomedicine besides its important role in agriculture [4, 5]. These traits make the pig a suitable model organism to investigate the risks and side effects of GE and generate significant data for potential agricultural and therapeutic applications.

The goal of this work was to evaluate the feasibility, efficiency, and safety of GE in pigs. Oedema disease (OD) was chosen as the target for GE due to its global significance regarding animal welfare and economics. OD is caused by E. coli F18 bacteria that bind to the intestinal mucosa and produce Shiga toxin 2e (Stx2e) which causes oedema and central nervous system dysfunction [6]. The respective receptor is synthesised by the enzyme α-1,2 fucosyltransferase encoded by the FUT1 gene [7, 8]. One gene variant leads to an amino acid exchange (p.T103A). The FUT1-AA genotype leads to a loss of fucosyl structures on the receptor which prevents E. Coli F18 binding and thereby mediates OD resistance [9]. Gene editing could be used to increase the incidence of the favourable FUT1 variant within the breeding population.

The second part of this work focuses on investigating the frequency of off-target mutations caused by CRISPR/Cas9, the most widely used GE tool. Concerns regarding the safety of this technology, particularly the potential for inducing off-target mutations prevent its more widespread application [10]. Off-target analysis is typically performed by screening *in silico* predicted off-target sites or by whole genome sequencing [11]. The fundamental problem with these approaches is that single nucleotide variants (SNVs) that occur during embryogenesis cannot be distinguished from mutations caused by GE technology [12].

The aim of this study was to analyse the frequency at which the CRISPR/Cas9 system generates off-target mutations *in vivo* using a novel approach termed genome-wide off-target analysis by two-cell embryo injection (GOTI). This method is based on the generation of mosaic foetuses containing edited and non-edited cells by microinjection of CRISPR/Cas9 expression vectors into one blastomere of two-cell stage embryos. After separation of both cell types, they can be

sequenced to determine the mutation frequency and off-target sites. The non-edited cells are the best possible control group because both cell populations stem from the same embryo [12].

The GGTA1 gene which encodes the enzyme α-1,3-galactosyltransferase was chosen as the target for gene editing because its inactivation leads to a loss of the α-1,3-Gal glycosylation [13]. The presence or absence of this cell surface epitope facilitates the precise separation of edited and non-edited cell populations [14]. For the first time, this proof-of-principle experiment provides comprehensive insights regarding off-target events in livestock species irrespective of natural mutations.

2. REVIEW OF THE LITERATURE

2.1. Oedema Disease

2.1.1. Clinical Signs and Lesions

Oedema Disease is an enterotoxaemia that mainly occurs in healthy, rapidly growing pigs seven to ten days after weaning [15]. OD epidemics characteristically begin and end suddenly on average lasting less than eight days [16]. Morbidity rates of 30-40% are coupled with high mortality rates up to 90% [17]. Enzootic persistence frequently leads to the recurrence of the disease within affected populations [18].

The main clinical signs of OD are oedema and dysfunction of the central nervous system (CNS) which reflect vascular damage. Acute cases are characterised by constipation and subcutaneous oedema typically in the eyelids and abdomen (see Figure 1) [19]. The progressive loss of CNS function leads to staggering gait followed by complete paralysis, lateral recumbency, dyspnoea, and generalised muscle tremors [20]. Death usually occurs one to three days after onset of symptoms and within 24 hours if neurological symptoms arise [21]. Subacute and chronic cases of OD result in decreased weight gain and therefore lower slaughter weight [22]. Peracute OD is characterised by sudden death without any preceding signs of illness [21]. Other possible pathological findings include haemorrhagic necrosis of intestinal mucosa, hydropericardium, pulmonary oedema, and altered vocal expression due to laryngeal oedema [23].

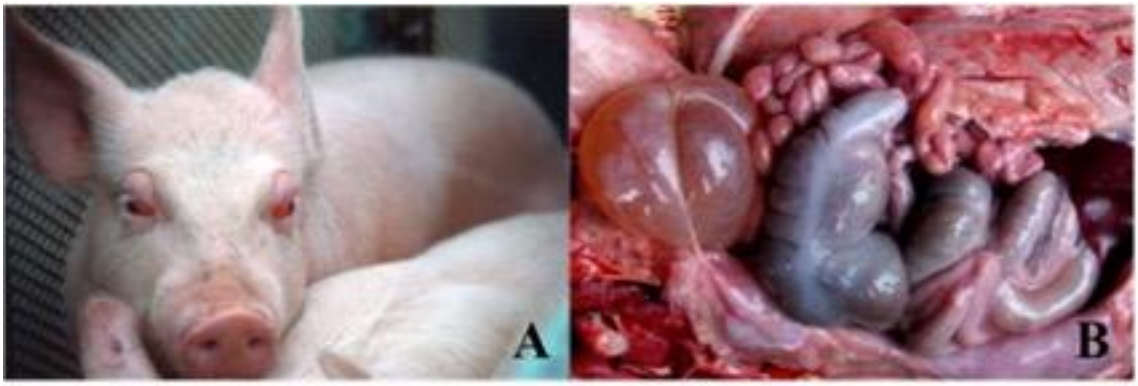

Figure 1: Typical clinical signs of OD. **A)** Periocular oedema and **B)** Oedema in the submucosa of the stomach and mesocolon (from Swine Disease Manual 4th Edition Ed. Neumann [24]).

2. REVIEW OF THE LITERATURE

Microscopically, degenerative angiopathy of small arteries and arterioles can be observed [20]. Fibrinoid necrosis of the smooth muscle cells in the tunica media is accompanied by perivascular haemorrhage [25]. This severe vascular damage causes microthrombus formation, infarction, and perivascular oedema resulting in ischaemic necrosis of the surrounding parenchyma [26]. These vascular lesions are most prominent in the brain stem, intestine, and kidneys. Focal encephalomalacia and infarction of the brain stem is characteristic and the main cause of death among affected pigs [20].

2.1.2. Aetiology and Pathogenesis

OD is caused by pathogenic serogroups of *Escherichia coli* (*E. Coli*) that produce Shiga toxin 2e (Stx2e) [6]. Stx2e is absorbed into the bloodstream from the intestine and binds to the vascular endothelium, inhibits protein synthesis, and causes cell death which leads to the characteristic vascular lesions (see Figure 2) [27]. The causal relationship between Stx2e and OD has been confirmed through intravenous injection of Stx2e which exactly replicates the microscopic lesions and symptoms of OD [28]. Some serotypes of *E. Coli* also produce enterotoxins which cause severe diarrhoea in addition to the usual symptoms of OD [29, 30]. Adhesion to the intestinal mucosa is mediated by F18ab fimbriae that bind to the *E. Coli* F18 receptor (ECF18R) located on the membrane of enterocytes [31]. During the acute phase of infection up to 10^9 colony forming units (CFU) of *E. Coli* per cm^2 can be found in the small intestine of pigs [32].

The initial source of infection is usually the sow or contaminated environment [26]. The decay of maternal antibodies and stress associated with the sudden change in diet and mixing of the pigs are responsible for the high susceptibility of pigs in the post-weaning period [33]. A highly nutritious and energy-rich diet is another predisposing factor for OD [6]. This explains the high incidence of OD especially among the fastest-growing pigs in intensive large-scale animal farming facilities [34].

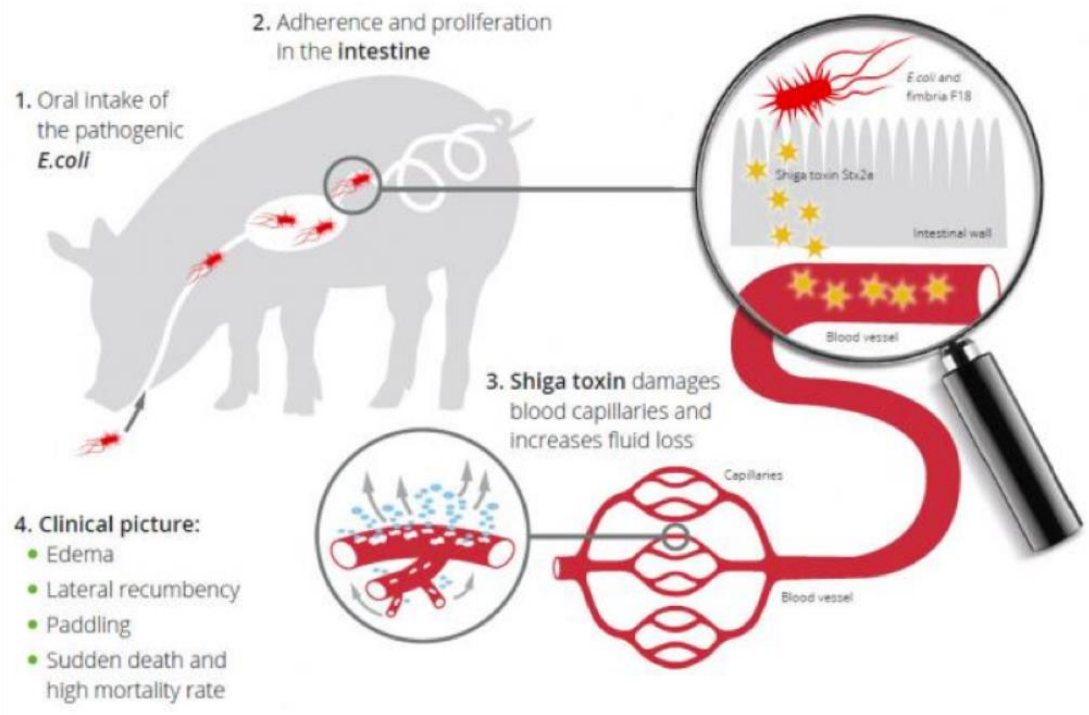

Figure 2: Pathogenesis of OD. Pathogenic E. Coli are orally ingested, proliferate in the intestine, and produce Stx2e Shiga toxin which damages the capillaries and causes the typical symptoms of OD (from "Disease of Swine" 11th edition [35]).

A single (G>A) nucleotide polymorphism (SNP) at base pair (bp) 307 of the α-1,2-fucosyltransferase (FUT1) gene located on the porcine Chromosome 6 controls F18ab mediated binding of *E. Coli* [7, 8]. Resistance and susceptibility to OD are thus determined by a single recessively inherited gene variant at a particular genomic locus [9]. The prevalence of the resistant FUT1-AA genotype within the pig population is breed specific and ranges from 6% in German Landrace (GL), 25% in Piétrain (PI) up to 75% in Large White (LW) [36]. OD resistant individuals in the pig population can be detected by Polymerase Chain Reaction (PCR) and then used for breeding [37]. There are concerns about selecting for OD resistance because this genotype was shown to be correlated with susceptibility to Porcine Stress Syndrome (PSS) in Swiss Landrace pigs [38]. However, this correlation could not be confirmed in other breeds [26, 39].

2.1.3. Diagnosis and Treatment

The characteristic set of neurological symptoms and its occurrence in a specific age group make typical outbreaks of OD easy to diagnose. Differential diagnoses are Pseudorabies (Aujeszky's disease), meningitis (usually *Streptococcus suis*), Glasser's Disease (*Haemophilus parasuis*), and water deprivation [40]. Isolation and characterisation of *E. Coli* is the standard procedure for a definitive diagnosis but can be difficult as the pathogen is often absent by the time of death [26]. Detection and quantification of virulence factors like F18ab fimbria and Stx2e by quantitative PCR is a sensitive and specific method to diagnose OD [31, 41].

Due to the sudden onset and rapid course of OD disease prevention is more effective than treatment [42]. Reducing the energy content of feed after weaning decreases the incidence of OD regardless of fibre and protein content. However, these dietary measures impair growth and weight gain before reaching significant clinical efficacy [34]. Pre-emptive administration of antibiotics in the post-weaning period is another effective measure to prevent OD. However, this approach has potential for the selection of strains with antimicrobial resistance and prevents active immunisation [43]. Immunisation against Stx2e and F18ab is another effective but costly control strategy. A variety of vaccines has been developed for this purpose including inactivated Stx2e toxoid, non-toxigenic F18ab *E. Coli,* and passive vaccination with F18ab antibodies [42, 44, 45].

Selective breeding for OD resistance is another control strategy that has proven to be effective in reducing the incidence of OD in breeding populations when systematically implemented [6, 7]. The low prevalence of the resistant gene variant in certain breeds makes this approach hard to realise without compromising genetic diversity and long-term productivity [7, 36]. Similarly, the incorporation of the OD resistant gene variant from other breeds and related species by trait-selective breeding is likely to result in a loss of productivity regarding other desired performance parameters [46]. Several studies have highlighted the potential of gene editing to incorporate genetic traits into productive, genetically diverse animals [39, 46, 47]. For example, GE technology has already been used to generate Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) resistant pigs [48].

2.2. Off-Target Effects in CRISPR/Cas9 mediated Gene Editing

2.2.1. The CRISPR/Cas9 System

The clustered regularly interspaced short palindromic repeat (CRISPR) / CRISPR associated nuclease (Cas) system was originally discovered as part of the adaptive immune system in archaea and bacteria [49]. CRISPRs are short, repeating sequences of viral DNA that are integrated into the bacterial genome and function as an immunological memory system in prokaryotes [50]. Upon reinfection, transcripts of these repeats guide the Cas endonuclease to the complementary sequence of the pathogen and induce DNA double strand breaks (DSBs) [51]. Three types of CRISPR systems are known in prokaryotes but only the CRISPR type II system which includes CRISPR associated nuclease 9 (Cas9) has been adapted for GE (see Figure 3) [52, 53]. The implementation of the CRISPR/Cas system for GE in mammalian cells requires only three components: Cas9, CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) [54, 55]. CrRNA and tracrRNA form an RNA complex that guides Cas9 to the target sequence [55]. For GE purposes this complex has been replaced with a synthetic single guide RNA (sgRNA) [56]. The first 20 nucleotides of the sgRNA determine the target DNA sequence. They can be adapted to recognize any target sequence with a subsequent 3' "NGG" protospacer adjacent motif (PAM) [51, 57]. Targeting of multiple genetic loci is enabled by applying several different sgRNAs simultaneously [58].

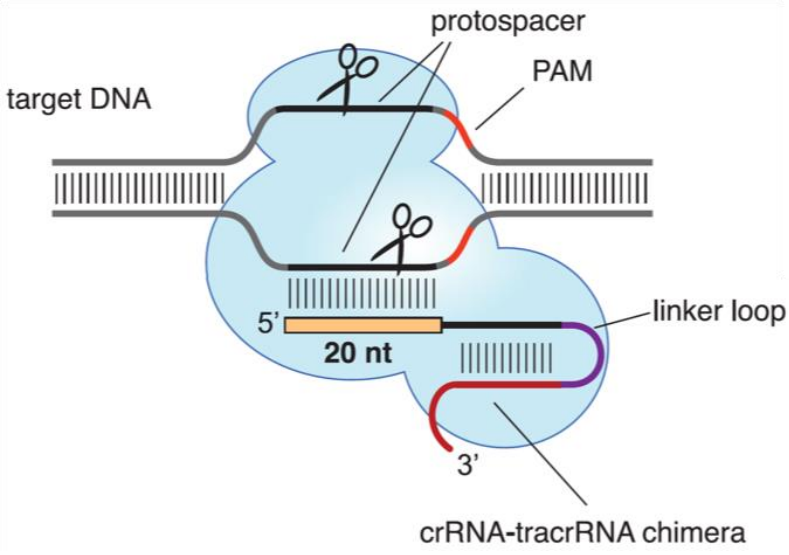

Figure 3: The CRISPR/Cas9 system as a tool for genome engineering. The 20bp sgRNA which determines the target sequence is connected to the tracrRNA with a linker loop to form a sgRNA (adapted from Jinek *et al.* [56]).

Upon recognition of the target site, Cas9 induces a double-strand break (DSB) which activates the cell's intrinsic DNA repair mechanisms, such as non-homologous end joining (NHEJ) and homology directed repair (HDR) (see Figure 4) [51, 59]. The repair of DSBs by NHEJ frequently causes insertions or deletions (indel mutations) which disrupt regulatory elements or lead to frameshift mutations in coding regions and gene inactivation [60]. In HDR DSBs are repaired consulting homologous sequences to facilitate accurate repair [61]. By providing DNA donor templates flanked with sequences homologous to the target site HDR can be utilised for the targeted integration of exogeneous DNA [62, 63].

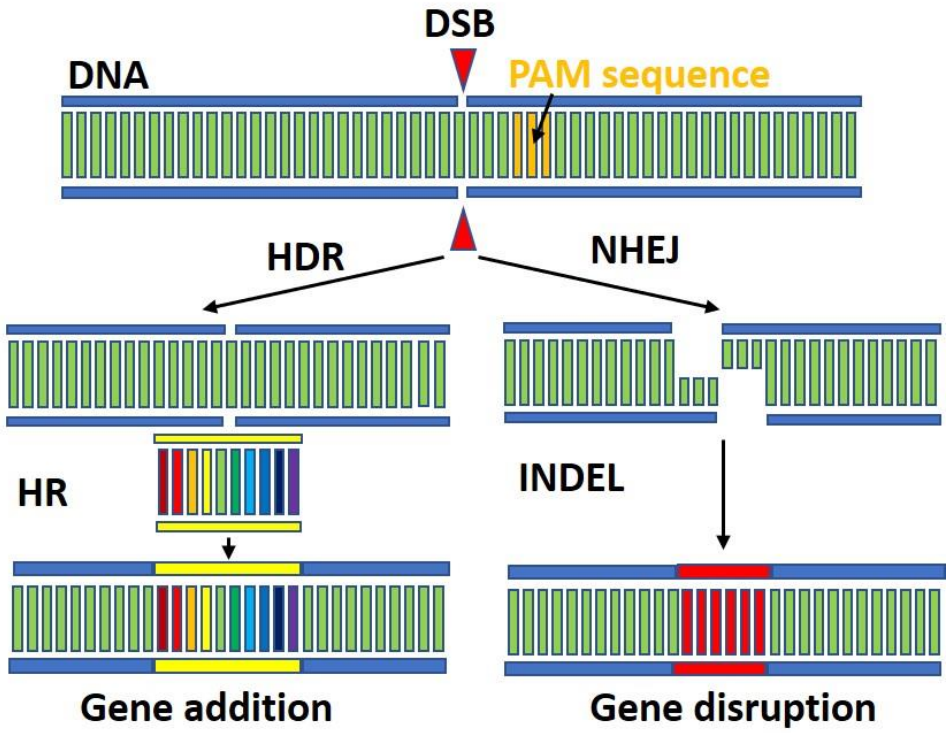

Figure 4: Repair of DSBs through HDR or NHEJ. NHEJ frequently leads to indel mutations causing frameshift and thus the inactivation of the target gene. By providing DNA donor templates with homologous arms to the target site HDR facilitates the targeted integration of exogeneous DNA.

2.2.2. Off-Target Mutations

Its high efficiency, cost-effectiveness, accuracy, and ease of use have made CRISPR/Cas the most widely used GE technology worldwide [64, 65]. The CRISPR/Cas system has been used to successfully generate GE plants, livestock, and humans [66-68]. However, the potential for the introduction of off-target mutations that could result in adverse phenotypic consequences limits the use of GE technology [69].

The specificity of the CRISPR/Cas9 system is tightly determined by the 20 nucleotides long sgRNA sequence followed by the presence of the PAM site in the genome [70]. The sgRNA can be subdivided into the 5' non-seed sequence and the 10 to 12 bp long 3' PAM proximal seed sequence [71]. Recent findings suggest that the seed region comprises only three to five bp adjacent to the PAM motif [72]. The seed sequence is critical in determining the specificity of the gRNA as correct sequence alignment next to the PAM is essential for DNA targeting [73, 74]. Off-target cleavage can however occur in regions with up to 5 base pair mismatches from the target site in the 5' non-seed segment of the sgRNA [75]. The frequency at which a region complementary to the respective "seed + PAM" sequence exists in the genome determines specificity [76]. U-rich seed sequences can induce the termination of transcription and can therefore result in increased specificity [72]. SgRNAs with very high or low GC content in the seed sequence lead to reduced activity of the CRISPR/Cas9 system [77]. The length of the sgRNA influences targeting efficiencies and the frequency of off-target mutations [81, 82].

Other than the usual NGG the CRISPR type II system also recognise NAG as a PAM sequence [75]. However, the design of CRISPR/Cas9 sequences using a NAG PAM reportedly reduces binding specificity by up to 80% compared to an NGG PAM [78, 79].

The way of application of the CRISPR/Cas9 system plays an important role in the generation of off-target effects. Delivery of Cas9 protein and sgRNA as ribonucleoprotein (RNP) complexes facilitate genome editing without requiring transcription and translation resulting in faster cleavage of the target sequence [80]. RNP complexes are degraded quickly after they cleave the target sequence which reduces the number of off-target mutations compared to the utilisation of plasmids encoding both components [81].

The frequency of off-target mutations is linked to the integrity of the cell's internal DNA repair pathways and therefore highly cell-type dependant [82]. The frequency of off-target mutations

is relatively high in cancer cells [83]. However, there is a scientific consensus that in cells with intact DNA repair pathways CRISPR/Cas9 technology causes very few off-target mutations [62, 72, 78, 82, 84, 85].

2.2.3. Detection of Off-Target Mutations

Comprehensive and sensitive detection and quantification of off-target mutations remains a major challenge [86]. Methods for the identification of off-target mutations can be categorised as *in silico* prediction tools, sequencing-based *in vitro* methods, and *in vivo* methods [87].

2.2.3.1. *In silico* prediction tools

The most common method for off-target analysis in the literature is the screening of *in silico* predicted off-target sites. A variety of algorithms and web-based tools such as CRISPOR (http://crispor.org), CCTop (http://crispr.cos.uni-heidelberg.de) and Cas-OFFinder (http://www.rgenome.net/cas-offinder/) have been developed for this purpose [88-90].

These tools predict potential off-target sites based on the number and location of mismatches compared to the target site. One built-in limitation of this approach is that it neglects off-target sites with less sequence similarity [91]. Furthermore, all algorithms rely on the availability of a high-quality reference genome for each specific species and genotype [92]. Overall, *in silico* tools can identify potential off-target sites but currently still fail to accurately predict *in vivo* mutations [75, 93].

Another field of application for prediction algorithms is to facilitate the design of sgRNAs that minimise potential off-target mutations [87]. Several tools such as CRISPR-P2.0 (http://cbi.hzau.edu.cn/CRISPR2), E-CRISP (http://www.e-crisp.org) and Breaking-Cas (http://bioinfogp.cnb.csic.es/tools/breakingcas) were developed specifically to aid the design of sgRNAs for GE [94-96].

2.2.3.2. *In vitro* methods

There is a wide spectrum of in *vitro methods* for the detection and quantification of off-target mutations that each have their individual benefits and drawbacks. The following segment describes the most relevant methods.

The first valid method for the detection of off-target mutations was the T7 endonuclease I (T7E1) assay [97]. This assay utilises the ability of T7E1 to recognise single bp mismatches

and cut DNA at the respective site. In case a wild-type DNA strand binds to a mutated one they form a heteroduplex at the mismatched site which can be recognised by T7E1 [98]. The resulting DNA fragments can be detected by gel electrophoresis. Drawbacks to this approach are its poor sensitivity and high cost [98, 99].

Whole genome sequencing (WGS) is an unbiased and comprehensive method for the assessment of off-target mutations [100]. Mutations are detected by WGS of cells prior to and after GE followed by comparison to the unedited control group. Precise investigation of the genetic background is critical for this approach [101]. WGS has been used for the detection of off-target mutations in a variety of plants and mammalian species [69, 84, 87]. WGS is suited for the analysis of single cells and genome-edited animals but lacks the sensitivity required for the detection of low-frequency off-target mutations in cell pools [84]. Similar to other methods, the screening of off-target effects via WGS found the frequency of mutations caused by CRISPR/Cas9 technology to be relatively low [85]. However, WGS cannot distinguish single nucleotide variants (SNVs) caused by GE from those that occur naturally or are caused by sequencing errors [12, 87].

Genome-Wide, Unbiased Identification of DSBs enabled by Sequencing (GUIDE-Seq) facilitates the genome-wide analysis of off-target mutations based on the integration of tagged DNA fragments into DSB breaks followed by their amplification and sequencing [102]. GUIDE-Seq is very sensitive and can detect off-target mutations that occur at frequencies as low as 0,12%. However, GUIDE-Seq requires the integration of additional exogeneous DNA which can affect gene editing outcomes and is inefficient in primary cells [103].

CIRCLE-Seq is another method for the genome-wide identification of off-target mutations [104]. CIRCLE-Seq is based on next generation sequencing technology and excels at the detection of cell-type-specific SNPs [105]. CIRCLE-Seq does not require a reference genome which allows the detection of off-target mutations in species where the full genomic sequence is not available [104].

Digested Genome Sequencing (Digenome-Seq) is an unbiased and cost-effective method for the detection of off-target mutations [106]. Digenome-Seq is a two-step process in which genomic DNA is isolated from modified cells and a control group followed by *in vitro* digestion and WGS. The resulting DNA fragments are aligned and compared to the reference genome to detect on- and off-target cleavage sites [106]. Digenome-Seq can detect off-target mutations that occur at frequencies as low as 0.1% [107]. Its high sensitivity and the fact that it is based

on DNA cleavage rather than the integration of exogeneous DNA make Digenome-Seq the gold standard to evaluate the specificity of GE tools at the time of writing [87, 91].

2.2.3.3. Detection of off-target mutations *in vivo*

In vitro methods are highly sensitive at detecting off-target mutations but fail to accurately predict mutations that occur *in vivo* [75, 98, 108]. The scientific literature suggests that off-target mutations occur at much lower frequencies *in vivo* compared to *in vitro* experiments. Animal experiments in mice [109, 110], pigs [66], and monkeys [111, 112] have not revealed any off-target mutations at the predicted off-target sites so far. However, in most publications off-target detection has been limited to the screening of *in silico* predicted off-target sites [91, 100]. Comprehensive, sensitive, unbiased screening of off-target mutations at the animal level is necessary to evaluate the target specificity of CRISPR/Cas9 and other GE tools in vivo [113]. The two most relevant methods for this purpose are described in this paragraph.

Verification of *in vivo* off-targets (VIVO) is a sensitive method for the genome-wide detection of off-target mutations *in vivo [114]*. VIVO utilises CIRCLE-Seq to identify potential off-target cleavage sites followed by the *in vivo* examination of target tissues for indel mutations. VIVO facilitates the detection and quantification of off-target mutations within whole organisms [87]. It has been used to confirm *in vivo* GE in mice using CRISPR/Cas technology without detectable off-target mutations [114].

Genome-wide off-target analysis by two-cell embryo injection (GOTI) is an approach for the evaluation of off-target mutations in a cell population derived from a single GE blastomere [12]. GOTI was conducted in mouse embryos by editing one blastomere of two-cell embryos and then comparing the whole genome sequence of cells derived from edited and non-edited blastomeres (see Figure 5) [12].

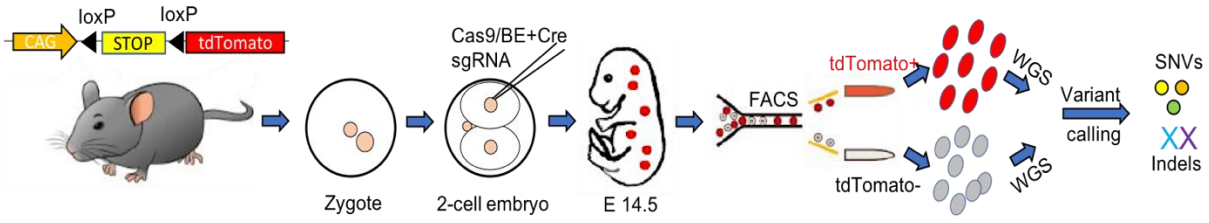

Figure 5: Experimental design of GOTI. CRISPR-Cas9 or base editors together with Cre mRNA are injected into one blastomere of two-cell embryos derived from LoxP-Stop-LoxP-tdTomato mice. Edited and non-edited cells

are separated, sorted by FACS based on tdTomato expression followed by WGS and analysis of Indels and SNVs (from Zuo *et al.* [12]).

The distinguishing feature of this method is that it facilitates the evaluation of off-target mutations caused by GE tools without the interference of SNPs present in distinct individuals [115]. GOTI revealed the frequency of off-target SNPs in embryos edited by CRISPR/Cas9 or adenine base editors to be close to the spontaneous mutation rate whereas cytosine base editors induced SNPs 20 times more frequently [12]. These findings are however limited to mouse embryos because so far, no data for non-rodent species has been published.

2.2.4. Minimising off-target effects

There are various strategies to reduce the incidence of off-target effects caused by CRISPR/Cas9 technology [106].

First, off-target mutations can be reduced by decreasing the concentration of CRISPR/Cas9 components *i.e.*, Cas9 and sgRNA but this approach also reduces on-target cleavage efficiencies [116]. Therefore, a compromise between efficient on-target cleavage and the reduction of off-target mutations is inevitable.

Shorter tissue culture time can reduce off-target mutations by lowering the duration of nuclease expression and thereby the risk for the accumulation of off-target mutations [117]. Delivering the components of the CRISPR/Cas9 system as Cas9 protein or mRNA together with the sgRNA can reduce off-target mutations compared to DNA GE vectors encoding both components [107, 118].

Optimal sgRNA design can further improve Cas9 specificity [78]. Several online tools have been developed specifically to design optimal sgRNA sequences (see 2.3.1.) [94-96]. Truncated small sgRNAs with shorter complementary regions to the target site (17-18bp) can further reduce undesired mutations [119].

CRISPR/Cas9 technology is rapidly evolving and a variety of modified Cas9 variants with high fidelity have been developed. Such optimised Cas9 variants include Base Editing [120], Nickases [121], Prime Editing [122], SpCas9-HF1 [123], eSpCas9 [124] and HypaCas9 [125].

Base editing is an approach that converts specific bases into another utilising a deaminase enzyme fused to an inactive Cas9 protein [126]. This method does not require the generation of DBS which theoretically should decrease the occurrence of off-target mutations [120]. However, base editors were shown to generate considerable off-target SNPs *in vitro* and *in vivo* [12, 127].

Nickases are another modification to the CRISPR system that causes single strand DNA breaks [121]. The so-called 'double nicking' approach uses paired Nickases guided by two sgRNAs to generate DSBs with high precision which increases specificity compared to the original version [128]. The frequency of off-target mutations can be further reduced by combining 'double nicking' with truncated sgRNAs [128].

'Prime editing' is a new approach that uses a reverse transcriptase enzyme connected to a catalytically inactive Cas9. The target site plus the intended modification are both specified by a prime editing gRNA. First reports claim high efficiencies and reduced off-target effects with this method [122].

SpCas9-HF1 is a Cas9 variant specifically designed to improve specificity [123]. SpCas9-HF1's on-target activity is comparable to SpCas9, but nonspecific DNA interactions are greatly reduced [123]. Similarly, eSpCas9 is an optimised Cas9 nuclease that was rationally designed via structure-guided protein engineering to improve specificity [124]. The HypaCas9 system offers higher genome-wide fidelity combined with precise on-target genome editing [125].

In summary, GE technology is evolving at a rapid pace and more accurate, efficient, and reliable tools are developed at a fast pace.

2. REVIEW OF THE LITERATURE

3. OBJECTIVE

Genome engineering could complement traditional breeding techniques to enhance the potential of the pig for agriculture and biomedical research. However, the safety and accuracy of this approach must be confirmed prior to its practical implementation.

The main goal of this project was to establish and evaluate methods for precise, efficient, and safe CRISPR/Cas9 mediated gene editing in pigs using the example of Oedema disease. This entails the design and optimisation of Cas9 vectors and repair templates to edit the FUT1 gene *in vitro* in somatic cells and *in vivo* in porcine embryos. The generation of genetically modified animals was not planned in this project.

The objective for the second part of this work was to precisely investigate the frequency at which CRISPR/Cas9 technology causes off-target mutations. For this purpose, mosaic foetuses were to be generated by microinjection of gene editing vectors into one blastomere of porcine two-cell-stage embryos. The next goal was to separate the edited and non-edited cells and perform whole genome sequencing to distinguish off-target mutations from single nucleotide variants that naturally occur during embryogenesis.

3. OBJECTIVE

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Apparatuses

Accu-jet pro	Brand, Dietenhofen, GER
Blue light table	Serva, Heidelberg, GER
Bunsen burner "Gasprofi 2"	WLD-TEC GmbH, Arenshausen, GER
Camera AxioCam MR (Axiovision)	Carl Zeiss Jena GmbH, Jena, GER
Centrifuge „Sigma 3-16KL "	Sigma, Osterode, GER
Dry block heater "PCH-2"	Grant instruments, Royston, GBR
Electrophoresis system	Peqlab Biotechnologie, Erlangen, GER
Electroporation cuvettes	Peqlab Biotechnologie, Erlangen, GER
Electroporator: BTX ECM 830	BTX, Holliston, MA, USA
Flow cytometer "FACSCalibur"	BD Bioscience, Franklin Lakes, USA
Freezer -20°C: "GS 2481"	Liebherr, Bulle, SUI
Freezer -80°C: "Forma 900 Series "	Thermo Fisher Scientific, Waltham, MA, USA
Gel documentation imaging system "Quantum ST5"	Vilber Lourmat, Eberhardzell, GER
Gel electrophoresis chamber	Bio-Rad Laboratories GmbH, Munich, GER
Glasware	Marienfeld GmbH, Landa, GER
Heating plate HT 200	Minitube, Tiefenbach, GER
Hera Safe clean bench	Heraeus Instruments, Hanau, GER
Ice machine	Manitowoc Ice, Manitowoc, WI, USA
Incubator (Heracell VioS 160i)	Thermo Fisher Scientific, Waltham, MA, USA
Incubator Steri-cycle CO2	Thermo Fisher Scientific, Waltham, MA, USA
Magnet „Dynamag 15"	Life Technologies, Carlsbad, USA
Magnetic stirrer "AREC_X"	VELP Scientific, Usmate, ITA
Microinjector: CellTram pro	Eppendorf, Hamburg, GER

Microscope “Axiovert 40CLF”,	Carl Zeiss GmbH, Jena, GER
Microwave “MW17M70G-AU”	MDA Haushaltswaren, Barsbüttel, GER
Mr. Frosty freezing container	Thermo Fisher Scientific, Waltham, MA, USA
Neubauer cell counting chamber	Brand GmbH, Wertheim, GER
Orbital shaker	Thermo Fisher Scientific, Waltham, MA, USA
P97-micropipette puller	Sutter Instrument, CA, USA
PCR cycler “PeqStar”	Peqlab Biotechnology, Erlangen, GER
Pipettes “Pipetman “2ul, 20ul, 1000ul”	Gilson, Middleton, WI, USA
Power supply EC105	Electron GmbH, Dreieich, Germany
Refrigerator “TSE1283”	Beko, Neu-Isenburg, GER
Safety Workbench Hera safe class 2H	Heraeus Instruments, Munich, GER
Stereomicroscope Stemi 508	Carl Zeiss, Göttingen, Germany
Table centrifuge	Sigma-Aldrich GmbH, Steinheim, GER
Thermos container	Alfi GmbH, Wertheim, GER
Transfer man NK2 micromanipulator	Eppendorf, Hamburg, GER
Transportable incubator	Minitube, Tiefenbach, GER
Vortex mixer “Vortex Genie 2”	Scientific industries, Bohemia, NY, USA
Water bath	Memmert, Schwabach, GER

4.1.2. Consumables

Borosilicate glass with filament	Sutter Instruments, CA, USA
Cover slips (24x60mm)	Menzel, Braunschweig, GER
Cryo-vials	Corning Inc., Corning, NY, USA
Electroporation cuvettes (2mm, 4mm)	Peqlab Biotechnology, Erlangen, GER
FACS 96-well plates	Sarstedt, Nürnbrecht, GER
Filter pipette tips	Fisher Scientific, Hampton, NH, USA
Gas cylinders (CO2, N2, O2)	Westfalen AG, Münster, GER
IVF 4-well plates	Fisher Scientific, Waltham, MA, USA
Pasteur pipettes	Brand, Wertheim, GER
PCR tubes	Starlab, Hamburg, GER

Petri dishes	Greiner Bio-One, Frickenhausen, GER
Plastic pipettes „Costar Stripette“(1-50ml)	Corning Inc., Corning, NY, USA
Syringes	BD Bioscience, Le Pont De Claix, FRA
Tissue culture flasks (T25, T75, T150)	Corning Inc., Corning, NY, USA
Tissue culture plates (6-, 12-, 24-well)	Corning Inc., Corning, NY, USA
Tubes (15ml, 50ml)	Corning Inc., Corning, NY, USA
Vacutip	Eppendorf, Hamburg, GER

4.1.3. Chemicals

Agarose	Sigma-Aldrich, Steinheim, GER
BSA (fraction V)	Biomol, Hamburg, GER
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, Steinheim, GER
CutSmart Buffer	New England Biolabs, Ipswich, USA
Cysteine	Sigma-Aldrich, Steinheim, GER
Deoxynucleotide solution	New England Biolabs, Frankfurt, GER
Dimethyl-sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, GER
Dynabeads biotin binder	Thermo Fisher, Waltham, MA, USA
Ethanol (EtOH)	Fisher Scientific, Loughborough, GBR
Ethylene diamine tetra-acetic acid (EDTA)	AppliChem, Darmstadt, GER
Foetal calf serum (FCS)	PAA laboratories, Pasching, Austria
Gel loading dye	New England Biolabs, Frankfurt, GER
Glucose ($C_6H_{12}O_6$)	Sigma-Aldrich, Steinheim, GER
Glutamine	Invitrogen GmbH, Darmstadt, GER
Glycerol ($C_3H_8O_3$)	AppliChem, Darmstadt, GER
HEPES buffer	Sigma-Aldrich, Steinheim, GER

Isolectin B4 (biotin conjugate)	Enzo Life Sciences, Lörrach, GER
$MgSO_4$	Sigma-Aldrich, Steinheim, GER
Mineral oil	Sigma-Aldrich, Steinheim, GER
Penicillin-Streptomycin	Sigma-Aldrich, Steinheim, GER
PeqGREEN	VWR International, Ismaning, GER
PE-streptavidin	BD Bioscience, St. Jose, USA
Phosphate buffered saline (PBS)	Sigma-Aldrich, Steinheim, GER
Polysorbat 20	Sigma-Aldrich, Steinheim, GER
Polyvinyl alcohol (C_2H_4O)	Sigma-Aldrich, Steinheim, GER
Sodium bicarbonate (NaHCO3)	Sigma-Aldrich, Steinheim, GER
Sodium chloride (NaCl)	Sigma-Aldrich, Steinheim, GER
Sodium hydroxide (NaOH)	Sigma-Aldrich, Steinheim, GER
Sodium pyruvate	Sigma-Aldrich, Steinheim, GER
Tris-HCL	Sigma-Aldrich, Steinheim, GER
Triton X100	Omnilab-Laborzentrum, Bremen, GER
Trypan blue	Thermo Fisher, Waltham, MA, USA

4.1.4. Enzymes

GoTaq G2 DNA polymerase	Promega, Mannheim, GER
DNA Polymerase I (Klenow Fragment)	New England Biolabs, Ipswich, USA
Hyaluronidase	Sigma-Aldrich, Steinheim, GER
Proteinase K (20mg/ml)	Sigma-Aldrich, Steinheim, GER
Q5 Hot Start DNA polymerase	New England Biolabs, Ipswich, USA
Restriction endonucleases	New England Biolabs, Ipswich, USA
T4 DNA Ligase	New England Biolabs, Ipswich, USA

4.1.5. Kits

DNeasy Blood and tissue kit	Quiagen GmbH, Hilden, GER
GenElute DNA miniprep kit	Sigma-Aldrich, Steinheim, GER
Guide-it™ Long ssDNA Production System	Takara Bio, Göteborg, SWE
MEGAclear kit	Ambion, Austin, TEX, USA
Mix2Seq kit	Eurofins, Ebersberg, GER
mMESSAGE mMACHINE T7 kit	Ambion, Austin, TEX, USA
NucleoBond Xtra Midi kit	Macherey-Nagel, Düren, GER
PlateSeq DNA kit	Eurofins, Ebersberg, GER
Poly-A tailing kit	Ambion, Austin, TEX, USA
PureYield™ Plasmid Miniprep kit	Promega, Mannheim, GER
SurePrep RNA/DNA/protein purification kit	Fisher Scientific, Hampton, NH, USA
Wizard SV gel and PCR clean-up kit	Promega, Mannheim, GER

4.1.6. Cells

E. coli ElectroMAX DH10B	Thermo Fisher Scientific, Waltham, MA, USA
Porcine foetal fibroblasts (Isolate 170220)	Chair of Livestock Biotechnology, TUM, Freising, GER
Porcine kidney fibroblasts (Isolate 250515)	Chair of Livestock Biotechnology, TUM, Freising, GER
Porcine oocytes	Schlachthof Ingolstadt GmbH, Ingolstadt, GER
Porcine semen	Bayerngenetik GmbH, Altenbach, GER

4.1.7. Oligonucleotides

4.1.7.1. Primers

FUT1 F1	5‘ CCTCCGATTCCTGTCCCAAG 3‘

FUT1 F2	5‘ TTAGACCTGCTGGCCCTGTG 3‘
FUT1 R1	5‘ CGGACATCCAGTCGTGAAGC 3‘
FUT1 R2	5‘ CGGAGGTGGTGGAAGAAGGT 3‘
FUT1 Scr F1	5‘ CGGGCTGCACTTATGACTGG 3‘
FUT1 Scr R1	5‘ TTGGGCATCACACGCAGATA 3‘
FUT1 tar amp F1	5‘ CCGCCACCTCTGTCTGACCT 3‘
FUT1 tar amp R1	5‘ GTGGTCGTGCAGGGTGAACT 3‘
Gal Scr E8 T4 F1	5‘ TCCCAGAGGTTACATTTACCCCA 3‘
Gal Scr E8 T4 R1	5‘ GCACATCCTGGCCCACATCC 3‘
GAPDH S. scrofa F	5‘ TTCCACGGCACAGTCAAGGC 3‘
GAPDH S. scrofa R	5‘ GCAGGTCAGGTCCACAAC 3‘
PX330 FUT 1 G20 R1	5‘ AAACGCAGCGTGGCATACTGTC 3‘
PX330 FUT1 G13 F1	5‘ CACCGCTGCCATGCACGCCGTCC 3‘
PX330 FUT1 G13 R1	5‘ AAACGGACGGCGTGCATGGCAGC 3‘
PX330 FUT1 G2 F1	5‘ CACCGACTATTTACCCGGATGGC 3‘
PX330 FUT1 G2 R1	5‘ AAACGCCATCCGGGTAAATAGTC 3‘
PX330 FUT1 G20 F1	5‘ CACCGACAGTATGCCACGCTGC 3‘
PX330 seq F1	5‘ GGGAGAAAGGCGGACAGGTA 3‘
PX330 seq R1	5‘ GCGGCATCAGAGCAGATTGT 3‘
PX330FUT1 G1 F1	5‘ CACCGTACCCGGATGGCCGGTTT 3‘
PX330FUT1 G1 R1	5‘ AAACAAACCGGCCATCCGGGTAC 3‘

4.1.7.2. gRNA oligonucleotides

PX330 FUT1 G1 F1	5‘ CACCGTACCCGGATGGCCGGTTT 3‘
PX330 FUT1 G1 R1	5‘ AAACAAACCGGCCATCCGGGTAC 3‘
PX330 FUT1 G2 F1	5‘ CACCGACTATTTACCCGGATGGC 3‘
PX330 FUT1 G2 R1	5‘ AAACGCCATCCGGGTAAATAGTC 3‘
PX330 FUT1 G13 F1	5‘ CACCGCTGCCATGCACGCCGTCC 3‘
PX330 FUT1 G13 R1	5‘ AAACGGACGGCGTGCATGGCAGC 3‘
PX330 FUT1 G20 F1	5‘ CACCGACAGTATGCCACGCTGC 3‘

PX330 FUT1 G20 R1	5‘ AAACGCAGCGTGGCATACTGTC 3‘
PX330 Gal-E8-T4-F	5‘CACCTAATAATACGACTCACTA 3‘
PX330 Gal-E8-T4-R	5‘AAACGGAGATATCATCCACCAT 3‘

4.1.8. Vectors

pX330-U6-Chimeric_BB-CBh-hSpCas9	Addgene, Cambridge, MA, USA
pX330-U6-Chimeric_BB-CBh-hSpCas9-Gal-E8-T4	Dr. Konrad Fischer, Chair of Livestock Biotechnology, TUM, Freising, GER

4.1.9. DNA Ladders

1 kb DNA ladder	New England Biolabs, Frankfurt, GER
100 bp DNA ladder	New England Biolabs, Frankfurt, GER
2-log DNA ladder	New England Biolabs, Frankfurt, GER

4.1.10. Tissue culture media and supplements

Accutase	Sigma-Aldrich, Steinheim, GER
Cell culture water	Sigma-Aldrich, Steinheim, GER
Collagenase	Sigma-Aldrich, Steinheim, GER
DMSO	Sigma-Aldrich, Steinheim, GER
Dulbecco’s Modified Eagle’s Medium	Sigma-Aldrich, Steinheim, GER
Foetal calf serum	PAA Laboratories, Pasching, Austria
G418 sulphate	Genaxxon Bioscience, Ulm, GER
GlutaMAX	Gibco, BRL, Paisley, UK
Hygromycin	AppliChem, Darmstadt, GER
Hypo-osmolar buffer	Eppendorf, Hamburg, GER
Lipofectamine 2000	Thermo Fisher Scientific, Waltham, MA, USA
MEM non-essential amino acids 100x	Sigma-Aldrich, Steinheim, GER
Opti-MEM	Gibco Life Technologies, Carlsbad, CA, USA
Penicillin/Streptomycin	Sigma-Aldrich, Steinheim, GER

Phosphate-buffered saline	Sigma-Aldrich, Steinheim, GER
Puromycin	InvivoGen, San Diego, CA, USA
Sodium pyruvate	Sigma-Aldrich, Steinheim, GER
Trypan blue	Gibco Life Technologies, Paisley, GBR
Trypsin-EDTA	PAA Laboratories, Pasching, Austria

4.1.11. Bacterial culture media and supplements

Ampicillin	Sigma-Aldrich, Steinheim, GER
Chloramphenicol	Sigma-Aldrich, Steinheim, GER
LB medium (Luria-Bertani)	Becton Dickinson, Aalst, BEL

4.1.12. Embryo culture media and supplements

Amphotericin B	Sigma-Aldrich, Steinheim, GER
BSA	Sigma-Aldrich, St. Louis, MO, USA
Cysteine	Sigma-Aldrich, St. Louis, MO, USA
Epidermal growth factor (EGF)	Sigma-Aldrich, St. Louis, MO, USA
FBS Superior	Biochrom GmbH, Berlin, GER
Fibroblast growth factor (FGF)	Sigma-Aldrich, St. Louis, MO, USA
Hyaluronidase	Sigma-Aldrich, St. Louis, MO, USA
Insulin like growth factor (IGF)	Sigma-Aldrich, St. Louis, MO, USA
Suigonan (PMSG/ECG)	MSD-Tiergesundheit, Unterschleißheim, GER
Leukaemia inhibitory factor (LIF)	Sigma-Aldrich, St. Louis, MO, USA
Mineral oil	Sigma-Aldrich, St. Louis, MO, USA
Ovogest (HCG)	MSD-Tiergesundheit, Unterschleißheim, GER
Polyvinyl alcohol	Sigma-Aldrich, St. Louis, MO, USA
Porcine fertilisation medium (PFM)	Fujihira Industry, Tokyo, JAP
Porcine zygote medium 5 (PZM5)	Fujihira Industry, Tokyo, JAP
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO, USA

Sodium pyruvate	Sigma-Aldrich, St. Louis, MO, USA
Tissue culture medium 199	Sigma-Aldrich, Steinheim, GER

4.1.13. Solutions and buffers

Type	Components	Quantity
FACS wash buffer	BSA	500 mg
	NaN3	100 mg
	PBS	Add to 100 ml
TAE 10x	Tris	242 g
	0.5 M EDTA	100 ml
	C2H4O2	57.1 ml
	H2O	Fill up to 5 l
TBE 10X	Tris	545 g
	H3BO3	275 g
	EDTA	39.2 g
	H2O	Fill up to 5 l
TE buffer	Tris-HCl	158 mg
	EDTA	29 mg
	H2O	Fill up to 100 ml

4.1.14. Veterinary equipment and products

Ampitab® (Ampicillin)	Vetoquinol GmbH, Ismaning, GER
Careflow Catheter 5F, 300mm	Merit Medical, Jordan, UT, USA
Cellulose swabs	B. Braun AG, Melsungen, GER
Electric cauter	HBH Medizintechnik, Tuttlingen, GER
Suigonan®	MSD-Tiergesundheit, Unterschleißheim, GER
Ketanest® (Ketamine)	Elanco GmbH, Bad Homburg, GER
Needle holder	Omega Medical, Winnenden, GER

Ovogest®	MSD-Tiergesundheit, Unterschleißheim, GER
Razor	B. Braun AG, Melsungen, GER
Regumate® (Altrenogest)	MSD-Tiergesundheit, Unterschleißheim, GER
Scalpels	Braun, Melsungen, GER
Stresnil® (Azaperone)	Elanco GmbH, Bad Homburg, GER
Surgical drape	B. Braun AG, Melsungen, GER
Surgical gloves (Peha-taft)	Omega Medical, Winnenden, GER
Surgicryl 910 HS 48, 5 (2), 90cm	Omega Medical, Winnenden, GER
Surgicryl DS 24, 3.0 (2/0) 75cm	Omega Medical, Winnenden, GER
Syringes, (1ml,5ml,10ml,20ml)	B. Braun AG, Melsungen, GER
XYLAPAN® (Xylazine)	Vetoquinol GmbH, Ismaning, GER

4.1.15. Software

Benchling	https://www.benchling.com/
Chromatogram viewer" Finch TV"	Digital world biology LLC, CA, USA
Crispr design tool	http://crispor.tefor.net/crispor.py
Flow cytometry software "FlowJo"	FlowJo LLC, Ashland, OR, USA
Gel documentation software "Quantum ST5"	Vilber Lourmat, Eberhandzell, GER
Genome database "Ensembl"	https://www.ensembl.org/index.html
Microscope software "Axio Vision"	Carl Zeiss, Göttingen, Germany
Sequence alignment tool "Clustal Omega"	https://www.ebi.ac.uk/Tools/msa/clustalo/
TIDE: Tracking of Indels by DEcomposition	https://tide.deskgen.com/
Vector design software "Everyvector"	http://www.everyvector.com/

4.2. Methods

4.2.1. Molecular biology

4.2.1.1. Isolation of genomic DNA

Genomic DNA was extracted from eukaryotic cells using QuickExtract DNA extraction solution. Cells were detached with accutase, centrifuged at 300 x g for 5 minutes and resuspended in 30 µl QuickExtract DNA extraction solution. This solution was heated to 68 °C for 15 minutes followed by 98 °C for 8 minutes.

If genomic DNA of higher purity was needed, the SurePrep DNA/RNA/Protein purification kit was used according to the manufacturer's protocol.

DNA from mammalian tissues or sperm was isolated by phenol-chloroform extraction. Approximately 1g of sperm pellet or tissue sample was incubated overnight in 1ml of lysis buffer at 55° C (see Table 1). The lysate was incubated with an equal amount of phenol-chloroform-isoamyl alcohol (25:24:1) for 10 minutes at room temperature and centrifuged at 13.000 x g for 10 minutes (same settings for all following centrifugation steps). The supernatant was mixed 1:1 with chloroform (99%) and centrifuged. DNA precipitation was achieved by supplementation of 10% v/v sodium acetate (5M) and 0.7% v/v isopropanol followed by thorough shaking. The pellet was centrifuged, rinsed with 70% ethanol, and centrifuged again. The resulting DNA pellet was air-dried and dissolved in 20 µl sterile TE buffer.

Table 1: Tissue lysis buffer

Tris-HCL	83 mM
SDS (sodium dodecyl sulphate)	0.8%
EDTA	0.2 M
NaCL	0.2 M
Proteinase K	100 µg/ml
H_2O	-

4.2.1.2. Isolation of plasmid DNA

Depending on the required purity and quantity, different methods were used for the isolation of plasmid DNA from bacterial cultures.

Plasmid bearing E. Coli bacteria were cultured overnight in LB-medium. If only small amounts of DNA were required a mini prep was performed using the PureYield™ Plasmid Miniprep kit according to the manufacturer's instructions.

Larger amounts of DNA for transfection, cloning or microinjection were extracted using the NucleoBond Xtra Midi Kit. The high-copy midi-prep protocol was carried out using 100ml of overnight culture according to the manufacturer's instructions.

4.2.1.3. Isolation of DNA from blastocysts

DNA was isolated from blastocysts or single blastomeres to evaluate the efficiency of gene engineering. Blastocysts or blastomeres were rinsed twice with PBS and incubated in 10 µl of blastocyst DNA extraction buffer (see Table 2) for one hour at 65 °C, followed by ten minutes at 95°C.

Table 2: Blastocyst DNA extraction buffer

KCL	50 mM
$MgCl_2$	1.5 mM
Nonidet P-40	0.5% (w/v)
Proteinase K	100 µg/ml
Tris-Cl (pH 8.0)	10 mM
Tween-20	0.5% (v/v)

4.2.1.4. Determination of DNA and RNA concentration

The measurement of DNA and RNA concentrations was performed using the NanoDrop Lite spectrophotometer according to the manufacturer‘s protocol.

4.2.1.5. Polymerase chain reaction

Polymerase chain reaction (PCR) was conducted to amplify specific DNA sequences from plasmid, genomic or cDNA templates. Different polymerases and protocols were chosen depending on the DNA template and length of the desired sequence. Amplification of short fragments from plasmid, genomic or cDNA templates was performed using GoTaq G2 polymerase or FastGene Optima HotStart ready mix. Q5® high fidelity polymerase was used if proofreading was necessary or only low concentrations of DNA were available. The PCR extender system was used for long-range PCR. For thermal cycling conditions and PCR reaction compositions see Table 3-5.

Table 3: GoTaq® G2 polymerase PCR reaction composition and thermal cycling conditions

GoTaq® G2 polymerase					
PCR reaction composition		**Thermal cycling conditions**			
Component	**Final concentration**	**Step**	**Temperature**	**Time**	**Cycles**
DNA	< 250 ng	Initial Denaturation	95°C	2 min	1
5x buffer	1x	Denaturation	95°C	30 sec	35-40
dNTPs	200 µm each	Annealing	58-62°C	45 sec	
Primer F	0.2 µM	Extension	72°C	1 min/kb	1
Primer R	0.2 µM	Final extension	72°C	5 min	
Polymerase	0.03 U/µl	Storage	8°C	Indefinite	
H2O	Add to 25 µl				

Table 4: PCR extender system reaction composition and thermal cycling conditions

PCR extender system					
PCR reaction composition		**Thermal cycling conditions**			
Component	**Final concentration**	**Step**	**Temperature**	**Time**	**Cycles**

DNA	100 ng	Initial Denaturation	93°C	3 min	1
10x tuning buffer	1x	Denaturation	93°C	30 sec	
dNTPs	500 µm each	Annealing	60-64°C	1 min	10
Primer F	0.4 µM	Elongation	68°C	1 min/kb	
Primer R	0.4 µM	Denaturation	93°C	30 sec	
PCR extender polymerase mix	0.04 U/µl	Annealing	60-64°C	1 min	30
H2O	Add to 50 µl	Elongation	68°C	1 min/kb + 20 sec/cycle	
		Final elongation	68°C	5 min	1
		Storage	8°C	Indefinite	1

Table 5: Fastgene Optima HotStart PCR reaction composition and thermal cycling conditions

FastGene Optima HotStart ready mix

PCR reaction composition		**Thermal cycling conditions**			
Component	**Final concentration**	**Step**	**Temperature**	**Time**	**Cycles**
DNA	50 ng	Initial Denaturation	95°C	3 min	1
FastGene Optima HotStart ready mix	1x	Denaturation	95°C	15 sec	35-40
Primer F	0.5 µM	Annealing	58-62°C	15 sec	
Primer R	0.5 µM	Elongation	72°C	1 min/kb	
H2O	Add to 25 µl	Final elongation	72°C	5 min	1
		Storage	8°C	Indefinite	

Table 6: Q5® high-fidelity DNA polymerase PCR reaction composition and thermal cycling conditions

Q5® high-fidelity DNA polymerase

PCR reaction composition	**Thermal cycling conditions**

Component	Final concentration	Step	Temperature	Time	Cycles
DNA	1 ng -1 µg	Initial Denaturation	98°C	30 sec	1
Q5® 5x buffer	1x	Denaturation	98°C	10 sec	
Q5® high GC enhancer (optional)	1x	Annealing	58-62°C	30 sec	35-40
dNTPs	200 µm each	Elongation	72°C	30 sec/kb	
Primer F	0.5 µM	Final elongation	72°C	2 min	1
Primer R	0.5 µM	Storage	8°C	Indefinite	1
Polymerase	0.02 U/µl				
H20	Add to 25 µl				

4.2.1.6. Agarose gel electrophoresis to separate DNA fragments

Agarose gel electrophoresis was performed to analyse the size of DNA-fragments. Analytical gels were prepared with 1xTBE and preparative gels with 1xTAE buffer each supplemented with 4 µl PeqGREEN dye per 100 ml. The concentration of agarose was adjusted between 0.8-2% according to the size of the expected DNA fragments. DNA fragments were mixed with loading dye, applied to the gel and 80-120V were applied until adequate separation was achieved (approximately 1 to 7 hours). The separated DNA fragments were visualised under UV light (254-366nm) using the Bio Imaging System Quantum ST5.

4.2.1.7. DNA isolation from agarose gels

DNA bands were visualised by UV light und cut out with a surgical blade. DNA purification was performed using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer’s protocol.

4.2.1.8. Proteinase K digest

Proteinase K digest was conducted to inactivate nucleases and prevent the degradation of DNA during purification. The DNA was co-incubated with SDS (0.5%) and Proteinase K (1 µg/µl) for 30 minutes at 50°C.

4.2.1.9. DNA purification

Purification of DNA fragments and PCR products was conducted using the Wizard® SV Gel and PCR Clean-Up System or SurePrep DNA purification kit according to the manufacturer's instructions.

Prior to sequencing PCR products were enzymatically purified to remove residual primers and dNTPs. The PCR product was co-incubated with 0.2 µl exonuclease I and 0.4 µl antarctic phosphatase at 37°C for 30 min, followed by 15 minutes at 80°C.

DNA templates for *in vitro* transcription were purified by modified phenol-chloroform extraction. DNA was mixed 1:1 with phenol-chloroform-isoamyl alcohol, vortexed and incubated in a phase lock gel for 10 minutes followed by centrifugation at 14.000 rpm for 10 minutes. The DNA containing supernatant was incubated with two volumes of 100% ethanol and 1/10 volume 5M ammonium acetate at -20°C for 2 hours. The DNA was pelleted by centrifugation and the pellet was air dried and resuspended with 20 µl nuclease-free water.

4.2.1.10. Sequencing of DNA fragments

DNA sequencing was performed by Eurofins Genomics (Ebersberg, GER). Samples were prepared for sequencing following the manufacturer's instructions using the Mix2Seq kit.

4.2.1.11. Oligonucleotide annealing

Complementary single-stranded oligonucleotides were diluted to a final concentration of 10ng/µl with TE buffer. Double-strand hybridisation was achieved by heating the solution to 100°C for 5 min and then letting it cool to room temperature.

4.2.1.12. Restriction digestion

Analytical restriction digests were performed to verify plasmid size. Preparative restriction digests were conducted to linearize targeting vectors for cloning. All restriction digests were conducted at the optimal temperature for each enzyme according to the manufacturer's instructions. To prevent re-ligation the 5' phosphates were removed from the vector backbone by incubation by alkaline phosphatase for 30 minutes at 37°C. The conditions for analytical and preparative restriction digestions are shown in Table 7.

Table 7: Conditions for analytical and preparative restriction digestions

Analytical digest		**Preparative digest**	
Component	**Concentration**	**Component**	**Concentration**
DNA	0.2-3 µg	DNA	7-40 µg
10x NEB Buffer	1x	10x NEB Buffer	1x
Enzyme	3-5 U/ µg	Enzyme	5 U/ µg
H_2O	add to 20-50 µl	H_2O	add to 50 µl

4.2.1.13. Blunting of DNA fragments

DNA Polymerase I Large (Klenow) Fragment was used to remove 3' overhangs and fill in 5' overhangs of DNA fragments with incompatible sticks ends prior to ligation. The reaction was performed according to the manufacturer's instructions. DNTPs (60 µM) were added to inhibit the 3'-5' exonuclease activity of the polymerase. All reagents were incubated at 25 °C for 15 minutes. The reaction was stopped by adding 10 mM EDTA and increasing the temperature to 75 °C for 20 minutes. Table 8 shows the conditions for blunting reactions.

Table 8: Conditions for blunting reactions

Component	**Final concentration**
DNA	5 µg
10x NEBuffer	1x

dNTPs (2mM)	50 µM
Enzyme	1U/ µg
H_2O	add to 50 µl

4.2.1.14. Ligation of oligonucleotides into vector backbones

Vector backbones and insert DNA fragments were ligated using T4 Ligase. Ligation reactions were set up according to the manufacturer's instructions and incubated for two hours at room temperature, followed by 4°C overnight. Ligation reaction conditions are shown in Table 9.

Table 9: Conditions for ligation reactions

Component	**Final concentration**
Hybridised oligonucleotides	1.5 µL
10x T4 Ligase buffer	1x
T4 DNA Ligase	1.5 µL
Digested vector backbone	100 ng
H_2O	add to 15 µl

4.2.1.15. Colony PCR

Colony PCR was performed to identify E. Coli colonies carrying the intended plasmid constructs. One primer was designed to bind to the plasmid insert, the other to the plasmid backbone. Single bacterial colonies were resuspended in 30 µl TTE buffer and incubated at 95°C for 5 minutes to generate template DNA for the GoTaq PCR reaction (see Table 3).

4.2.1.16. *In vitro* transcription

SgRNAs were generated by in vitro transcription of DNA templates using the MEGAshortscript T7 kit as instructed by the manufacturer. *In vitro* transcription of DNA templates encoding RNA transcripts of 0.3-5kb was performed using the mMESSAGE mMACHINE T7 kit according to the manufacturer's instructions. Purification and polyadenylation of the RNA

transcript was carried out using the Poly-A tailing kit and MEGAclear kit according to the manufacturer's protocol.

4.2.1.17. Generation of CRISPR/Cas9 components

CRISPR gRNAs with low predicted off-target effects were identified using an online CRISPR design tool (https://www.benchling.com/). Oligonucleotides containing a single G for U6 promoter transcription followed by the target guide sequence and overhangs compatible to the BbsI-digested vector backbone were purchased from MWG Eurofins Genomics (Ebersberg, GER). The single-stranded oligonucleotides were hybridised and ligated into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (see Figure 6). Bacterial cells were transformed , positive clones were confirmed by sequencing and plasmid DNA was extracted using the NucleoBond Xtra Midi kit and directly used for microinjection or DNA transfection.

For in vitro transcription the gRNA encoding sequence was amplified by Q5® high-fidelity polymerase followed by gel-purification and a 4-fold nested PCR. The PCR product was enzymatically purified, digested with proteinase K and purified by phenol-chloroform extraction. *In vitro* transcription was performed using the MEGAshortscript T7 kit followed by purification using the MEGAclear kit according to the manufacturer's instructions.

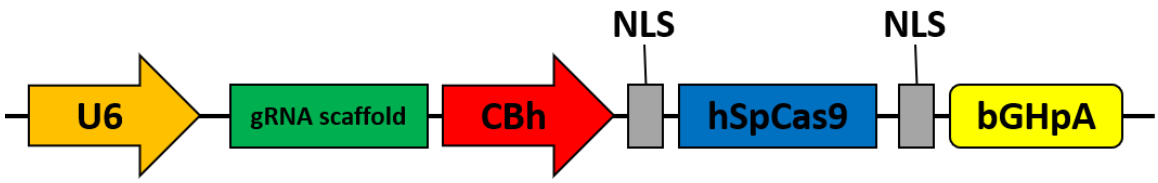

Figure 6: Generation of pX330-U6-Chimeric_BB-CBh-hSpCas9. The annealed oligonucleotides were cloned into the digested vector backbone. The vector contains the U6 promoter, sgRNA scaffold, CBh promoter, nuclear localization signals (NLS) flanking the hSpCas9 gene and bGH-poly-A sequence (adapted from Addgene [129]).

4.2.1.18. Tracking of Indels by Decomposition

Sequences from cell pools were analysed using the TIDE (Tracking of Indels by Decomposition) online tool. To facilitate the identification and quantification of insertions and deletions (indels) PCR amplification was performed across the target site followed by DNA sequencing of the PCR product. The frequency of mono- and biallelic mutations was determined by calculating the total proportion of edited cells. TIDE determines the spectrum and frequency of mutations at a specific sequence withing a cell pool from sequencing data. Only data with a statistical R_2 value above 0.9 which indicates low negative interference by sequencing noise or large deletions was considered in the analysis.

4.2.2. Microbiology

4.2.2.1. Bacterial cell cultivation

Bacterial cells were grown on agar plates supplemented with antibiotics or in LB medium on an orbital shaker. Single bacterial clones were picked from an agar plate and cultivated overnight at 37°C in LB medium supplemented with 100 μg/ml ampicillin or on agar plates. For blue-white screening plates were coated with X-Gal and Isopropyl-β-D-thiogalactopyranoside (IPTG).

4.2.2.2. Bacterial cell preservation

Bacteria carrying plasmids of interest were prepared for long-term storage by mixing 1ml of overnight culture 1:1 with glycerol (99%). Glycerol stocks were stored at -80°C.

4.2.2.3. Bacterial cell transformation

Electrocompetent bacteria were transformed by electroporation to introduce plasmids. Plasmids (10-30 pg) were co-incubated with 50 μL of ElectroMAX DH10B E. coli bacteria in an electroporation cuvette (electrode distance 2mm). Cells were electroporated at 2.5kV, 5ms and incubated in LB medium without antibiotics for 30 minutes at 37°C. Subsequently the transformed bacteria were plated on LB agar supplemented with antibiotics and cultivated overnight at 37°C.

4.2.3. Embryology

4.2.3.1. Oocyte isolation

Ovaries were sourced from a local slaughterhouse and transported at 38°C in PBS supplemented with Amphotericin B and Penicillin/Streptomycin (Pen/Strep) (1% each). They were rinsed twice with warm PBS solution and maintained at 38°C during the whole isolation process. A 10ml syringe and 18G needle was used to puncture follicles (Ø 3-6mm). Cumulus-oocyte-complexes with dark, evenly granulated cytoplasm covered by multiple compact layers of cumulus cells were selected under a stereomicroscope. To remove cell detritus the oocytes were rinsed twice in working medium (tissue culture medium 199 supplemented with 10% (FCS, 1% Pen/Strep and 1% Amphotericin B).

4.2.3.2. Oocyte maturation

Groups of 50 oocytes were transferred to four-well plates containing 500 µl of maturation medium (see Table 10) and placed in a triple gas incubator at 38,5°C humidified atmosphere for 45 hours. Successful maturation was confirmed by extrusion of the first polar body.

Table 10: Maturation medium

Cysteine	0.57mM
Epidermal growth factor	10ng/ml
Fibroblast growth factor II	40ng/ml
Glucose	3.05mM
HCG/ECG	1 IU/ml each
Insulin-like growth factor I	20ng/ml
Leukaemia inhibitory factor	20ng/ml
Polyvinyl alcohol	0.1% w/v
Sodium pyruvate	0.91mM
Tissue culture medium 199	add to desired volume

4.2.3.3. Parthenogenetic activation

Parthenogenesis was conducted to provide a control group for IVF experiments and to analyse the efficiency of gene editing after microinjection.

After *in vitro* maturation (45 hours) the cumulus cells were enzymatically removed (1mg/ml hyaluronidase). Zygotes showing extrusion of the first polar body were rinsed with activation medium (see Table 11), transferred to an activation chamber (ø 1mm) and activated with a single DC pulse (150V, 100µs). After parthenogenesis the oocytes were cultivated in PZM5 supplemented with 5µg/ml Cytochalasin B and 10µg/ml Cycloheximide for 4 hours. Afterwards they were rinsed in PZM5 and cultivated in PZM5 (6 days) to the blastocyst stage.

Table 11: Activation medium

CaCl2	0.05 mM
H_2O	Add to desired volume
Mannitol	280 mM
$MgSO_4$	0.1 mM
PVA	0.01 % w/v

Sterile filtrate (22µm), adjust PH to 7.2-7.4, adjust osmolarity to 300 Ω.

4.2.3.4. *In vitro* fertilisation

Groups of 50 oocytes were placed in 500 µl of equilibrated porcine fertilisation medium (PFM). Frozen sperm was thawed, then rinsed with sperm diluent (Androstar® Plus) and centrifuged (800G, 3 min) to remove cryo-protectants. The pellet was mixed with 500 µl of PFM and analysed concerning motility, morphology, and sperm concentration. *In vitro* fertilisation (IVF) was conducted by incubating 6000 motile spermatozoa per oocyte with 50 oocytes for seven hours.

4.2.3.5. Microinjection

Cumulus cells were enzymatically removed (1mg/ml hyaluronidase) and zygotes showing extrusion of the second polar body were selected for microinjection. Groups of 50 zygotes were placed in a 10 µl droplet of working medium covered by mineral oil. The gene editing vectors were diluted to a final concentration of 5 ng/µl with low-tris EDTA buffer (10 mmol/L Tris-HCL, pH 7.6 and 0.25 mmol/L EDTA, pH 8.0) and backfilled into microinjection needles.

The injection needle was gently opened by tapping it with the holding needle. Zygotes were fixated with the polar body located at the twelve or six o' clock position, the injection needle was gently inserted into the ooplasm and about 10 pl of solution were dispensed.

For two-cell stage injection zygotes were cultivated for 24 hours after IVF and embryos that had undergone the first cleavage division were selected for microinjection. Microinjection was performed as described above but the injection solution was only delivered into one out of two blastomeres. Injection pressure was reduced to accommodate the smaller size of the 2-cell-stage blastomere and approximately 5pl of injection solution were dispensed.

4.2.3.6. Embryo cultivation

Groups of 50 zygotes were rinsed with porcine zygote medium 5 (PZM5) and cultivated in the triple gas incubator in 500 µl of PZM covered by mineral oil. Zygotes destined for DNA extraction and analysis were cultured *in vitro* for six days until they reached the blastocyst stage. Zygotes designated for embryo transfer were cultivated for 12-36 hours *in vitro* and their viability was visually assessed prior to embryo transfer.

4.2.3.7. Production of microinjection needles

Microinjection needles were manufactured with a P-97 Flaming brown micropipette puller according to the manufacturer's protocol (see Table 12). Optimal melting temperatures for borosilicate glass tubing were determined by conducting a temperature ramp test. Holding pipettes (Vacutip I®, Eppendorf, Hamburg, GER) were purchased.

Table 12: Parameters for microinjection needle fabrication

Heat	750
Pressure	500
Pull	70
Time	210
Velocity	40

4.2.3.8. Embryo transfer

Synchronisation of gilts (aged 6 months, weight 120kg) was conducted by oral administration of Altrenogest (Regumate®) for 15 days followed by two intramuscular injections of 750 IU HCG/ECG (Suigonan®) one and five days later. Embryo transfer was conducted one to two days after the last injection.

Fasted pigs were anaesthetised by intravenous application of 20 mg/kg ketamine (Ketanest®) and 2mg/kg xylazine (XYLAPAN®) and given 15mg/kg ampicillin (Ampitab®) and 0.4mg/kg meloxicam (Metacam®). Recipients were fixated on the surgery table, the abdomen was cleaned with warm water and disinfected with iodine solution. The surgery area was covered with a surgical drape and the skin excision was made at the Linea Alba between the last two pairs of teats. The abdomen was opened, one ovary was displaced, and the correct state of the reproductive cycle was visually confirmed by the presence of fresh ovulation sites. Approximately 150 to 200 embryos were gently dispensed into the recipient’s oviduct with a sterile catheter (Careflow®). The surgical wound was stitched in three layers using Sultan's diagonal suture for peritoneum and muscle and the mattress suture technique for skin.

4.2.4. Cell culture

4.2.4.1. Cell isolation

Wild type porcine kidney fibroblasts (PKFs) were isolated from kidneys obtained from the TUM animal facility Thalhausen or from a local slaughterhouse. Approximately 1g or kidney tissue was rinsed twice with 80% ethanol and PBS solution. The tissue was chopped into small pieces and enzymatically dissociated by incubation in 10ml collagenase A (10mg/ml) at 37°C for 30 minutes on a magnetic stirrer. The cells were centrifuged for 5 minutes at 300 x g and the cell pellet was resuspended in warm cell culture medium supplemented with amphotericin B and pen/strep. After seven days cells were cultivated under antibiotic-free conditions. All steps were carried out in a laminar flow using sterile equipment.

For the isolation of porcine foetal fibroblasts (PFFs), the pregnancy was sonographically confirmed on and the surrogate pig was humanely killed. The foetuses were extracted from the uterus and approximately 1g of foetal tissue was used for cell isolation. All further steps were carried out a described above.

4.2.4.2. Cell cultivation

PKF and PFF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2mM Ala-Gln, 1mM sodium pyruvate, 1x MEM non-essential amino acid solution (NEAA) and 10% FCS at 5% CO_2, 37°C in humified atmosphere. Medium was exchanged every three days. Cells were passaged when reaching 90% confluence by detaching them with accutase and transferring them to a new tissue culture vessel.

4.2.4.3. Cell counting

Cells were counted using the Countess™ automated cell counter according to the manufacturer's protocol.

4.2.4.4. Cell transfection

Cells were transfected with DNA by electroporation or lipofection.

Prior to electroporation cells were detached from the cell culture flask with accutase, $1x10^6$ cells were counted and centrifuged for 5 minutes at 300 x g. The cell pellet was resuspended in 400µl hypoosmolar buffer and gently mixed with 5-6 µg linearized vector DNA. The mixture was incubated at room temperature for 5 to 10 minutes in an electroporation cuvette (electrode gap 4mm). Electroporation was conducted by applying a 1200 V pulse for 85µs and the cell suspension was divided into three cell culture vessels with fresh medium.

For lipofection cells were seeded at 50% confluency, rinsed with PBS, and cultured in Opti-MEM medium in 10cm cell culture plates. A transfection and DNA solutions were prepared by diluting 6µl Lipofectamine 2000® and 5 µg DNA with OptiMEM to a final volume of 300 µl respectively. Both solutions were incubated at room temperature for 5 minutes, gently combined, and co-incubated for another 20 to 25 minutes. The solution was gently applied onto the cells and cultivated. After 5 hours DMEM medium was added, the cells were cultured overnight, and a medium change was conducted on the next day.

4.2.4.5. Antibiotic selection

Cell clones were selected for stable transgene integration using cell culture medium supplemented with the corresponding antibiotic for the plasmid's resistance cassette. The appropriate concentration of antibiotics for each cell type was determined in a killing curve experiment. Selection medium was performed until single cell clones without background became apparent. In this project selection was conducted using G418 (Geneticin) and puromycin.

4.2.4.6. Isolation of single cell clones

Separated cell colonies were marked and cloning rings were fixated over each colony with silicon grease. Accutase was dispensed into each cloning ring to detach the cells. The cell suspension was transferred into 6-well cell plates and cultivated.

4.2.4.7. Cell cryopreservation and thawing

Cells were detached with accutase and centrifuged at 300 x g for 5 minutes. The cell pellet was resuspended in 1.5ml of cryopreservation medium (70% FCS, 20% DMEM and 10% dimethyl sulfoxide). This suspension was transferred into cryo-vials, and frozen at -80 °C in Mr. Frosty® cell freezing containers. For long-term storage cells were conserved in the gas phase of liquid nitrogen containers.

Thawing of cells was conducted by transferring the cryo-vials to a water bath (37°C) until the medium was nearly thawed. The cells were pelleted by centrifugation (300 x g, 5 minutes), resuspended in prewarmed DMEM medium and cultivated (5%CO_2, 37°C).

4.2.4.8. Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) was performed to separate galactose-α-1,3-galactose (α-Gal) negative and positive cells. PFF cells were isolated as described in 4.2.4.1., rinsed with PBS and centrifuged (300 x g, 5 minutes). The cell pellet was resuspended with 50µl isolectin B4 (0.5 mg/ml, biotin conjugated) and incubated on ice for 15 minutes. The cells were rinsed with PBS, centrifuged (300 x g, 5 minutes) and co-incubated with 200 µl purified, streptavidin coated magnetic beads (Dynabeads®, biotin binding) on ice for 30 minutes. A magnetic field was applied for 1 minute, and the supernatant containing the αGal-negative cells was collected whereas the αGal-positive cells remained bound to the magnetic beads. The purity of sorted cells was analysed by flow cytometry. Figure 7 schematically depicts the process for the separation of α-Gal positive and α-Gal negative cells.

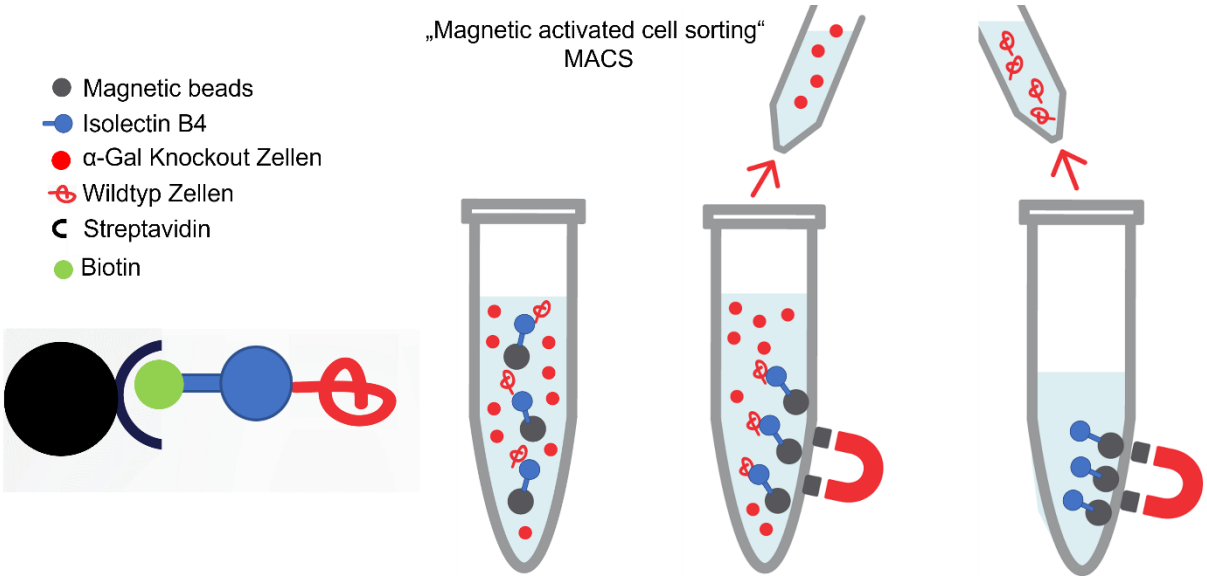

Figure 7: Separation of α-Gal positive and negative cells by magnetic-activated cell sorting. Streptavidin coated magnetic beads bind to the biotinylated isolectin B4. A magnetic field is applied, and the α-Gal negative cells are removed whereas the α-Gal positive cells remain bound to the magnetic beads.

4.2.4.9. Flow cytometry

For flow cytometry $1x10^6$ PFF cells were transferred to each well of a 96-well plate, washed and resuspended in fluo buffer (PBS supplemented with 0.1mg/ml sodium-azide and 1% BSA). Cells were incubated with Isolectin B4, biotin conjugated for 20 minutes on ice. Then they were washed with fluo buffer and incubated with PE-streptavidin for 20 minutes on ice. Finally, cells were washed and resuspended in 300 µl of fluo buffer and flow cytometry measurements were conducted using the Attune NxT Flow Cytometer. Data analysis was performed using the FlowJo software.

4. MATERIALS AND METHODS

5. RESULTS

The goal of this thesis was to evaluate the feasibility, efficiency, and safety of gene editing in pigs by the example of Oedema disease.

For this purpose, guide RNAs with low predicted off-target activity were identified, Cas9 vectors were generated, primary porcine cells were transfected, and the most efficient gRNAs were determined by TIDE analysis. Primary porcine cell isolates were screened for their FUT1 genotype and gene editing was performed using single stranded oligonucleotides and CRISPR/Cas expression vectors. Single cell clones were selected, expanded and the efficiency of gene editing was analysed (described in 5.1.).

Precise *in vivo* editing of the FUT1 gene was attempted directly in porcine embryos. Cas9 vectors and single stranded DNA repair templates were delivered to *in vitro* fertilised porcine zygotes by microinjection. Genetically modified embryos were generated, and the editing efficiency was analysed by PCR and DNA sequencing (addressed in 5.2.).

In the second part of this project the frequency at which the CRISPR/Cas9 system generates off-target mutations *in vivo* was analysed using a novel approach termed GOTI never before applied in livestock. This approach facilitates a differentiation between natural mutations that occur during embryogenesis and off-target mutations.

Cas9 expression vectors were microinjected into one blastomere of a two-cell stage embryo to generate mosaic foetuses containing both edited and non-edited cells simultaneously. An essential prerequisite is the ability to separate edited and non-edited cells from a single foetus. Therefore, the GGTA1 gene was chosen as the target for genome engineering. Its inactivation leads to the absence of the α-Gal surface epitope which allows the precise separation of edited and non-edited cells. The microinjected embryos were transferred to surrogate sows, one pregnancy was established and terminated on day 38. Fourteen foetuses were isolated, three of them mosaic and foetal fibroblasts were isolated. Edited and non-edited cells were separated via magnetic-activated cell sorting. Whole genome sequencing was performed to analyse the frequency of off-target mutations caused by CRISPR/Cas9 mediated *in vivo* gene editing in livestock, irrespective of naturally occurring mutations (outlined in 5.3.).

5.1. *In vitro* editing of the FUT1 gene

Genetics play a decisive role in animal health. Gene editing technology could complement traditional breeding techniques to improve animal welfare. This project aimed to establish methods for precise, efficient, and safe gene editing in pigs to improve animal welfare by the example of Oedema disease.

Animals carrying a specific variant of the FUT1 gene (Chr.6:49826013A>G) which mediates an amino acid exchange (p.T103A) are resistant to Oedema disease. Gene editing was used to precisely elicit this single A>G base pair exchange and thereby convert FUT1-GG into FUT1-AA cells.

5.1.1. Identification of FUT1 gRNA sequences

Four different gRNAs for the FUT1 gene (FUT1 G1, G2, G3, G20) with high predicted on-target and minimal predicted off-target activity were identified using an online CRISPR design tool (http://crispor.tefor.net/crispor.py). Each gRNA was cloned into a Cas9 expression vector containing a puromycin resistance cassette to facilitate transient selection in primary porcine cells.

Porcine kidney fibroblasts (PKFs) from eight donor animals were screened for their FUT1-GG, -GA, or -AA genotype by PCR and DNA sequencing. Seven cell isolates had the FUT1-GG genotype, one cell isolate the FUT1-AG genotype. Only cell isolates with the FUT1-GG genotype were used to facilitate a precise determination of gene editing efficiencies.

PKFs were transfected with each expression vector and selected with puromycin. DNA was isolated from each transfected cell pool and PCR amplification was conducted across the target site followed by DNA sequencing. The frequency of insertions and deletions at the target site was determined by TIDE analysis (see Figure 8).

The most efficient gRNA (FUT1 G20) caused the highest frequency of indel mutations at the target site (37,1%) and was therefore used for all further experiments.

5. RESULTS

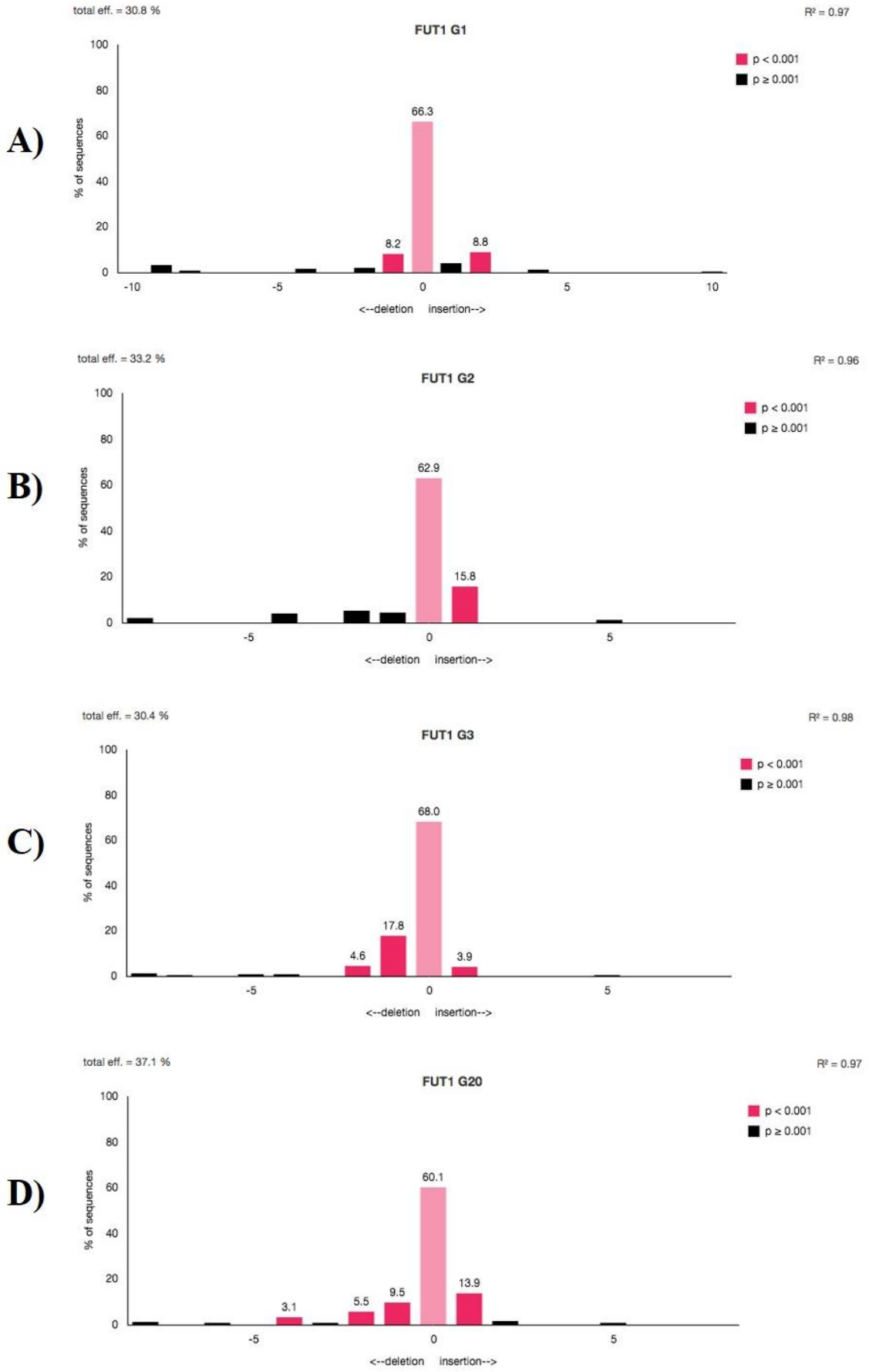

Figure 8: The spectrum and frequency of insertions and deletion at the FUT1 target site determined by TIDE analysis. **A)** FUT1 G1 (30,8%), **B)** FUT1 G2 (33,2%), **C)** FUT1 G3 (30,4%), **D)** FUT1 G20 (37,1%)

5.1.2. Evaluation of FUT1 repair templates

Single-stranded DNA repair templates with different lengths (700-3000 bps) were synthesized using the Guide-it™ Long ssDNA Production System according to the manufacturer's protocol. The repair templates were individually co-transfected with the most efficient Cas9 expression vector (G20) into porcine somatic cells. DNA was extracted from the cell pool and analysed by DNA sequencing. The single-stranded DNA repair template ssOliFUT1 (700bp) led to the highest repair efficiencies (>90%) in the cell pool and was therefore used for all further experiments (see Figure 9).

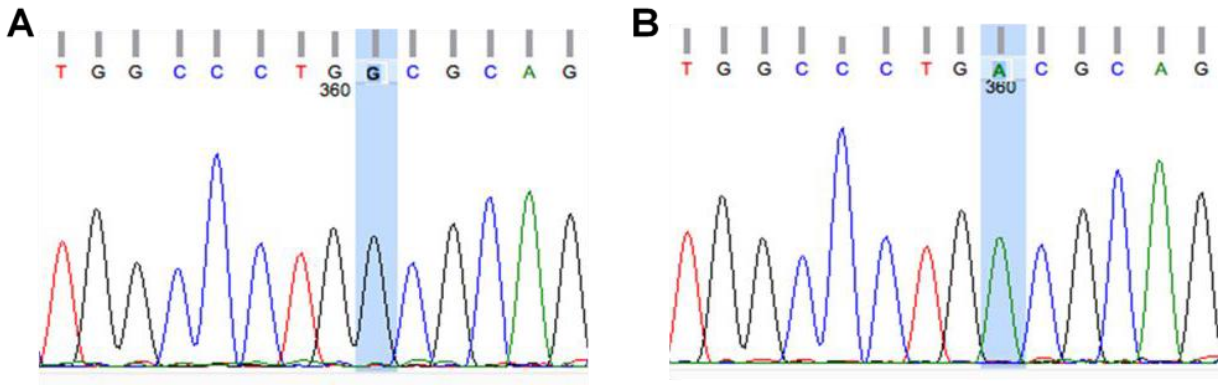

Figure 9: A) Sequencing of the FUT1 gene in the original cell population (FUT1-GG genotype, highlighted in blue), **B)** Sequencing of the FUT1 gene after transfection with the CRISPR/Cas9 FUT1G20 expression vector and ssOliFUT1 ssDNA repair template (cell pool DNA). Sequencing data reveal a distinct signal for adenosine (FUT1-AA genotype, highlighted in blue). A double peak would be observed here if there was still a quantifiable proportion of cells with the FUT1-GG or GA genotype in the cell pool).

Single cell clones with the FUT1-AA genotype were isolated and could be used as nuclear donor cells for somatic cell nuclear transfer. However, the generation of genetically modified animals was not planned within this project.

In summary, the goal of precisely and efficiently correcting a gene variant with adverse effects on animal health (FUT1-GA or FUT1-GG) by gene editing could be met. Cas9 expression vectors and DNA repair templates were designed and tested *in vitro*. In porcine somatic cells the desired G>A base exchange could be conducted with an efficiency of more than 90% which was confirmed by DNA sequencing.

5.2. *In vivo* editing of the FUT1 gene

The Cas9 expression vector and single stranded DNA repair template that had been identified and tested *in vitro* were used to perform *in vivo* gene editing in porcine embryos.

5.2.1. Preliminary experiments in parthenotes

The efficiency of gene editing and embryotoxicity was assessed using parthenogenetically activated embryos. Ovaries were sourced from a local slaughterhouse, oocytes were extracted and matured *in vitro*. The GE vector (5ng/µl) and DNA repair template (10ng/µl) were microinjected into the cytoplasm of 100 *in vitro* matured oocytes followed by parthenogenesis and *in vitro* cultivation. 100 non-injected embryos were parthenogenetically activated and cultivated as a control group. In the microinjected group 29/100 embryos developed to the blastocyst stage compared to 42/100 in the control group. The efficiency of gene editing was analysed by isolating DNA from each blastocyst followed by PCR amplification across the target site and DNA sequencing.

In the microinjected group the FUT1-AA genotype was detected in 27/29 embryos (93%). However, in the non-injected control group the FUT1-AA genotype could also be detected in 28/42 embryos (66.6%). This shows that within the local pig population a higher proportion of animal than previously anticipated based on the literature already had the resistant FUT-1 AA genotype.

The evaluation of sequencing data from 12569 pigs revealed allele frequencies for the FUT1-AA, -AG and -GG genotypes of Piétrain (PI) (7%/41%/52%), Deutsches Edelschwein (DE) (44%/41%/15%) and German Landrace (DL) (0%/11%/89%). The incidence of the FUT1-AA genotype was higher than previously known, particularly in PI (7%) and DE (44%) (unpublished data, Prof. Fries, Chair of Animal Breeding, TUM).

5.2.2. Editing of the FUT1 gene in IVF zygotes

5.2.2.1. Identification of FUT1-GG sperm isolates for IVF

The FUT1 genotype of *in vitro* matured oocytes is unknown because they are sourced from a diverse group of donor animals at the slaughterhouse. Therefore, FUT1 GG sperm is required for IVF to prevent the generation of FUT-1 AA embryos by breeding. In total, 15 samples of frozen sperm were obtained from Bayern Genetik. DNA was isolated by phenol-chloroform extraction followed by PCR amplification across the target site and DNA sequencing (see Table 13).

Table 13: FUT1-Genotype of 15 sperm isolates.

Name	Breed	FUT1-genotype
Cadura	Piétrain	GG
Fadros	Piétrain	AG
Fadros	Piétrain	AG
Iberico	Piétrain	GG
Madura	Piétrain	AG
Mozzi	Piétrain	GG
Orloki	Large White	AG
Pablura	Piétrain	GG
Ryder	Large White	AA
Wadtbandt	Piétrain	AG
Wadthose	Piétrain	AA
Wadtlist	Piétrain	AG
Wadtpill	Piétrain	GG
Wadttext	Piétrain	AG

Out of 15 sperm isolates, two were FUT1-AA, eight FUT1-AG and six FUT1-GG. Sperm from all six FUT1-GG boars was examined for its IVF suitability. The best results (16% blastocyst development) were obtained using sperm from the boar Cadura which was therefore used for all further experiments.

5.2.2.2. Gene editing in IVF zygotes

IVF was conducted (as described in 4.2.3.4.) using FUT1-GG sperm followed by microinjection of the Cas9 expression vector and single-stranded DNA repair template that had been previously tested in cell culture and parthenotes. 100 microinjected embryos and a control group of 100 non-injected embryos were cultivated for 6 days. In the microinjected group 16/100 embryos and in the control group 20/100 embryos developed to the blastocyst stage (see Figure 10).

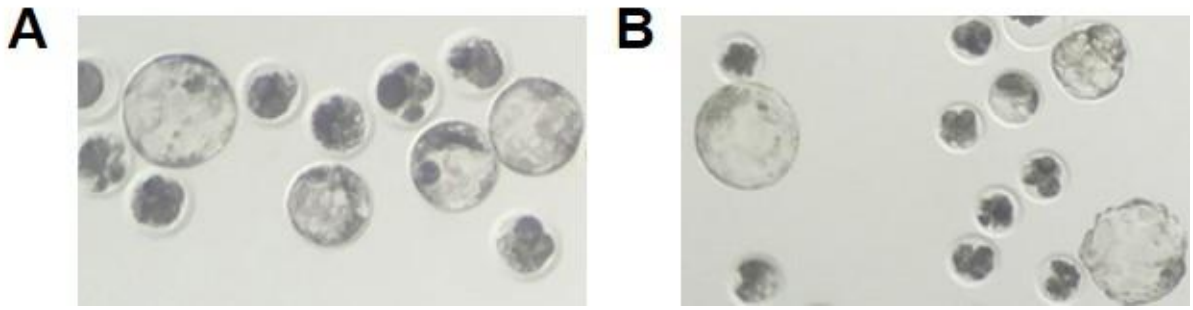

Figure 10: A) Blastocyst development after IVF and microinjection with the gene editing vector and ssDNA repair template. **B)** Non-injected control group

The FUT1 genotype of the blastocysts was determined by performing DNA isolation followed by PCR amplification across the target site and DNA sequencing. In the injected group 15/16 blastocysts (94%) had the FUT1-AA and one blastocyst (6%) the FUT1-AG genotype (see Figure 13). In the non-injected control group, six blastocysts (30%) had the FUT1-AG and 14 blastocysts (70%) the FUT1-GG genotype but as expected no FUT1-AA blastocysts were observed.

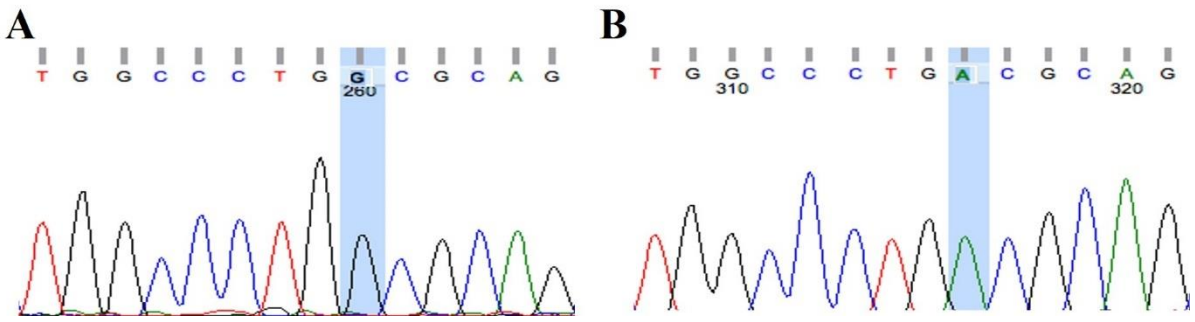

Figure 11: A) Sequencing of the FUT1 gene in a blastocyst from the non-injected control group (FUT1-GG genotype). **B)** Sequencing of the FUT1 gene in a blastocyst after microinjection with the GE vector plus repair template (FUT1-AA genotype).

In summary, efficient in vivo gene editing was successfully conducted in porcine zygotes. However, preliminary work in parthenotes revealed that the frequency of the FUT1-AA genotype within the local pig population was higher than anticipated based on the literature. These findings were later confirmed by new sequencing data provided by the Chair of Animal Breeding, TUM. Based on these facts no further *in vivo* experiments were conducted to edit the FUT1 gene.

5.3. Off-target analysis by two-cell-stage microinjection

Off-target mutations are usually analysed by screening of *in silico* predicted off-target sites or by WGS [87]. The fundamental problem with this these approaches is the differentiation between off-target mutations caused by the CRISPR/Cas9 system and natural mutations that occur during embryonic development (~60-80 in each individuum) [12].

Here, mosaic foetuses containing both edited and non-edited cells were generated by two-cell-stage microinjection. Both cell types were separated and analysed by whole genome sequencing (WGS) to facilitate the comprehensive analysis of off-target mutations. The comparison between edited and non-edited cells from the same individual promises a precise differentiation between off-target mutations and natural mutations. Figure 12 schematically depicts the experimental setup.

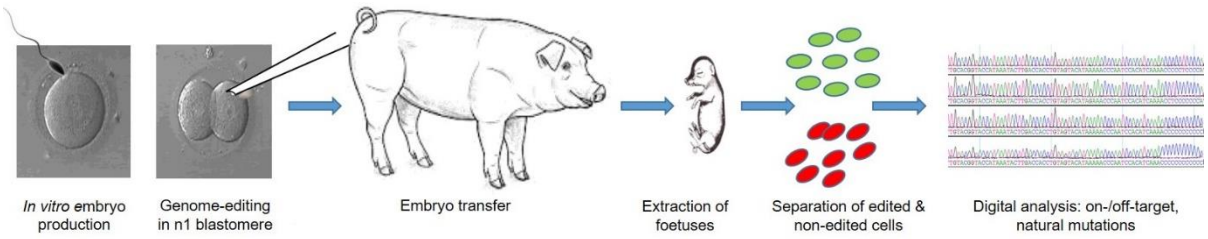

Figure 12: Genome-wide „off-target“ analysis by microinjection into two-cell stage embryos. Mosaic foetuses were generated by two-cell stage microinjection followed by embryo transfer. The pregnancy was aborted, foetuses were extracted and edited, and non-edited cells were separated. The Frequency of on-target, off-target and natural mutations was analysed.

5.3.1. Generation of mosaic foetuses by two-cell stage microinjection

The GGTA1 gene which codes for the enzyme α-1,3 galactosyltransferase was chosen as the target for gene editing. Inactivation of the GGTA1 gene leads to a loss of the α-1,3-galactosyl (α-Gal) cell surface epitope. This allows the precise separation of the α-Gal positive (non-edited) and α-Gal negative (edited) cell populations. The required Cas9 expression vectors had been previously tested and successfully used to generate α-Gal negative pigs (provided by Dr. Konrad Fischer, Chair of Livestock Biotechnology, TUM) [130].

Ovaries were collected at a local abattoir, oocytes were extracted, *in vitro* matured, and *in vitro* fertilised. The zygotes were cultivated for 24 hours until they had reached the two-cell stage. The CRISPR/Cas9 expression vector was delivered into only one of two blastomeres by intracytoplasmic microinjection (see Figure 13).

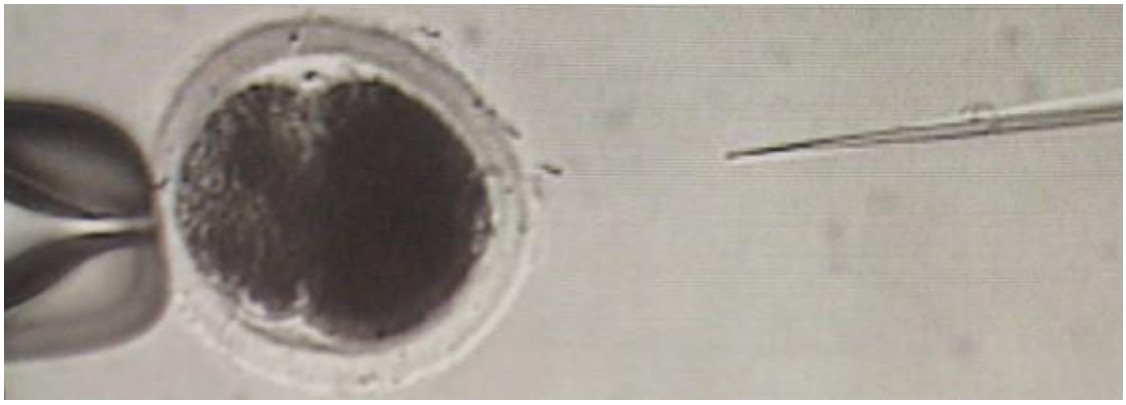

Figure 13: Two-cell stage microinjection. To generate mosaic foetuses the CRISPR/Cas9 expression vector was delivered to one blastomere of a two-cell stage embryo.

Two embryo transfers were conducted in which 160 - 180 microinjected embryos were transferred to hormonally synchronised recipients. One pregnancy was sonographically confirmed on day 28. The sow was euthanised on day 38 and 14 foetuses were extracted (see Figure 14).

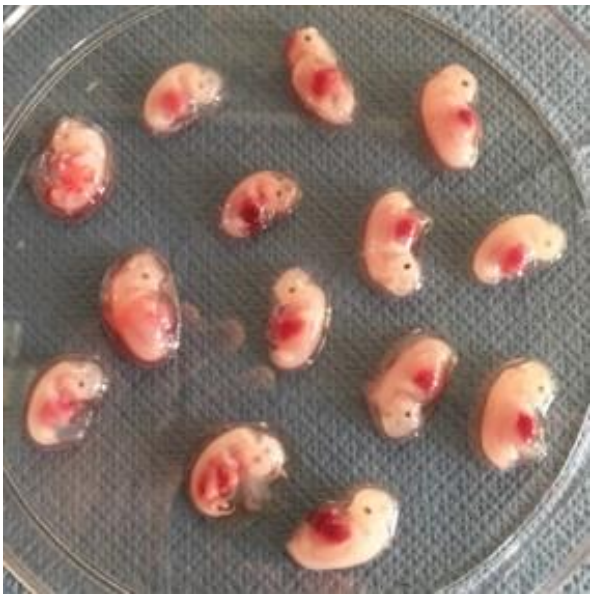

Figure 14: Fourteen foetuses resulting from two-cell stage microinjection.

Porcine foetal fibroblasts were isolated from each foetus. DNA was extracted from each individual cell population, followed by PCR amplification across the target site and DNA sequencing to ascertain the genotype of the embryos.

Eight foetuses were wild type and the GGTA1 gene was modified in 6/14 foetuses (42%). Three of the gene edited foetuses had a homozygous inactivation of the GGTA1 gene and three foetuses were mosaic as desired.

5.3.2. Separation of edited and non-edited cell populations

Edited and non-edited cell populations from each individual mosaic embryo were separated by magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS).

5.3.2.1. Fluorescence-activated cell sorting

FACS was performed to separate the different cell populations within each mosaic embryo (carried out by Kristiyan Kanev, Chair of Animal Physiology and Immunology, TUM). An efficient separation of α-Gal positive and negative cells could be achieved but FACS reduced cell viability (see Figure 15).

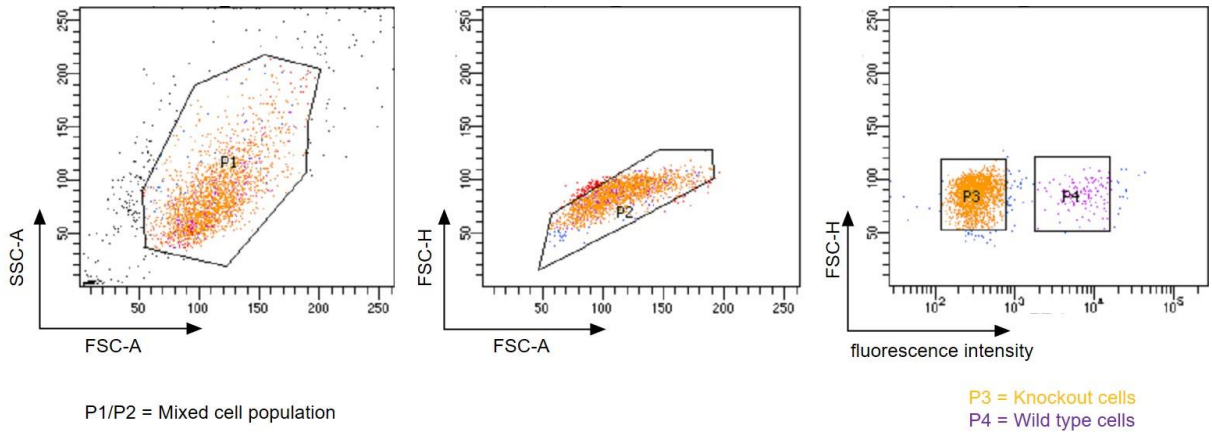

Figure 15: Separation of α-Gal positive and negative cells via FACS. From left to right: Gating of cell populations. P1/P1: mixed cell population; P3: α-Gal negative (GGTA1 knockout) cells; P4: α-Gal positive (wild type) cells

5.3.2.2. Magnetic-activated cell sorting

MACS was established as a method for the separation of α-Gal positive and negative cell populations because the viability of cells after separation was higher with this method compared to FACS. Cells with intact α-Gal glycosylation were marked with biotinylated Isolectin B4 that binds to streptavidin coated magnetic beads. Separation of cell populations was conducted under a magnetic field. The efficiency of separation was analysed by TIDE analysis (see Figure 16).

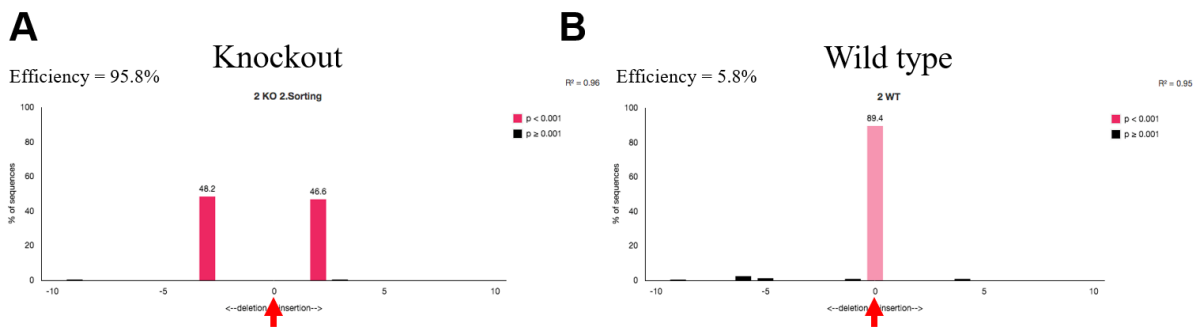

Figure 16: Analysis of indel mutations at the target site in two different cell populations after magnetic-activated cell sorting. **A)** Cells with an inactivation of the target gene **B)** Wild type cells

A high rate of indel mutations (95.8%) was ascertained in the knockout cell group but not in the wild type population. These results indicate that both MACS and FACS facilitate the efficient separation of cell populations with high purity. The viability of cells was higher after MACS which was therefore used for all further experiments. Cells from all three mosaic foetuses were separated with this method and used for genome sequencing to analyse the frequency of off-target mutations caused by CRISPR/Cas9 mediated gene editing.

5.3.3. Analysis of off-target mutations

A comparative analysis of edited and non-edited cell populations was performed by WGS at mean coverages of 30 times (conducted by Dr. Christine Wurmser, Chair of Animal Breeding, TUM). The α-Gal$^-$ and α-Gal$^+$ cell populations of embryos number 5 and six were analysed. The amount of DNA that could be isolated from embryo number 2 was not sufficient to establish a library for WGS because only 6% of cells were wild type. SNVs and indel mutations were identified using three different variant calling algorithms (Lofreq, Strelka and Mutect2 for SNVs and in addition Scalpel for Indels). The intersection of three algorithms were considered as true SNVs and the intersection of four algorithms as true indel variants.

WGS analysis revealed a low level of α-Gal$^-$ cells in the wild type group probably due to false-negative cell sorting (<5%). PCR amplification was conducted prior to WGS to obtain the necessary amount of DNA for library preparation. Therefore, only variants with allele frequencies >60% were considered in the analysis to exclude false positive results. In embryo number five 65 SNVs and 1 indel mutations (at the target site) and in embryo number six 17 SNVs and 1 indel mutation (at the target site) were found. The comprehensive data of SNV and indel analysis are shown in Appendix 10.1. The amount of SNVs is shown in Figure 17 and Indel Mutations are depicted in Figure 18.

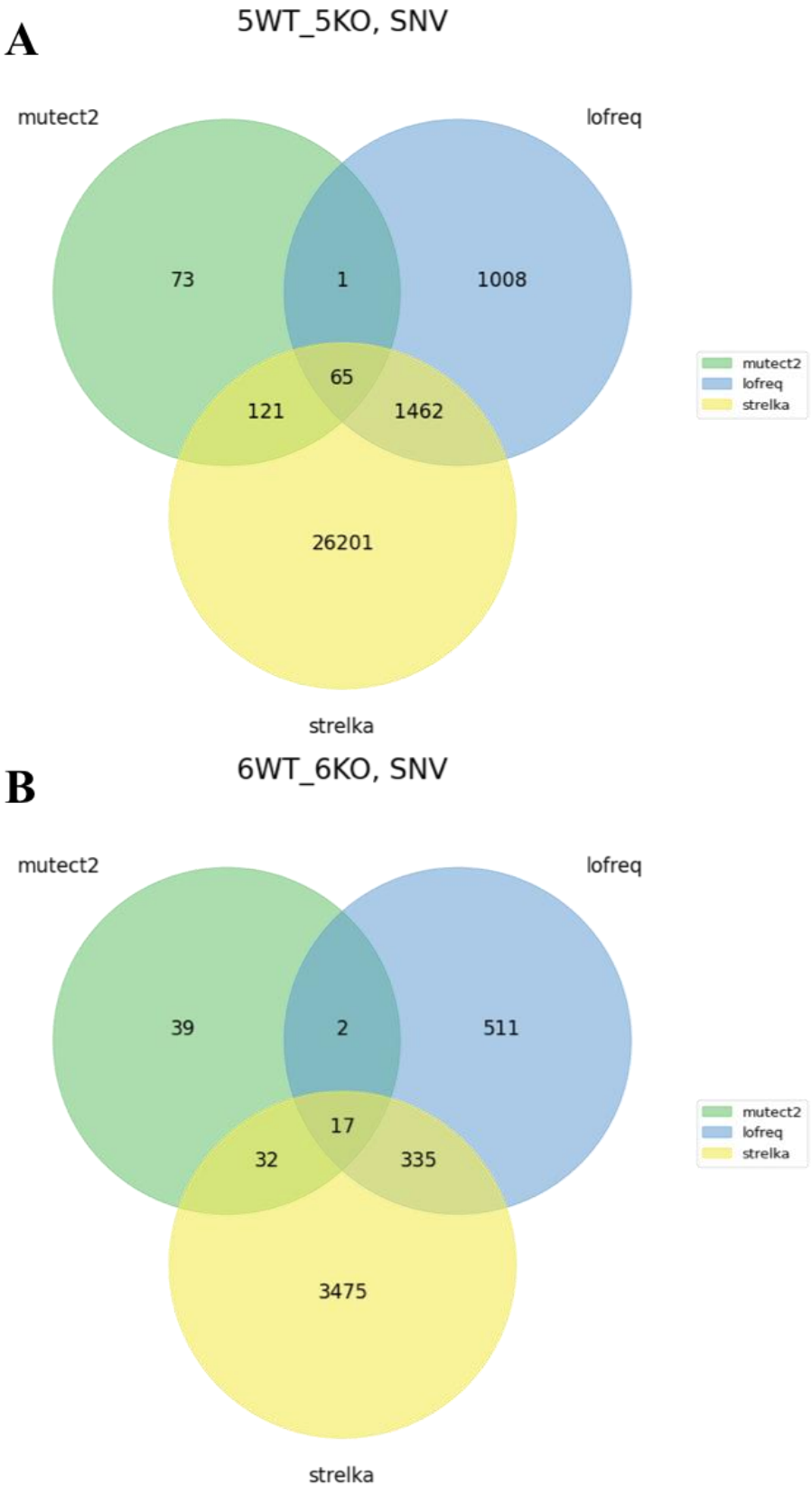

Figure 17: Comparative analysis of SNVs **A)** Embryo number 5; **B)** Embryo number 6; The overlap between all three algorithms were considered true SNVs. Only variants with an allele frequency >60% were considered.

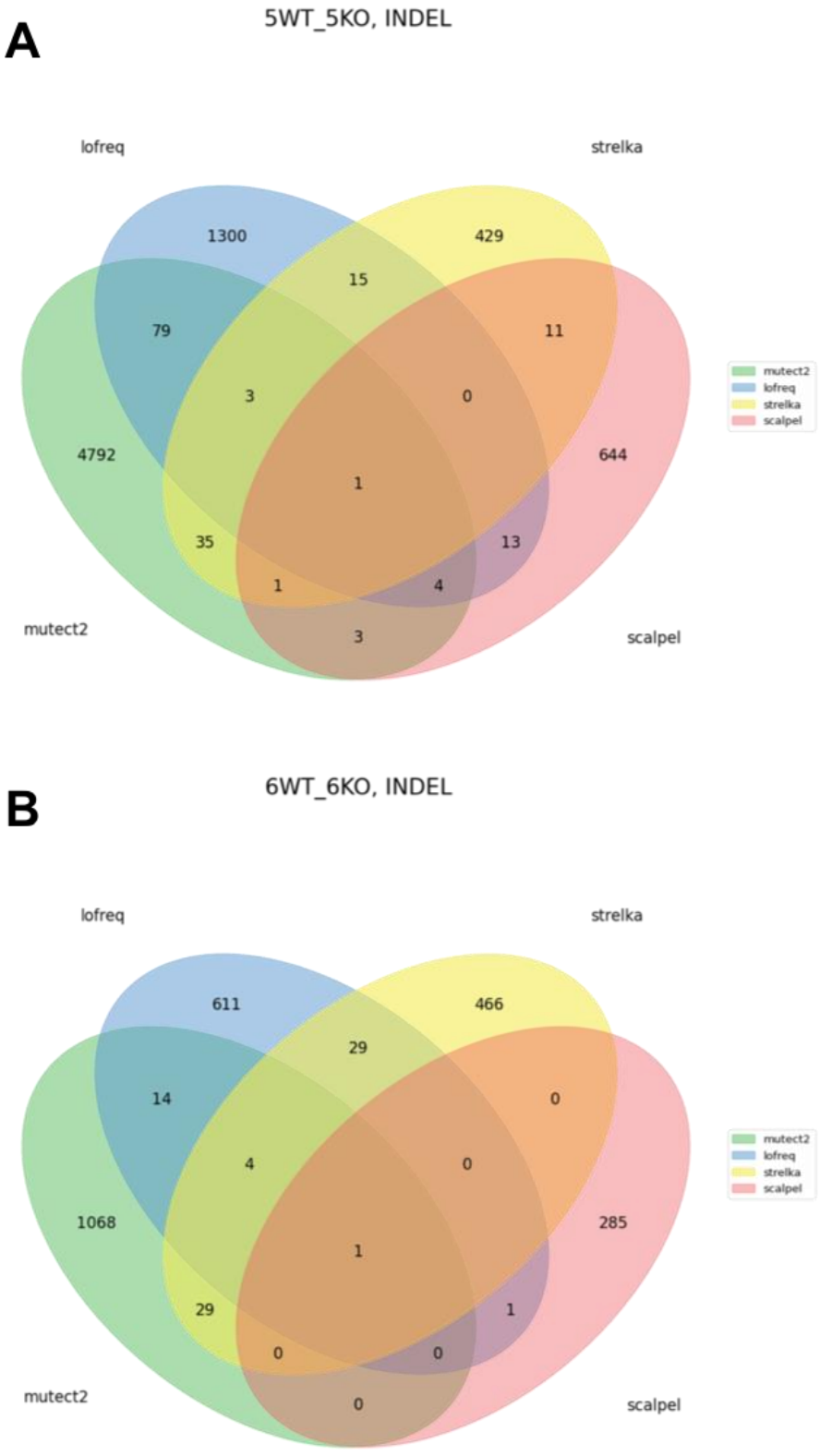

Figure 18: Comparative analysis of indel mutations **A)** Embryo number 5; **B)** Embryo number 6; The overlap between all four algorithms were considered true indels. Only variants with an allele frequency >60% were considered.

All somatic mutations were compared with the 14 most common off-target sites predicted by the CC-Top tool (see Table 14) but no sequence alignment was detected.

Chromosome	Start	End	Target Sequence
1	261513789	261513809	ATGGTGGATGATATCTCC
3	65036737	65036757	TTGATGAATGATATCTCC
4	96093961	96093981	GTGGGGGCTGATATCTCC
1	160233447	160233467	AAGGTTGAAGATATCTCC
6	35239072	35239092	AAGATGGATAATATCTCC
8	73016080	73016100	GTGGTTGATGGTATCTCC
5	60921081	60921101	AGAGTGGATGAAATCTCC
8	68667083	68667103	AAGCTGGATGAGATCTCC
15	37912014	37912034	AGGCTGGATGACATCTCC
14	45132914	45132934	ATGCTGGATGATTTCTCC
9	53893405	53893425	GTGATGGATGATTTCTCC
1	114459908	114459928	ATGGGAGATGATATATCC
14	100405146	100405166	CTGGGGGATGATATCTTC
3	121616432	121616452	ATCGTTGATGATATCTGC

Table 14: The 14 most common off-target sites predicted by the CC-Top tool. No sequence alignment was detected between the somatic mutations and the predicted off-target sites.

All cell populations were screened for the integration of the plasmid vector. None of the alignments with the plasmid sequence was found to be above the sequencing background noise (see Figure 19). The plasmid sequence could not be detected in any cell population.

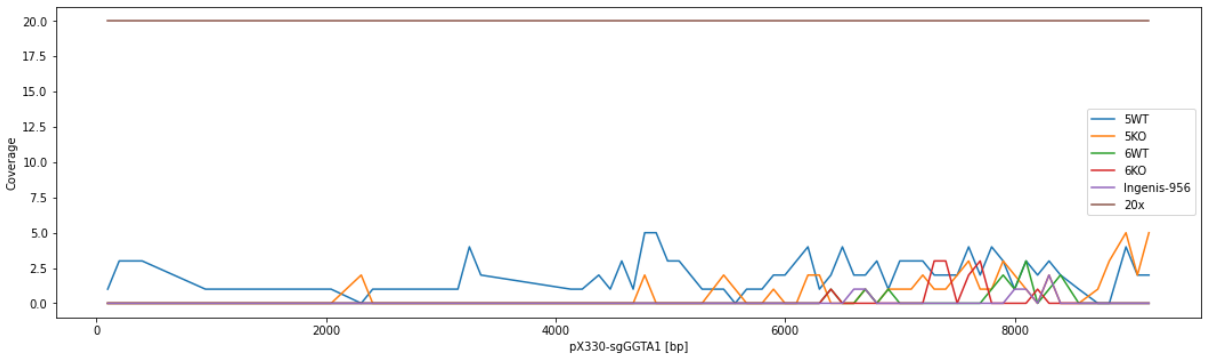

Figure 19: Sequence alignment with the plasmid sequence. The plasmid sequence could not be detected in any cell population. All alignments are below the background sequencing noise (brown line). Ingenis-956 is the pig reference genome.

In summary, mosaic foetuses containing edited and non-edited cells were generated by intracytoplasmic microinjection of CRISPR/Cas9 vectors targeting the GGTA1 gene into one blastomere of *in vitro* derived porcine two-cell-stage embryos. Embryos were surgically transferred into synchronised surrogate pig mothers, one pregnancy was established and aborted on day 38. Fourteen foetuses were isolated and 3 of them were identified as mosaic. Edited and non-edited cell populations within those individuals were separated using magnetic beads, followed by WGS. The non-edited cells were used as the control group to distinguish naturally occurring mutations from off-target mutations. In embryo number five 65 SNVs and one indel mutation were detected and in embryo number six 17 SNVs and one indel mutation at the target site were detected. None of the somatic mutations aligned with the predicted off-target sites and no integration of the plasmid sequence was detected.

6. DISCUSSION

The major goal of this work was to evaluate the feasibility, efficiency, and safety of gene editing in pigs. Section 6.1 discusses the modification of the FUT1 gene and gene editing as a tool to improve animal welfare. The analysis of off-target mutations by two-cell stage microinjection is examined in segment 6.2.

6.1. Gene editing as a tool to improve animal welfare

Globalisation, falling prices and increasing demands on animal welfare coupled with novel threats for animal health imposed by climate change (heat stress, spread of pathogens) pose new challenges for modern livestock production [2]. Animal genetics play a decisive role for animal health, but traditional livestock breeding is limited by long breeding cycles and the availability of genetic resources [131]. Gene editing could allow us to overcome these bottlenecks by facilitating the incorporation of genetic traits from other breeds, related species or laboratory findings [46].

6.1.1. Practical implications of gene editing for animal welfare

Due to the lack of germline transmittable embryonic stem cells gene targeting was extremely challenging in livestock species in the past [132]. Modern gene editing technology facilitates the efficient generation of gene targeted farm animals [133]. In the future gene editing could supplement traditional breeding techniques to meet the goal of producing healthier and more productive livestock [134].

The goal of this project was the establishment and evaluation of methods for the base-specific, efficient, and safe modification of the porcine genome by the example of Oedema Disease. We were able to precisely and efficiently (>90%) perform the desired G to A base exchange *in vitro* in somatic cells using a CRISPR/Cas9 expression vector and ssDNA repair template. The *in vivo* experiments in porcine zygotes confirmed the efficiency of this approach. Most publications describe *in vivo* targeting efficiencies of about 60-70% [66, 135]. Here, the desired FUT1 AA genotype could be confirmed in 93% of parthenotes and 94% of IVF zygotes after

microinjection. These results compare favourably to the literature and confirm that base specific edits can be performed *in vitro* and *in vivo* with high efficiencies. Differences in the effectiveness of gene editing at different genomic loci can be explained by chromatin state and secondary sgRNA structure [136].

In the case of the FUT1 gene one single genetic modification mediates Oedema Disease resistance. However, many traits of interest are polygenic and require multiple edits simultaneously to achieve the desired effect which is technically more challenging [62]. Multiplex gene editing can be conducted in one step using multiple sgRNAs, but each edit is a separate stochastic event which decreases the likelihood of obtaining all desired modifications simultaneously [58, 135]. Therefore, somatic cell nuclear transfer (SCNT) is the method of choice for multiplex gene editing because it allows for the selection and screening of donor cells prior to the generation of animals to ensure they carry the desired set of modifications [137].

Analysis of wild type embryos revealed that the FUT1-AA genotype appeared at a much higher frequency within the local pig population than previously known. Based on comprehensive literature research the favourable allele was expected to be prevalent at a frequency of 5-10% [39, 46]. Our results and new sequencing data indicate that selection for the favourable FUT1-AA allele was successful in Deutsches Edelschwein (DE, 44%) but this was not the case in German Landrace (DL, 0%) and Piétrain (PI, 7%). These data demonstrate that breeding for OD resistance in DE, particularly in Switzerland was successful beyond expectations. A similar timeframe of several decades would be necessary to increase the frequency of the desirable allele in other breeds (PI, DE) to similar levels. Gene editing has the potential to accelerate this process significantly.

The high frequency at which the FUT1-AA allele was ascertained in slaughterhouse derived oocytes (66%) is plausible as they stem from hybrid pigs containing DE genetics (DLxDExPI) [138]. The low frequency at which the favourable FUT1 allele occurs in DL and PI pigs makes the incorporation of effective selection into standard breeding regimes hard due to the risk of inbreeding and associated long-term productivity loss [139]. Genome editing could contribute in such circumstances by allowing the introduction of the favourable gene variant into a specific breeding population in one generation while keeping other desirable traits unsolicited [131, 140]. However, a large number of genetically modified founder animals needs to be generated to avoid inbreeding and maintain background genetic variation [139].

6. DISCUSSION

In the context of disease resistance, a clear distinction must be made between full resistance and disease resilience. The generation of pigs resilient to African Swine Fever Virus infection by interspecies allele introgression using gene editing is exemplary [141]. Resilient animals could act as reservoirs of infection and therefore their use would be prohibited in many jurisdictions [46]. However, such animals could be useful in regions where the specific disease is endemic.

One potential risk that gene editing has in common with other disease mitigation strategies such as vaccination is the limited shelf life of disease resistance due to the emergence of escape mutants [142]. This is especially problematic for pathogens with extremely high mutation rates such as RNA viruses like PRRSV [143] but also a justified concern for genetically mediated OD resistance.

Gene editing has the potential to solve many problems encountered in traditional livestock breeding but with any new technology, caution should be taken prior to its large-scale application. Tracking and registration of gene edited animals are difficult because the changes are footprint-free [144]. However, unlike genetically modified crops gene edited farm animals are easier to contain which prevents the dispersal of contaminated altered genes from genetically engineered organisms to natural organisms [145].

All current gene editing technologies (ZFNs, TALENs, CRISPR/Cas9) have the potential to induce off-target mutations in the genome [93, 98, 146]. These can harm the health of individuals which is a major obstacle for therapeutic gene editing [147]. Therefore, is imperative to carry out a thorough phenotypic and genotypic characterisation of gene edited animals. Another concern is the possibility of unwanted integration of the gene editing vector into the genome. This risk can be eliminated by using Cas9 mRNA or protein together with the gRNA instead of a gene editing vector encoding both components [62].

Low public acceptance and the regulatory framework are the biggest obstacles for gene editing in livestock species. Approval of genetically modified animals is handled differently in each jurisdiction and depends on legislation which is currently still in its early stages [148]. Some jurisdictions such as Argentinia or Brazil have ruled that modifications that do not require the integration of new genetic material into the genome are exempt from regulation [149]. In the US gene edited livestock are regulated as drugs. The approval of the first gene edited animal for sale on the open market (AquAdvantage® salmon) took 25 years [150]. The recent approval of GalSafe® GGTA1 knockout pigs by the FDA might pave the way for other gene edited livestock [151]. The European Court of Justice ruled that organisms derived by gene editing are

subject to the same regulations as genetically modified organisms (GMOs) [149]. Barring a change in legislation, this makes the approval of genetically modified livestock for sale in the European Union unlikely for the foreseeable future [149].

6.1.2. Ethical considerations

Certain genetic modifications align animal welfare and economic interests because they improve animal health and increase productivity simultaneously. Disease resistant animals such as PRRSV resistant pigs [152], Avian Leukosis Virus resistant chickens [153, 154], or tuberculosis resilient cattle are exemplary [155]. Pigs resistant to Oedema Disease fall into this "win-win" category because the disease-resistant genotype which occurs naturally within the pig population is not associated with any adverse phenotypic consequences [39]. Genetic modifications that allow animals to avoid undergoing painful procedures such as dehorning [156] or castration [157] also fall into this "win-win" category.

Gene editing can also be used to improve livestock performance, but this often contradicts animal welfare. One example is the generation of cattle [140], goats [158], and pigs [159] with dramatically elevated muscle growth caused by the inactivation of the myostatin gene. These animals suffer from gastric ulcers, high stress sensitivity, and lameness due to abnormal leg development which highlights the limitations of this approach [160].

The principle for the conservation of welfare formulated by the philosopher Bernard Rollin in 1995 states: "Any animals that are genetically modified through the use of genetic technology, for purposes other than research, should be no worse off, in terms of suffering, than the parent stock was prior to genetic alterations" [161]. Both, gene editing and traditional animal breeding have the potential to create phenotypes that result in suffering and lead to health problems e.g., fast-growing broilers [162]. Increasing performance at the expense of animal welfare is highly questionable from an ethical and veterinary perspective. [163].

6. DISCUSSION

In summary, gene editing facilitates the efficient and precise modification of the genome in livestock species. However, many relevant traits are polygenic which requires multiplex gene editing. Genetically mediated disease resistance has similar weak spots as other disease mitigation strategies i.e., mutation of pathogens. Gene editing to improve animal welfare is ethical if the animals are no worse off in terms of suffering than prior to the genetic alteration. The biggest obstacles for gene edited livestock are low consumer acceptance and the regulatory framework.

6.2. Off-target analysis by two-cell stage microinjection

Traditional methods for off-target analysis are based on screening of *in silico* predicted off-target sites or whole genome sequencing. These approaches fail to distinguish between single nucleotide variants (SNVs) that occur in each individual during embryogenesis and off-target mutations caused by gene editing technology [117, 164]. Our goal was to detect off-target mutations by editing one blastomere of two-cell stage pig embryos followed by whole genome sequencing. Comparison of sequencing data between progeny cells of edited and non-edited blastomeres allows the differentiation between SNVs and off-target mutations *in vivo*.

This approach termed genome-wide off-target analysis by two-cell embryo injection (GOTI) was developed in mice [12]. The original study was conducted in Ai9 mice (B6.Cg-Gt ROSA 26Sortm9, CAG-td-Tomato) with conditional tdTomato expression silenced by a floxed “stop” cassette. The conditional mutation was activated by co-injection of Cre mRNA together with the components of various gene editors to achieve tdTomato expression and later facilitate cell separation by FACS. A similar reporter pig line with constitutive expression of a red fluorescence marker (Tomato) and Cre-inducible expression of a green fluorescence marker (eGFP) is available but no pregnancies could be obtained using sperm with this genotype for IVF. The GGTA1 gene was chosen as an alternative target for gene editing because the presence or absence of the α-Gal surface glycosylation facilitates the precise and unequivocal separation of cell populations [14]. Another advantage of this approach is that it does not depend on the availability of transgenic reporter lines with conditional expression of fluorescent proteins and can be performed in species where such animals are not available.

The fact that 14 foetuses were obtained suggests high viability of embryos after microinjection at the two-cell-stage. All embryos were injected with the gene targeting vector but still, eight wild type embryos were obtained. This means that either no gene editing had taken place, the blastomere was damaged beyond repair during microinjection, or simply did not contribute to the embryo proper [165]. The homozygous inactivation of the GGTA1 gene in three embryos implies that only the edited blastomere contributed to the embryo proper. In the three mosaic foetuses edited and non-edited blastomeres contributed to the embryo proper during embryogenesis.

Two different methods were established for the separation of the different cell populations. Both magnetic- and fluorescence assisted cell sorting resulted in the separation of α-Gal positive and negative cells with high purities (>95%). However, consistent with the literature the magnetic approach resulted in higher viability of cells after separation [166].

There are many gene editing tools and nucleases and new ones are developed at a rapid pace [167]. A comprehensive safety evaluation including off-target analysis is mandatory prior to the application of each new technology [10]. In the future, advances in gene editing technology could improve the specificity and safety of gene editing to facilitate its large scale agricultural and therapeutic application [11]. The required level of comprehensiveness of off-target analysis depends on the intended application of gene editing.

For research, the primary concern is whether off-target mutations might confound the interpretation of biological phenotypes. This can be ruled out by performing control experiments such as using multiple sgRNAs to introduce the same mutation [117]. For clinical applications of gene editing safety is the predominant concern because even low mutation frequencies can lead to detrimental outcomes. Therefore, the most sensitive, comprehensive, and unbiased approach should be used to identify all potential off-target sites [168]. Multiple redundant approaches are required to analyse off-target effects for this purpose because a fully comprehensive and sensitive method currently does not exist [117]. Off-target mutations are a concern for the use of gene editing as a novel breeding tool in farm animals as well. The large-scale utilisation of gene edited animals with adverse phenotypes caused by off-target mutations would have far reaching economic consequences. However, over time such mutations would likely disappear by genetic drift or be selected against if they result in adverse phenotypic consequences [131].

The original study revealed that cytosine base editors generate a substantial number of off-target mutations in mice but for the CRISPR/Cas system off-target events occur at a frequency close to the spontaneous mutation rate (on average 12 SNVs per embryo) [12]. These findings are consistent with a similar study conducted in rice plants [169].

We detected 17 and 65 SNVs per embryo which is within the rate of approximately 60-80 mutations that naturally occur during embryogenesis [170]. These findings agree with a clone-based study in cattle that compared the mutation frequency and spectra in bovine cells and calves cloned from these edited and non-edited cell lines [171]. The study showed that gene

edited cells and calves did not carry a higher mutation load than the non-edited control group [171].

A recent study revealed that even monozygotic twins differ on average by 5 early developmental mutations and that 15% of them have a high number of mutations specific to only one twin [172]. A median number of 48 postzygotic mutations specific to only one twin was detected in high coverage (152x) pairs [172]. This indicates that while cells from the same embryo are the best possible control group available there is still the possibility of genomic sequence differences between them [172].

Another possible explanation is the short period of *in vitro* cultivation which was necessary to remove cell detritus prior to sorting. *In vitro* cultivation is known to cause somatic mutations in mammalian cells at a rate ranging from $2x10^{-6}$ to $0.7x10^{-7}$ [173, 174]. Due to population averaging these can be removed from the analysis by only considering SNVs with high allele frequencies. However, amplification of a next generation sequencing library is performed by PCR [175]. This is a potential source of mutations because even high fidelity polymerases have a certain error rate [176]. This step can introduce artefacts into sequencing libraries by amplifying natural mutations and increases their allele frequency [175]. Therefore, only variants with an allele frequency >60% were considered in the analysis.

PCR-free WGS avoids these drawbacks and is the method of choice for future GOTI experiments [177]. However, because the required, larger amount of sample DNA was not available only traditional PCR based WGS could be performed here. No sequence similarity was observed between the adjacent sequences of the SNVs and the predicted off-target sites. Indel mutations were only observed at the target site. Therefore, it is likely that some SNVs observed here are artefacts caused by a combination of *in vitro* cultivation or spontaneous mutation in conjunction with PCR amplification rather than true off target mutations.

In summary, we provide first proof of principle that GOTI is a useful method to analyse the off-target effects caused by gene editing tools in livestock without the interference of SNVs. The rate of mutations detected here is close to the natural mutation rate and none of the SNVS aligned with the predicted off-target sites. Therefore, it is highly likely that they are artefacts caused by PCR amplification during library generation. PCR-free WGS technology could facilitate a more precise evaluation of off-target mutations in the future. A final statement

regarding the frequency of off-target mutations cannot be made because even cells from the same embryo have certain genomic differences.

6. DISCUSSION

7. SUMMARY

Efficiency and safety of gene editing in pigs: Evaluated in an experimental and in a disease relevant model

In this thesis, the feasibility, efficiency, and safety of gene editing technology were analysed in the pig. Gene editing has the potential to complement traditional breeding techniques and help improve animal health and welfare. However, concerns regarding the safety of this new technology prevent its application outside of research.

Because pigs are physiologically very similar to humans and play an important role in agriculture, they are a suitable model to analyse the potential and risks of gene editing for applications in agriculture and potential therapeutic applications in patients. In this study the FUT1 gene which mediates resistance to Oedema disease was edited *in vitro* in somatic cells and *in vivo* in porcine embryos. The desired base specific G>A nucleotide exchange at position 307 was performed precisely and efficiently using CRISPR/Cas9 expression vectors in combination with ssDNA repair templates. The generation of OD resistant animals by GE was not planned within the scope of this project.

The second part of this project focused on the investigation of off-target mutations caused by CRISPR/Cas9 technology, the most widely used GE tool. One blastomere of porcine two-cell-stage embryos was microinjected with CRISPR/Cas9 expression vectors to generate mosaic foetuses. The edited and non-edited cell populations from two mosaic foetuses were separated and analysed by whole genome sequencing. This approach facilitates the differentiation between natural mutations that occur during embryogenesis and off-target mutations caused by gene editing technology. The frequency of SNVs in embryos edited by CRISPR/Cas9 was higher than expected but within the spontaneous mutation rate. Because none of the mutations aligned with the predicted off-target sites they are likely artefacts caused by PCR amplification during library generation. A more precise evaluation of off-target mutations could be obtained by avoiding the cell culture step and by utilising PCR-free Next Generation Sequencing technology. Cells from the same embryo are the best possible control group but a final distinction between off-target effects and natural mutations remains difficult because even these have certain genomic differences.

7. SUMMARY

8. ZUSAMMENFASSUNG

Effizienz und Sicherheit der Genom-Editierung beim Schwein: Evaluierung in einem experimentellen und einem krankheitsrelevanten Model

In dieser Arbeit wurde die Machbarkeit, Effizienz und Sicherheit der Genom-Editierung beim Schwein analysiert. Genom Editierung könnte in Zukunft traditionelle Züchtungsmethoden ergänzen und somit zur Verbesserung der Tiergesundheit und des Tierwohls beitragen. Allerdings verhindern Zweifel bezüglich der Sicherheit dieser neuen Technologie ihre Anwendung außerhalb der Forschung.

Schweine sind Menschen physiologisch sehr ähnlich und spielen eine wichtige Rolle in der Landwirtschaft. Deshalb sind sie ein geeignetes Model, um die potenziellen Risiken der Genom-Editierung vor einer möglichen Anwendung in der Landwirtschaft oder Humanmedizin zu erforschen. Hier wurde das FUT1 Gen, welches die Resistenz gegenüber der Ödemkrankheit vermittelt *in vitro* in somatischen Zellen und *in vivo* in Schweine Embryos editiert. Der erwünschte G>A Basenaustausch an Position 307 konnte präzise und effizient durchgeführt werden. Hierzu wurden CRISPR/Cas9 Expressionsvektoren in Verbindung mit einzelsträngigen DNA-Reparaturvorlagen verwendet. Die Generierung gegen Ödemkrankheit resistenter Tiere mittels Genom-Editierung war im Rahmen dieses Projektes nicht vorgesehen.

Der zweite Teil dieses Projektes beschreibt die Erforschung von Off-Target Mutationen durch CRISPR/Cas9, das am häufigsten Verwendete Werkzeug für die Genom-Editierung. Eine Blastomere eines Zwei-Zell Embryos wurde mit CRISPR/Cas9 Expressionsvektoren mikroinjiziert, um mosaike Föten zu erzeugen. Die editierten und nicht editierten Zellpopulationen wurden separiert und durch Sequenzierung des gesamten Genoms analysiert. Diese Vorgehensweise erlaubt eine Abgrenzung zwischen natürlichen Mutationen, welche während der Embryogenese entstehen und jenen, welche durch die Genom-Editierung verursacht werden. Die Rate an Polymorphismen in den editierten Embryos war höher als erwartet, lag aber innerhalb der spontanen Mutationsrate. Keine der Mutationen stimmte mit den vorhergesagten off-target Sequenzen überein. Vermutlich handelte es sich hierbei um Artefakte, welche durch die PCR Amplifikation zur Erstellung der Sequenzierungsbibliothek verursacht wurden.

Eine präzisere Evaluierung von Off-Target Mutationen könnte in Zukunft durch Vermeidung des Zellkultur Schrittes und durch Verwendung PCR-freier Sequenzierungstechnologie erreicht werden. Zellen vom selben Embryo stellen die bestmögliche Kontrollgruppe dar, doch selbst diese weisen gewisse genomische Unterschiede auf. Deshalb bleibt eine klare Abgrenzung zwischen natürlichen und off-target Mutationen schwierig.

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10.1. Comprehensive data of Indel and SNV analysis

10.1.1. Embryo number five

CONTRAST	VAR_ID	CHROM	POS	REF	ALT	VAR_TYPE
5WT_5KO	1_19956402	1	19956402	G	A	SNV
5WT_5KO	1_19956404	1	19956404	G	A	SNV
5WT_5KO	1_100314080	1	100314080	C	T	SNV
5WT_5KO	1_100541077	1	100541077	A	G	SNV
5WT_5KO	1_114471023	1	114471023	C	T	SNV
5WT_5KO	1_233320503	1	233320503	G	A	SNV
5WT_5KO	2_86152857	2	86152857	C	T	SNV
5WT_5KO	2_89158233	2	89158233	T	C	SNV
5WT_5KO	2_151913137	2	151913137	G	A	SNV
5WT_5KO	2_151914662	2	151914662	G	A	SNV
5WT_5KO	4_11017683	4	11017683	G	A	SNV
5WT_5KO	4_41235415	4	41235415	A	T	SNV
5WT_5KO	4_83262646	4	83262646	A	C	SNV
5WT_5KO	4_91448898	4	91448898	C	T	SNV
5WT_5KO	4_122822945	4	122822945	C	T	SNV
5WT_5KO	4_122822959	4	122822959	T	A	SNV
5WT_5KO	5_28335391	5	28335391	G	A	SNV
5WT_5KO	5_63089804	5	63089804	G	T	SNV
5WT_5KO	6_19202381	6	19202381	G	A	SNV
5WT_5KO	6_98219308	6	98219308	C	G	SNV
5WT_5KO	6_153339592	6	153339592	T	C	SNV
5WT_5KO	6_153415359	6	153415359	G	A	SNV
5WT_5KO	6_153880565	6	153880565	G	A	SNV
5WT_5KO	6_156876283	6	156876283	C	T	SNV
5WT_5KO	6_168433719	6	168433719	A	G	SNV
5WT_5KO	7_28553420	7	28553420	C	A	SNV
5WT_5KO	7_50584652	7	50584652	T	C	SNV

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5WT_5KO	8_6670047	8	6670047	C	A	SNV
5WT_5KO	8_6670088	8	6670088	C	T	SNV
5WT_5KO	8_6670508	8	6670508	G	A	SNV
5WT_5KO	8_19949515	8	19949515	C	T	SNV
5WT_5KO	8_19949631	8	19949631	A	G	SNV
5WT_5KO	9_10663139	9	10663139	G	C	SNV
5WT_5KO	9_44025220	9	44025220	A	C	SNV
5WT_5KO	9_44025231	9	44025231	A	G	SNV
5WT_5KO	10_7344347	10	7344347	G	A	SNV
5WT_5KO	10_7892865	10	7892865	C	T	SNV
5WT_5KO	10_47280531	10	47280531	T	G	SNV
5WT_5KO	10_49214815	10	49214815	T	C	SNV
5WT_5KO	10_54061045	10	54061045	G	T	SNV
5WT_5KO	10_57292809	10	57292809	C	A	SNV
5WT_5KO	11_71939325	11	71939325	G	A	SNV
5WT_5KO	13_69909188	13	69909188	C	T	SNV
5WT_5KO	13_192198706	13	192198706	G	A	SNV
5WT_5KO	14_10471903	14	10471903	C	T	SNV
5WT_5KO	14_38002934	14	38002934	A	T	SNV
5WT_5KO	14_38002983	14	38002983	G	A	SNV
5WT_5KO	14_106337952	14	106337952	G	C	SNV
5WT_5KO	14_106885043	14	106885043	G	A	SNV
5WT_5KO	14_126542587	14	126542587	A	G	SNV
5WT_5KO	15_29892487	15	29892487	T	A	SNV
5WT_5KO	15_124333310	15	124333310	A	G	SNV
5WT_5KO	15_124333352	15	124333352	G	A	SNV
5WT_5KO	15_124366967	15	124366967	A	G	SNV
5WT_5KO	15_126685289	15	126685289	C	A	SNV
5WT_5KO	15_128312005	15	128312005	T	C	SNV
5WT_5KO	16_5711008	16	5711008	C	T	SNV
5WT_5KO	16_5711041	16	5711041	C	A	SNV
5WT_5KO	16_33081802	16	33081802	G	A	SNV
5WT_5KO	17_9770542	17	9770542	C	T	SNV
5WT_5KO	17_28825055	17	28825055	A	G	SNV
5WT_5KO	17_45266890	17	45266890	G	A	SNV
5WT_5KO	17_54104513	17	54104513	C	G	SNV

5WT_5KO	18_46804674	18	46804674	A	G	SNV
5WT_5KO	X_78378597	X	78378597	T	A	SNV
5WT_5KO	1_261513792	1	261513792	GGA	G	INDEL

10.1.2. Embryo number six

CONTRAST	VAR_ID	CHROM	POS	REF	ALT	VAR_TYPE
6WT_6KO	1_96821088	1	96821088	C	T	SNV
6WT_6KO	1_96821093	1	96821093	C	T	SNV
6WT_6KO	1_221995969	1	221995969	C	T	SNV
6WT_6KO	3_114440106	3	114440106	A	G	SNV
6WT_6KO	4_11510992	4	11510992	A	G	SNV
6WT_6KO	5_46279560	5	46279560	A	G	SNV
6WT_6KO	5_52027092	5	52027092	T	C	SNV
6WT_6KO	8_124861724	8	124861724	C	T	SNV
6WT_6KO	9_5106971	9	5106971	T	C	SNV
6WT_6KO	11_47175839	11	47175839	G	A	SNV
6WT_6KO	13_16612136	13	16612136	A	G	SNV
6WT_6KO	13_91140310	13	91140310	A	G	SNV
6WT_6KO	14_15975979	14	15975979	A	G	SNV
6WT_6KO	14_35527918	14	35527918	A	G	SNV
6WT_6KO	15_44297475	15	44297475	C	T	SNV
6WT_6KO	15_125543798	15	125543798	C	T	SNV
6WT_6KO	16_19704995	16	19704995	C	T	SNV

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