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Modification of the porcine genome using nuclease-based targeting tools

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Meiner Familie

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AAV	adeno-associated virus
APS	ammonium persulfate
BAC	bacterial artificial chromosome
BMD	Becker muscular dystrophy
bp	base pairs
BRCA1	breast cancer associated gene 1
CCR5	chemokine receptor 5
CFTR	cystic fibrosis transmembrane conductance regulator
CiA	chloroform isoamylalcohol
dATP	deoxyadenosine triphosphate
ddH_2O	double-distilled water
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DAPI	4',6-diamidino-2-phenylindole
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
DSB	double-strand break
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
el	elimination limit
eNOS	endothelial cell nitric oxide synthase
ENU	*N*-ethyl-nitrosourea
ESCs	embryonic stem cells
EtOH	ethanol
FCS	fetal calf serum

FISH	fluorescence *in situ* hybridization
Flp	flippase
FRT	flippase recognition target
G418	geneticin
GGTA1	alpha-1,3-galactosyltransferase 1
GFP	green fluorescent protein
GIP	glucose-dependent insulinotropic polypeptide
GIPR	GIP-receptor
HCl	hydrochloric acid
HR	homologous recombination
indels	insertions and deletions
Ins2	insulin 2 gene
iPSCs	induced pluripotent stem cells
IPTG	isopropyl-beta-D-thiogalactopyranoside
KCl	potassium chloride
kb	kilo base pairs
KH_2PO_4	di-potassiumhydrogenphosphate
KOAc	potassium acetate
lacZ	β-galactosidase
$MgCl_2$	magnesium chloride
mvc	mean relative copy number
NaCl	sodium chloride
$Na_2HPO_4+2H_2O$	sodiumdihydrogenphophate-1-hydrate
NaOH	sodium hydroxide
NHEJ	nonhomologous end joining
OD_{600}	optical density measured at a wavelength of 600 nm
OPEN	oligomerized pool engineering
pA	polyadenylation site
PAC	P1 artificial chromosome
PBS	phosphate-buffered saline without calcium and magnesium
PCiA	phenol-chloroform-isoamylalcohol
PEG	polyethylenglycol

PKG	phosphoglycerate kinase
rAAV	recombinant adeno-associated viral vector
RNase A	ribonuclease A
RVDs	repeat variable di-residues
SCID	severe combined immunodeficiency
SCNT	somatic cell nuclear transfer
SDS	sodiumdodecylsulfate
SOC	super optimal broth
ssDNA	single-strand DNA
TALE	transcription activator-like effector
TALEN	transcription activator-like effector nucleases
TEMED	N,N,N',N'-tetramethylethylendiamine
TFO	triplex forming oligonucleotides
Tris	tris-(hydroxymethyl)-aminomethan
UV	ultraviolet
X-Gal	5-bromo-4-chlor-3-indoxyl-β-D-galactopyranosid
ZF	zinc finger motifs
ZFN	zinc finger nuclease
ZiFiT	Zinc Finger Targeter

1 INTRODUCTION

Genetically modified animals were shown to be a powerful tool, creating a better understanding of gene function and the nature of genetic diseases [1]. Despite still being the most widely used and versatile animal models, not all biomedical questions can be addressed using rodents due to their size or short life span. Large animal models such as pigs are more suitable for many biomedical purposes and are able to mimic human genetic diseases to a much greater extent than rodents due to their size, physiology or metabolism [2, 3]. But feasible new pig models need to be generated by modifying the porcine genome in a site-specific manner.

In the last decades, different gene targeting tools were designed and established for site-specific modification of the DNA. However, many of them struggle with low targeting efficiencies or specificities. One promising approach to overcome the low targeting rate is the introduction of DNA double-strand breaks (DSBs) at the target site, which triggers the cellular repair machinery, struggling to eliminate this severe DNA lesion. Two major pathways are known to repair the DNA DSBs. Non-homologous end joining (NHEJ) is an error-prone repair system promoting the ligation of the two DNA strands, resulting mostly in sequence alterations like deletions, insertions or sequence rearrangements and was used to generate knock-outs of endogenous genes [4, 5]. The more accurate way to repair a DNA DSB is offered by homologous recombination (HR). This repair pathway uses a homologous DNA sequence as a template, the sister chromatid or an exogenous targeting vector, to restore the disrupted segment and has already been applied to correct a mutated gene [6-8].

For a targeted introduction of DSBs artificial proteins containing a DNA binding domain and a DNA cleavage domain, mostly consisted of the *Fok*I endonuclease that cut the DNA unspecificly, were developed. Zinc finger nucleases (ZFNs) and the novel transcription activator-like effector nucleases (TALENs) represent such chimeric proteins. Both carry the *Fok*I endonuclease for DNA cleavage, but differ in their DNA binding domains.

In this study, both nuclease technologies were applied to modify the porcine genome. We demonstrated the ability to alter the DNA of two porcine genes, the

cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and the porcine dystrophin gene (*DMD*). ZFN has been shown to mediate genome alterations by both mechanisms, the NHEJ and the HR repair pathway. In order to generate a transgenic pig model carrying a reporter gene driven by the *CFTR* promoter, we transfected porcine primary cells with *CFTR*-specific ZFNs and different targeting vectors, varying in the length of their homology arms. The notably high targeting efficiency, capable of inducing heterozygous and homozygous targetings, proves the potential of nuclease technologies for genome modification and the subsequent generation of a porcine animal model. The method applied in this work can be used for a variety of genomic loci within the porcine genome in the future and thereby help to elucidate fundamental mechanisms leading to human diseases.

2 REVIEW OF THE LITERATURE

2.1 Genetic engineering

Long before the modern science of genetics was established, mankind practiced selective breeding to modify plants and animals for its intent. After the discovery of DNA, genes and proteins and the existence of direct determination between them, humans were enabled to manipulate this genetic information by the means of molecular techniques. Today, two general pathways can be distinguished to modify the genome in a desired way, non-specific genome editing and gene targeting.

Non-specific genome modification is based on two major approaches chemical or insertional mutagenesis. Both provide tools to alter the genome, but a prediction of the resulting modifications is difficult.

Chemical mutagenesis can be achieved by chemical agents such as ethyl-nitroso-urea (ENU), which mostly causes single base-pair mutations throughout the genome. The application of this widely used mutagen in animals, e.g. in mice, results in mutant phenotypes, which can be screened and characterized [9, 10] and thus, can be exploited to display the connection between particular genes and proteins. Nevertheless, the identification of mutated genes responsible for observed phenotypes remains critical [11] as the observed phenotypes can be caused by several different mutations.

However, this problem can be overcome by additive gene transfer, which involves the transfer of exogenous genetic material into cells and its integration into the cell's genome, becoming a so called transgene. The vector commonly contains a promoter, the coding region of the gene of interest and a polyadenylation (pA) cassette to provide sufficient transcription of the transgene.

As the delivered gene integrates randomly into the genome, gene disruption, functional knockouts or other alterations can occur [12, 13]. Furthermore, the phenotype may result not only from the defined transgene, but also from differences in genetic and/or epigenetic background [14, 15].

Mostly, transgenes integrate into a genomic locus as a single copy, but they are also able to form tandem head-to-tail arrays that consist of few to several hundred

copies lined up one after the other [16] and result in some cases in unspecific inhibitory effects [17, 18]. In addition multiple copies of a transgene can decrease its expression [19] or cause even transgene silencing in some cases as shown in plants [20, 21], *Drosophila* [22] and mice [19]. Furthermore, integration site, chromatin accessibility and cell type are important factors for a stable transgene expression (reviewed in [23]).

In contrast to the random outcome of insertional mutagenesis, it might be desirable to aim at a more predictable outcome of the induced genetic modification. Site-specific genome modification, also known as gene targeting, meaning the mutation of a defined site in the genome, represents a valuable alternative in this respect.

2.2 Molecular tools for site-specific genome modifications

2.2.1 DNA-based targeting strategies

Gene targeting strategies are commonly based on homologous recombination (HR) between the target site in the genome and an artificially introduced vector, consisting of a transgene flanked by sequences homologous to the target site. Recombination occurs also as a natural cellular process at a very low frequency of one targeting event per 10^5 to 10^7 cells [24, 25]. Studies revealed that HR depends on cell cycle and damage-induced expression of proteins, which are main components of the recombination complex [26, 27]. Initial evidence for DNA damage induced HR was demonstrated by DNA-damaging agents (methylmethanesulfonate, UV light as well as SV40 virus), triggering homologous exchanges between sister chromatids [28]. HR is known to be a major DNA-repair pathway in mammalian cells, where DNA breakage events activates the cellular DNA damage response, whose enzymatic machinery repairs the break by sequence exchange with any available homologous template, i.e. usually the sister chromatid, but also an introduced gene targeting vector [6, 7]. Thereby, a resection of DNA takes place at the breakage site and the resulting single-stranded DNA (ssDNA) becomes coated by replication protein A forming a binding substrate for the RAD52 protein. Subsequently, RAD52 interacts with RAD51 and the active nucleoprotein filament binds to a homologous double-stranded DNA template forming a structure

called Holliday junction. With the help of RAD54 a sequence exchange is performed, followed by separation of the repaired DNA strand and the homologous template (reviewed in [29]).

The involvement of the recombination machinery in the repair of DNA double-strand breaks (DSBs) might also be used as a tool for the integration of a desired mutation into a defined site by providing the modification within a fragment that is homologous to the target site. This homology-based targeting strategy relies on the very rare moment of DSBs within the desired locus and extensive homology arms are needed to obtain sufficient targeting frequencies. The targeted recombination of an exogenous DNA with the host genome is very efficient in yeast [30], but in vertebrates targeting vectors will be rather inserted into the genome randomly than in a specific manner [31].

In order to distinguish the rare process of HR with exogenous DNA from random integrants, transfected cells need to be screened for targeted insertion of the transgene. One strategy to detect cell clones carrying a stably integrated transgene is a simple positive selection strategy. Thereby, the transgene consists not only of a gene of interest, but also of an antibiotic resistance cassette (neomycin, blasticidin, puromycin or hygromycin). However, this strategy allows the selection of cells with stably integrated vectors, either targeted or randomly inserted. Consequently, a large number of cell clones need to be screened by further methods to identified those that are correctly targeted [32]. Diverse strategies have been developed to overcome the low rate of HR in vertebrate cells, each of them providing distinct advantages, but also have their limits.

2.2.1.1 Negative selection

In order to enrich the number of cell clones with a correctly integrated transgene, a positive-negative selection can be applied to segregate these cells from cells with randomly inserted transgenes [33]. In addition to the positive selection marker a negative selection cassette such as thymidine kinase or diphtheria toxin A is placed outside of the homology arm on the linearized targeting vector. In case of a targeted recombination, the negative selection marker will get lost, making these clones resistant to both selective treatments. Randomly integrated cells will contain both

selection markers and will die as the negative selection produces toxic components [34]. However, this method is not applicable for all cell types and purposes.

2.2.1.2 Trapping approaches

A more powerful strategy is promoter-trap positive selection. The transgene contains a promoter-less positive selection marker, which utilizes the promoter of the target gene to drive its expression [25, 35], and thus, only targeted integrants will provide a cell survival. In contrast, random integrants remain sensitive to the selection agent as the promoter-less selection marker will not be expressed. The limit of this approach is that the promoter of the target gene must be active to provide a selection marker expression.

Similar to this strategy is the polyadenylation-trap positive selection, where the positive selection cassette lacking a termination signal needs the pA sequence of the target gene to be able to express the selection marker correctly [36]. In contrast, cells with a random integration express only an unstable transcript that gets degraded easily [37]. The pA-trapping strategy avoids the limitations of promoter trapping which is only applicable for genes that are active in the targeted cells as the function of the pA-signal is not dependent on transcriptional regulation.

2.2.1.3 Recombinase-based systems for targeted gene alteration

A variety of genetic tools has been developed for DNA modification and all of them struggle to achieve highly targeted sequence engineering. Site-specific recombinases recognize and mediate the recombination between short, defined DNA sequences, causing the integration, excision or inversion of DNA segments [38].

Simple recombinase systems, e.g. Cre and the flippase recombination enzyme (*Flp*), mediate recombination between identical target recognition sequences in actively dividing and postmitotic cells of most tissue types. Cre recombinase, from *E.coli* bacterionphage P1, recognizes and recombines 34 bp long target sites termed loxP [39]. The yeast Flp recombinase 48 bp flippase recognition target (FRT) sites that are similar to the loxP sites according to a 13 bp palindromic sequencer (inverted repeats), separated by an 8 bp asymmetric core sequence (spacer) [38]. In contrast to loxP sites, FRT contains a third 13 bp direct site [40]. The recombinase molecules,

each bound to one repeat, cleave the DNA and provide recombination in the central spacer region [41]. In general, first recombinase recognition sequence is introduced into a region of interest by conventional gene targeting and secondly, recombinases are used to induce a desired modification into their recognition sites, suggesting that the limitations of the first step make this strategy unsuitable for high-throughput gene targeting application.

2.2.1.4 Triplex forming oligonucleotides (TFOs) for induction of HR events

Increase of frequency of HR was observed in experiments which are based on the triplex forming oligonucleotides (TFOs) method, using 10 to 30 nucleotide long single stranded sequences that bind purine-rich target sequences of the major grove of DNA with high specificity and binding affinity. TFOs-mediated triple helix formation activates the cellular DNA repair machinery and leads to recombination events between homologous sequences [42]. Linked to a short DNA donor fragment that is homologous to the target site, TFOs were demonstrated to mediate gene correction with a frequency up to 2 % [43, 44]. In addition, further studies revealed that TFOs drive recombination of a donor fragment also without being linked to it, which would enable the use of larger donor DNA sequences delivered separately (reviewed in [44]). Nevertheless, regions suitable for high affinity TFO binding are rare, approximately once per kilobase of genomic DNA, and TFO delivery into the cell and its stability inside the cell also represent main limitations of this technique (reviewed in [24]).

2.2.1.5 Adeno-associated virus targeting vectors

The Adeno-associated virus (AAV) is a ssDNA helper-dependent parvovirus that replicates or causes a productive infection exclusively in association with a helper virus such as an adenovirus or herpesvirus. However, the ability to transduce a variety of cell types, dividing and non-dividing, makes AAV-based vectors an important tool for gene transfer and targeting [45, 46]. Wild-type AAV can integrate in a site-specific manner at the preferred AAVS1 locus on the long arm of chromosome 19 (19q13-qter) in humans, although, large homology does not exist between this site and the AAV genome [47] and thus, recombination is somehow

supported by parts of the viral genome. The Rep helicase of AAV has been found to mediate the recombination between the viral genome and a Rep binding element at the AAVS1, resulting in the integration of a small fraction of viral DNA [48].

AAV vectors were widely used for insertional mutagenesis, but targeted modifications have also been achieved including single base pair substitution, deletions and insertions either [49, 50] demonstrating targeting rates of 0.1 to 1.0 % [51]. AAV vector-mediated gene correction has been established for example in creating a mutated *lacZ* transgene, the β–glucuronidase gene, the fumarylacetoacetate hydrolase (*Fah*) gene [52, 53] and human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) were successfully modified [54]. Furthermore, cystic fibrosis transmembrane conductance regulator gene (*CFTR*) deficient pigs and *CFTR*-Δ*F508* heterozygous pigs were generated using recombinant AAV targeting vectors, containing inverted terminal repeats at both ends of the targeting construct as the only viral sequences [55]. Another group reported the generation of a breast cancer associated gene 1 (*BRCA1*) knock-out pig using recombinant AAV-mediated gene targeting as well, demonstrating that homology arms of isogenic DNA are required for a successful HR-based targeting [56, 57]. Nevertheless, the limited ability to harbor foreign DNA might restrict the application of AAV vectors in some gene targeting approaches [49].

2.2.1.6 Artificial chromosome type vectors

Extended homologous regions positively influence the targeting efficiency. However, conventional HR-based vectors have a limited packing ability of 20 kb foreign DNA, but other vector types are capable to accommodate larger DNA fragments, which enables the construction of large homologous sequences. Yeast artificial chromosomes (YACs), phage artificial chromosomes (PACs) and bacterial artificial chromosomes (BACs) were initially developed for analysis and mapping of complex genomes to provide libraries for genome sequencing. YACs, which are linear vectors, enable cloning of DNA fragments up to 1 Mb [58] and are mostly used for additive transgenesis in animals, e.g. to analyze genomic regulatory systems avoiding position effects [59] or to create mouse models [60], but difficulties with the manipulation of YAC libraries have been reported [61]. In contrast, PAC systems can

carry 100 kb long fragments and BACs, the most commonly used, harbor DNA fragments up to 300 kb [62].

The BAC cloning system is based on the well-characterized *E. coli* fertility (F-) factor, which is maintained in low copy number in *E. coli* cells to avoid recombination events between the homologous DNA fragments. Different manipulation methods [63, 64] allow the construction of BACs for targeting experiments as they can be designed to carry large homology sequences. In addition, their circular form shows high stability and facilitates their isolation and manipulation. Methods for a high throughput generation of BAC targeting vectors for a variety of genes, e.g. in murine ESCs with an average targeting rate of 3.8 %, has been introduced. Due to the size of several 10 kb, conventional screening methods using end-point PCR are not applicable. Instead, different screening techniques have been established so far, including DNA fluorescence *in situ* hybridization (FISH) and real-time quantitative PCR-based "loss-of-native-allele" assays [65]. In addition, this technique was successfully employed for site-directed mutagenesis by disrupting protein kinase *ATM* and tumor suppressor gene *p53* in human ESCs [66]. Furthermore, a CFTR deficient pig [67] was produced demonstrating targeting rates of > 1 % due to application of primary cells and proving the capability of this technique.

2.2.2 Designed nucleases for gene targeting strategies

One promising idea to overcome the poor efficiency of gene targeting events based on conventional HR strategies is the forced introduction of DSBs into a desired locus. The promoting effect of forced induction of DSBs on HR results in an up to 1000 – 5000-fold increased rate of recombination events, as reported for several gene targeting experiments [68, 69]. Designed nucleases have the capability to induce DSBs in a sequence specific manner, making them a promising tool for targeted gene modification (figure 2-1 [70]).

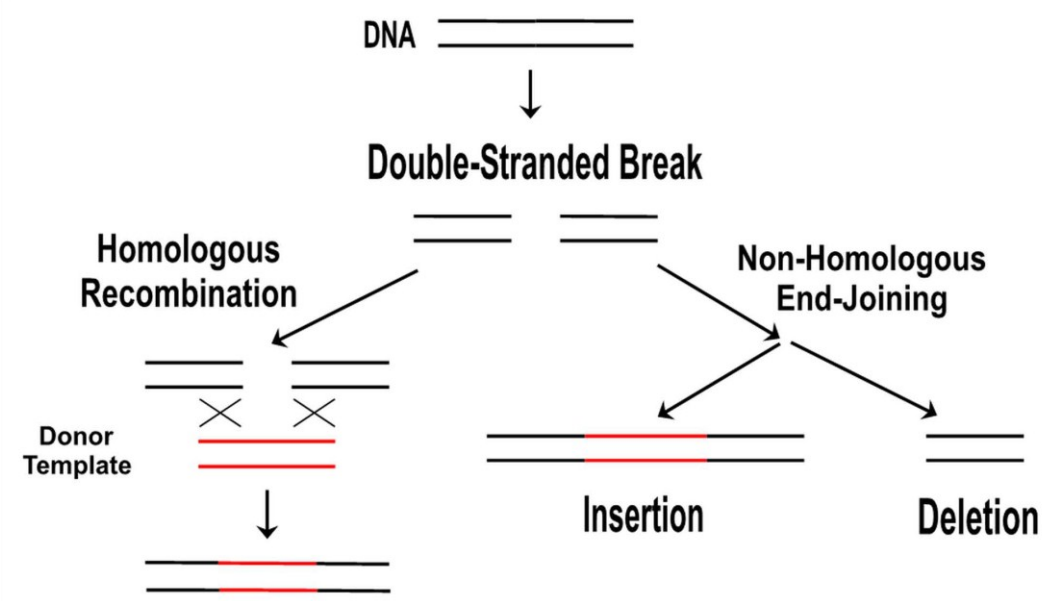

Figure 2-1: Genome engineering by non-homologous end-joining- (NHEJ) or homologous recombination-based repair of double-stranded DNA breaks. DSBs can be repaired by two major mechanisms: NHEJ (right) and HR (left). Both repair pathways can be used to modify a desired DNA locus in a specific manner.

An alternative approach of gene targeting, in addition to HR, is the second major mechanism for the repair of DSBs, non-homologous end joining (NHEJ). In mammals NHEJ is the preferred repair pathway for DSBs and functions throughout the cell cycle [71]. A protein complex consisting of Mre11, RAD50 and Nbs1 [72, 73] in combination with the ligase IV (Lig4) enzyme provides ligation of the broken DNA strands without any or very little (1-6 bp) homology [30]. While NHEJ normally ligates genomic DNA strands, exogenous DNA might also participate in this process [74], and thus become a stably integrated transgene. The exonuclease activity during NHEJ leads to resection of the cleaved DNA and causes minor modifications at the terminal ends, generating in ~70 % of the cases deletions of variable lengths, insertions or substitutions at the break site [4]. These sequence alterations result in gene disruption and has been proven to be a successful tool for gene knock-out [75]. In contrast to HR, which generates site-specific and defined modifications, NHJE at a double-strand break that has been introduced by a site-specific nuclease results in mutations that are introduced at a target site, but their constitution is not precisely predictable.

In the last decades, different tools for DSB-based gene targeting were identified and different nucleases were developed to bind and cleave the desired DNA sequence.

2.2.2.1 Homing endonucleases

First DSB-induced targeting strategies involved the use of endonucleases such as I-*SceI* isolated from the yeast *Saccharomyces cerevisiae* [76]. This intron-encoded homing nuclease (also called meganuclease) is responsible for copying an intron from an intron-containning allele of the 21S ribosomal gene to an intronless allele of the same gene [77]. The allele lacking the specific intron, contains an 18 bp recognition site (homing site), which is a cleavage substrate for this enzyme. The homing site on the intron-containig allele is disrupted by the intron preventing cleavage by the endonuclease. The produced DSBs at the homing site activate the HR-based repair pathway and thus, lead to an unidirectional gene conversion event (homing), which promotes a spread of the intron containing homing endonuclease gene to related alleles lacking the intron [69]. I-*Sce*I is a very rare cutting enzyme, that occurs randomly throughout the genome with a very low probability (approximately once every 7×10^{10} bp) and is predicted to be absent from most mammalian-sized genomes [76]. This aspect is important as unpredicted cleavage of any endogenous recognition site would result in unwanted genomic alterations with undesirable consequences.

I-*Sce*I-based gene targeting for genetic modification of cell lines has been used in a two step strategy [76]. First I-*Sce*I site needs to be inserted into the target locus in a classical HR step using a replacement vector and a selection marker, which is an extremely low-efficiency process. In the next targeting round the locus is retargeted using an I-*Sce*I expressing plasmid and a second replacement vector achieving recombination frequencies up to 5000-fold higher than in the first step [78].

Although I-*Sce*I was successfully applied in mammalian cells for generation of stably transfected cell lines with single copy integrations [79, 80], several approaches to modify the recognition site of the homing nucleases have been developed [81]. Even engineering of novel chimeric proteins [82] has been successfully used in some cases, but the technology remains challenging, making this approach unsuitable for high throughput targeted modification and broad application [83].

2.2.2.2 Zinc-finger nucleases (ZFN)

A further DSB-based targeting strategy involves a type of artificial enzyme called zinc-finger nuclease, which is a chimeric protein consisting of a DNA cleavage domain and a DNA binding domain. Initially, it was based on the observation, that *Fok*I, a type II restriction enzyme, has a DNA recognition domain and a separate cleavage domain, which has no sequence specificity. Furthermore, it was observed that alternative DNA sequences could be cleaved by replacing the natural occurring sequence recognition domain by alternative DNA-binding domains [84]. *Fok*I DNA cleavage domain was equipped with new binding domains for desired DNA sequences by using Cys_2His_2 zinc finger motifs (ZF) [85]. DNA-binding ZFs can be found in many proteins that regulate eukaryotic protein-nucleic acid interactions such as transcription. They fold into a ββα configuration and coordinate one zinc ion with two cysteine and two histidine residues to stabilize their folding structure [86]. The crystal structure of Zif268, a three-finger protein, revealed that each ZF recognizes specifically 3 bp of DNA by binding into the major DNA groove with its α-helix. It was suggested that defined sequences of 18 bp or more should be targeted to ensure specificity of appropriate sets of ZF [87] [88].

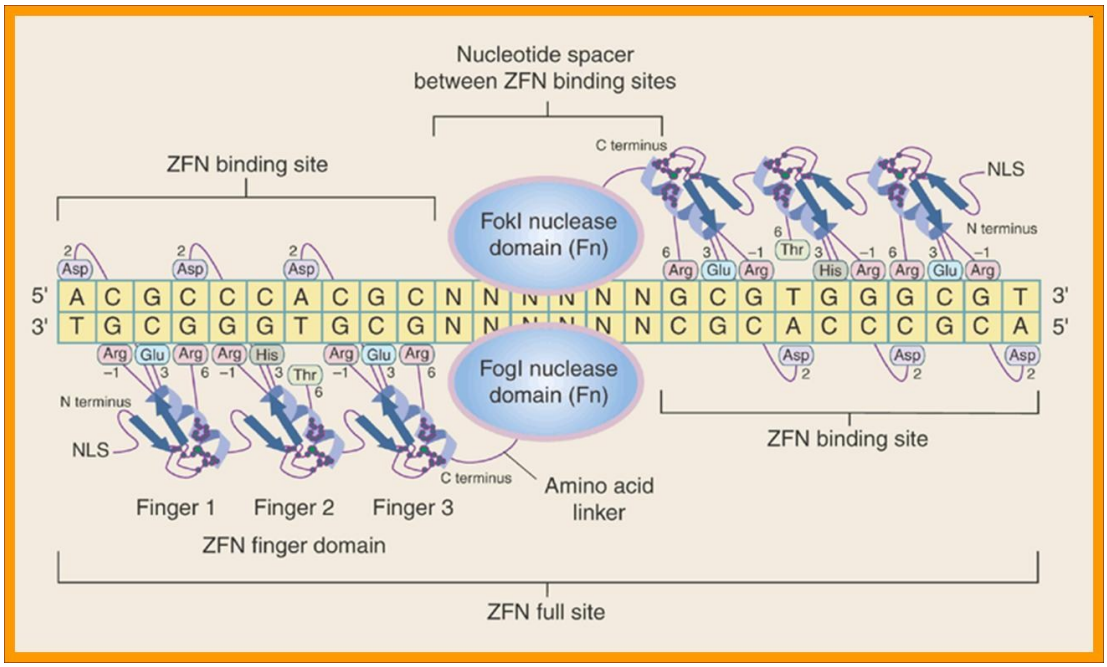

Figure 2-2: ZFN pair binding to a targeting DNA sequence. Each ZFN consists of three ZFs and is fused to a DNA cleavage domain. The two endonuclease domains need to form a dimer to be catalytically active.

For a successful introduction of DSBs into the targeted DNA sequence by ZFN, dimerization of the *Fok*I domain is essential (figure 2-2 [86]). Therefore a pair of ZFNs, each binding to the DNA sequence in opposite orientation with three ZFs, allows the *Fok*I domain to form a catalytically active cleavage dimer within the binding sites. However, in addition to the desired heterodimers, the ZFN monomers can also form homodimers and introduce DSBs in unwanted sites of the genome. Early experiments revealed that in some cell types ZFN showed cytotoxic effects due to cleavage of nontarget sequences, reflecting unspecificity of the ZFN and limiting the potential of the ZFN technology for gene targeting. This problem could be widely solved by creating *Fok*I variants with altered protein surfaces that preferentially heterodimerize [89].

2.2.2.2.1 Design of customized zinc-finger nucleases

Theoretically, ZFNs might be designed for any of the 64 DNA triplet combinations. Single fingers with pre-characterized specificities [88, 90] can then simply be joined together to form binding domains for any desired DNA sequence. This "modular assembly" is easy to perform, but only 6 % of ZFN pairs designed with this method

are functional [91], furthermore they show low activity and/or high cytotoxicity due to interaction between the single ZFs [92].

In order to overcome this problem pre-characterized multifinger domains, which show high DNA-binding affinities and specificities [93] can be selected for oligomerized pool engineering (OPEN). The Zinc Finger Consortium (www.zincfingers.com), a group of academic laboratories, established a publicly available platform containing an archive of engineered zinc-finger arrays that showed a higher success rate than enzymes obtained by modular assembly [94].

In addition, a web-based software, the Zinc Finger Targeter (ZiFiT), was established to provide a simple and rapid tool to scan a DNA sequence for potential ZFN binding sites, for engineering by modular assembly or OPEN, as well as to evaluate and validate ZFN targets [95]. A further publicly available database for ZFNs is the recently established ZFNGenome [70]. This platform provides information about potential ZFN target sites, their chromosomal localization and their targeting capability. In addition, ready-made ZFNs can be obtained from a commercial supplier that provides validated ZFNs for many targeting sites (http://www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology.html).

Nevertheless, the improvement of binding specificity and DNA affinity, both a further limitation of this technology, remains an important research topic.

2.2.2.2.2 Applications of ZFNs

In one of the initial experiments, ZFNs were used to disrupt the *yellow* gene of *Drosophila melanogaster* via NHEJ demonstrated targeting rates of 1:250 in the male germ line [96]. Later studies revealed ZFN targeting frequencies to 10 % and higher [5] making this method a promising tool for standard applications in the gene targeting research area.

Possible applications of engineered ZFNs involve targeted mutagenesis by NHEJ for the study of gene function. This gene strategy has been successfully performed in plants, animals for creating model organisms and in cell culture for therapeutic purposes, e.g. disrupting of chemokine receptor 5 (CCR5), a co-receptor for HIV by ZFNs to establish HIV-1 resistance in CD4+ T cells [97, 98].

ZFN technology also provides targeted gene correction by combination of forced DSBs induction and HR between cleaved genomic DNA and a template DNA. The method can be use to repair dysfunctional genes involved in recessive monogenetic diseases, but also for numerous other purposes. ZFN-based therapy approaches have already been reported for X-linked severe combined immunodeficiency (SCID), sickle cell anemia, cystic fibrosis and myotonic dystrophy [99, 100] and the permanent and heritable gene correction by ZFNs will find applications for other diseases as well. Gene-targeted insertions were successfully performed in cultured cells, e.g. human ESCs and iPSCs, where a green fluorescent protein (*GFP)* gene and a puromycin resistance gene were introduced and their expression controlled by the *OCT4* promoter [101], in plants to introduce missense mutations [102] as well as in *Drosophila* flies [103]. Furthermore, reporter genes might be integrated into a defined site in the genome to achieve ectopic expression. Genes such as *lacZ, GFP* or resistance genes (to puromycin, neomycin, blasticidin) act as easily-detectable markers to monitor cellular mechanisms or to study expression conditions when the marker is inserted under an endogenous promoter.

2.2.2.2.3 Potential barriers

Although the application of ZFN technology proved to be suitable for gene targeting in organisms ranging from plants, to insects and mammals, the cytotoxic potential of ZFNs in complex organisms is still under debate. The assumed reason of cellular cytotoxicity is the ability of ZFN to create off-target DSBs, as the degree of cytotoxicity is directly correlated with of extra DSBs that are being created [104-106].

While new software and platforms were established to provide a rapid and efficient way to design new ZF arrays, a certain proportion of ZFN pairs fail, no matter which method has been used for their construction. Unfortunately, binding specificity and affinity of chosen ZF sets are major issues and thus, it is still necessary to evaluate newly designed ZFNs extensively in time-consuming studies.

In order to facilitate adequate affinity, each ZFN should contain at least three zinc finger motifs. More ZFs can be added to the nuclease domain to increase the binding efficiency of the nuclease. However, this lowers the ZFN specificity by increasing the

possibility that some of the added ZFs can promote binding to off-target sites. Even the best designed ZF arrays show affinity for other sequences than they are supposed to. Moreover, it is also possible that some sequences or genomic regions are unsuitable for targeting at all due to their inherent particularly inaccessible chromatin structure or DNA modifications [107]. Since methods to measure accessibility are difficult, it is often easier to construct several ZFNs for different regions close to the desired targeting site.

Although ZFN technology is a precise gene targeting tool with many advantages, the discussed challenges might be relevant for certain approaches and for using this technique for therapeutic purposes in human. Therefore, it is crucial to develop direct and simple analysis methods for the detection of off-target cleavages.

2.2.2.3 Transcription activator-like effector nucleases (TALENs)

Statistically, a suitable ZFN binding site is only found every 500 bp [94, 108]. A novel class of designed nucleases has been engineered recently, the transcription activator-like effector nucleases, which could bind the DNA on average every 35 bp [109]. Similar to the ZFNs, these artificial enzymes contain a *Fok*I cleavage domain, but the DNA binding domain is comprised of transcription activator-like (TAL) effectors.

TAL effectors are proteins isolated from pathogens of the *Xanthomonas* genus, which infect plants including pepper, rice, citrus, cotton, tomato, and soybeans by injecting a variety of effector proteins via their type III secretion system. Once delivered into the host cell, TAL effectors translocate into the nucleus and bind to effector-specific sequences in host promoters and activate transcription [110]. TAL effector proteins contain a N-terminal translocation domain, central repeats (commonly 12 – 30) that provide sequence-specific DNA binding and a C-terminal nuclear translocation domain as well as a transcriptional activation domain (figure 2-3) [111]. The conserved 33 – 35 residues long repeats of the TAL effector DNA binding domain are arranged in tandem arrays and differ predominantly in residue position 12 and 13. These two positions, also called repeat variable di-residues (RVDs) preferentially recognize one of the four DNA bases forming a “one repeat to one base” code [112].

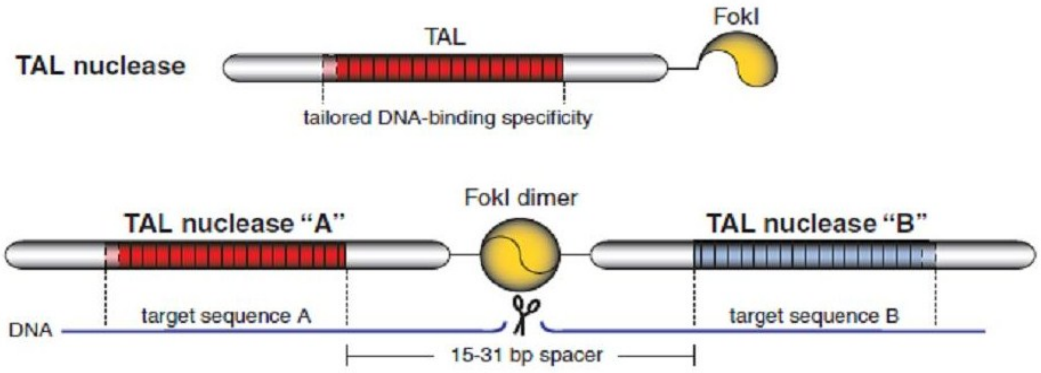

Figure 2-3: TALE protein fused to a *Fok*I endonuclease. TALEN can specifically bind DNA sequences with their repeats segment (shown in red and blue) and consequently introduce a DSB by the *Fok*I dimer into the DNA targeting site [113].

Consequently, repeats binding with high efficiency to a unique base have been described as well as repeats that are less specific and thus, bind to more or even all bases. The existence of a code facilitates the prediction of the DNA binding sites of naturally occurring TAL effectors and enables the design and construction of customized TAL effector repeat arrays for any DNA target sequence [114, 115] making these proteins invaluable for developing of new gene targeting tools. Unlike ZFNs, TALENs can be constructed by assembling repeats in a random manner, which already has been proven in initial studies [115]. However, designing TALE nucleases by standard cloning approaches revealed to be challenging due to their high homology and the high number of repeats, but these obstacles could be overcome by the newly developed Golden Gate cloning and software based strategies allowing the construction of TALENs for a large variety of targeting loci in *Arabidopsis*, tobacco, *Drosophila* and zebrafish [109, 116, 117]. Until today, successful *in vitro* targeting of *GFP*, *eGFP* and human *CFTR* and *HPRT1* genes has been achieved with comparable mutation rates as ZFNs [109, 112]. Although, these studies, which involved ZFNs and TALENs targeting the same locus, have revealed a lower cytotoxicity for TALENs [112] and also no target site overlapping or crosstalk between individual repeats in a TAL effector array has been reported yet, the technique still requires further characterization regarding its specificity and potential off-target effects.

2.3 Vector delivery systems

By which means ever a gene of interest is to be modified, the targeting tool has to be introduced into cells or embryos by an appropriate delivery system. The commonly used delivery systems are based on chemical, physical and viral approaches.

2.3.1 DNA transfer by viral vectors

Viruses have a natural ability to transfer their genome into cells efficiently. For gene delivery purposes viral vectors have been developed by removal of their genes required for viral propagation and replacing them by (trans-) genes of interest. Consequently, the deficient viral genome is able to transduced cells and in some cases integrate into the cell's genome as efficiently as its wild-type counterpart, but is, on the other hand, unable to proliferate and infect his host.

Retroviral vectors are capable to integrate foreign DNA into the host genome, but these vectors are limited by the fact that most of them infect only dividing cells, since they can reach the chromatin only when the nucleus membrane is disrupted during the division event.

Lentiviral vectors have the ability to target also non-dividing cells (e.g. neurons) through mitosis-independent transport of the viral DNA into the host nucleus [118]. Initially, unlike other viral vectors, they were thought, to escape epigenetic silencing [119, 120], however, later studies demonstrated that one-third of the transgenes integrated via lentiviral vectors showed low to undetectable expression levels [121] exhibiting a potential limitation of this delivery system.

Adenoviral vectors have the advantage of being highly infectious *in vitro* and *in vivo,* however, transfection experiments revealed a very low integration rate and consequently is limited to the transient presence of the vector in the cell, making this delivery system unsuitable for gene targeting purposes.

The AAV vectors have not only the ability to target a DNA region by integrating into the genome they also are capable of transducing cells and thus, represent an important delivery system. Although, AAV-mediated gene targeting experiments have shown promising targeting rates, as already described above, their targeting

frequency is even higher when combined with I-*Sce*I meganuclease-based technology [122, 123].

Nevertheless, all viral delivery systems show limitations due to their ability to induce immunological reactions, their preference for random integration and their low capacity to harbor foreign DNA (usually 10 kb maximum).

2.3.2 Non-viral delivery systems

Chemical-based delivery methods include cationic polymers, liposomes and calcium phosphate [124].

Synthetic cationic polymers (e.g. DEAE-dextran and polyethylenimine) bind the negatively charged DNA and thus, facilitate the cellular uptake via endocytosis [125]. Liposomes deliver DNA by encapsulating it with positively charged cationic lipids and subsequently fusing with the plasmalemma or the endosome [126], thus, facilitating a cellular uptake of the negatively charged DNA. Calcium phosphate, on the other hand, causes a DNA precipitation on the cell surface resulting in a spontaneous endocytosis of a small amount of DNA. Chemical methods show low transfection efficiency and are not applicable for all cell types and transfection purposes. However, commercially available systems for lipofection are pre-optimized and provide high transfection efficiency and application for a wide range of cell types.

Physical methods include DNA microinjection that can be performed into the cytoplasm or directly into the nucleus. Although, it represents the most efficient delivery method, DNA has to be injected individually into each cell making it rather suitable for one-cell embryo injection.

Electroporation is another physical delivery method that transfects a variety of cell types with high efficiency. An applied electric field creates transient pores in the cell membrane and increases the mobility of DNA added to the electroporation solution facilitating the uptake of DNA into the cells [127]. In order to overcome an increased apoptosis rate of electroporated cells, commercially available equipment with pre-optimized settings and chemical solutions for different cell types can be applied to deliver targeting DNA directly into the nucleus [128].

2.4 Nuclease-based animal models

Generation of genetically modified animals has proven to be a powerful tool to study gene function, analyze disease and produce animal models for therapeutical purposes. Although cultured cells are used as model systems for a variety of applications, not all complex questions can be answered using cell culture.

In order to generate new animal models being able to mimic human genetic diseases, the targeted knock-out of an endogenous gene is one possible and widely used strategy. Before the development of artificial nuclease technologies, the production of site-directed mutagenesis in animals was restricted to HR using conventional DNA vectors, a time consuming and inefficient technique, in addition to the introduction of a permanent selection marker such as an antibiotic cassette for screening and selection of targeted cells. Also, the gene knock-out strategy initially was restricted to mice and later to rats [129], because of lacking ESCs from other species. In recent studies, HR was performed also in other animals, using somatic cell nuclear transfer (SCNT) of genetically modified primary cells [3]. Nevertheless, regarding the highly skilled SCNT technology and the low efficiency of HR in primary cells, targeting in non-mouse species is a tremendous effort.

I-*Sce*I meganuclease was successfully used to produce transgenic animals, including several fish species, amphibia and ascidians [130, 131], but no mammalian model was generated using this technology yet.

ZFN-induced gene knock-out has been achieved in *Drosophila* flies, where the *yellow* locus was targeted [96]. Zebrafish were modified by ZFN technology targeting the *golden* and *no-tail/Brachyury* (*ntl*) genes [132]. In mice, site-specific ZFN-mediated gene insertion of a *GFP* gene succeeded [133] and furthermore, *IgM*-knock-out rats were produced. These rats were mature B lymphocytes- and heavy chain Ig-deficient and were generated to analyze the role of antibodies in a variety of pathological situations. While most of the 22 obtained founder animals carried deletions on only one allele, one founder showed biallelic mutations on both *IgM* alleles. In addition, some of the animals carried more than one deletion, indicating that ZFNs acted over a certain time period of the embryonic development and thus, generated mosaic animals [134].

As the TALEN technology has been established recently, not many animal models were reported yet. One group has been utilized this strategy *in vivo* and reported *IgM* knock-out rats [135]. The rat *IgM* locus was disrupted by TALENs designed for exon 2 of this gene and delivered as DNA or mRNA.

All mentioned animal models were generated by injecting the DNA or mRNA encoding ZFNs directly into the embryos resulting in a mutation frequency between 7 and 46 % for alterations on one allele. In addition, up to 50 % of these mutations were found to create biallelic knock-outs. However, mosaic animals were observed, using ZFN or TALEN technology due to a delay in activity of the designed nuclease or a cleavage of already-modified sequences [4, 135].

2.4.1 Pigs in biomedical research

Traditionally, mice have always been model organisms to study human physiology. Most human genes have orthologs in mouse, whose functions are closely related. Furthermore, the mouse is a small animal that is easy and relatively cheap to maintain and has a short breeding cycle.

Despite these advantages, mice represent only a limited model for humans due to their size, their short life span or their capability to reflect more complex human diseases. One example for the limitation of the mouse model is cystic fibrosis, which is caused in human by a mutation in the *CFTR* gene. Mice with a mutation on a corresponding gene do not show a related airway phenotype as humans [136].

In contrast, pigs represent a highly potential animal model for human diseases due to their size, physiology, anatomy, pathology and metabolism (reviewed in [2]). Since SCNT has successfully been established for porcine cells [137-139], it was possible to produce pig models for various human diseases.

For complementation of the deficient CF mouse model, *CFTR* knock-out pigs were generated by different gene targeting methods [55, 67] displaying similar defects as seen in human patients, including meconium ileus, exocrine pancreatic destruction, and focal biliary cirrhosis. Consequently, the adjusted phenotype of this model enables a better understanding of the pathogenesis of this disease and facilitates the development of new therapy strategies for cystic fibrosis.

In addition to the mouse model, pigs with an altered *BRCA1* gene were produced. Porcine fibroblasts were modified by AAV-mediated targeting of the *BRCA1* gene exon 11 [57]. Although the characterization and phenotype of these animals is not reported to date, *BRCA1* knock-out pigs might contribute to a better understanding of *BRCA1*-associated breast cancer in human.

In order to address different biomedical questions, further pig models were generated by additive gene transfer.

Pigs expressing mutated $GIPR^{dn}$ under the control of rat insulin 2 (*Ins2*) promoter were established due to a limited usage of mouse models for human type 2 diabetes mellitus [140]. Glucose-dependent insulinotropic polypeptide (GIP), secreted in response to fat and glucose, shows a reduced insulinotropic affect in type 2 diabetic patients due to variations in the GIP-receptor (GIPR). The $GIPR^{dn}$ transgenic pigs were used to analyze what role GIPR plays in maintenance of pancreatic islet function and structure. Moreover, two other transgenic porcine diabetes models were reported carrying a mutation in the *Ins2* and in the hepatocyte nuclear factor 1 alpha gene, respectively (reviewed in [3]).

Neurodegenerative disease models include for example pigs with a mutated amyloid precursor protein gene (APP^{sw}) resulting in an Alzheimer's disease phenotype [141]. Huntington's disease pigs were generated by inserting 75 CAG trinucleotid repeats into the triple region of exon 1 of the Huntington gene (*HTT*) [142] and a retinitis pigmentosa disease model was produced by additive gene transfer of a mutated rhodopsin gene (*RHO*) resulting in night blind pigs [143].

For analysis of muscle metabolism and cardiovascular regulation by endothelial cell nitric oxide synthase (eNOS) pigs with an inserted *eNOS* transgene were produced [144] demonstrating the variable application of transgenic pigs.

Due to their anatomical and physiological similarities, pigs represent also promising donor organisms for xenotransplantation. The hyperacute rejection of donor organs after a transplantation from pig to primate can be overcome by modification of alpha-1,3-galactosyltrasferase (*GGTA1*) gene, which encode the *GGTA* epitop, the major xenoantigen triggering immune rejection. Therefore, different *GGTA* knock-out pig models with mutations on different exons of this gene were generated by

different groups, demonstrating strategies to overcome incompatibilities of pigs and primates (reviewed in [145]).

2.4.2 Artificial nucleases for modification of porcine genome

The first group that showed how ZFN technology can be applied to pigs was Watanabe *et al,* 2010 [75]. Porcine *eGFP*-transgenic fetal fibroblasts were transfected *in vitro* with *GFP* specific ZFN mRNA via electroporation and screened for GFP negative cells revealing a targeting rate of approximately 15 %. First ZFN-based gene modified piglets were reported one year later demonstrating again the knock-out of *GFP* in adult porcine ear fibroblasts hemizygous for the *eGFP* transgene [146].

Another group established the first ZFN-based knock-out pig model by disrupting the *GGTA1* gene on both alleles. The study demonstrated the suitability of the ZF method for gene modification of endogenous genes in pigs without introducing a transgenic sequence or selection cassette into the genome [5].

Transgenic pigs generated via TALEN-mediated modification have not been reported so far.

2.5 Aim of the study

The examples discussed above illustrate the importance of pigs in biomedical research and at the same time demonstrate that further research is needed to generate suitable models for human diseases. In order to achieve this, it is necessary to select an optimal molecular tool that is convincing in targeting specificity and efficiency. The abundance of genetic tools for modifying the mammalian genome offers potential in order to find a targeting strategy for many different purposes. Although every individual strategy provides many advantages, it is essential to select the targeting tool carefully according to their respective limits. Furthermore, not every strategy can be used for modifying the pig genome, as no porcine ESCs are available to date.

To determine a targeting tool that provides a simple and fast generation of transgenic pigs, we examined the suitability of the relatively new ZFN in generating genomic modifications.

In this study, ZFNs were used to generate a functional gene knock-out of the porcine *CFTR* gene by transfecting porcine primary kidney cells with ZFN mRNA of plasmid DNA.

In addition, the combination of ZFN technology with already-established BAC vectors was investigated to determine potential increase in targeting efficiency and to compare the successfully used BAC technology with new developed targeting strategies.

The potential of ZFN to mediate gene replacement was determined by co-transfecting these proteins with a DNA template. Therefore, a targeting vector was designed carrying an exogenous *lacZ* gene to replace exon 1 of the *CFTR* gene and to insert *lacZ* under the *CFTR* promoter for generation of *lacZ*-transgenic pigs.

We also evaluated the newly designed TALENs for their application in porcine primary cells and their targeting ability. Furthermore, ZFNs and TALENs were constructed for the same exons of the porcine dystrophin (*DMD*) gene in order to compare their targeting efficiencies.

The examination and establishment of new gene targeting technologies is crucial for generation of new pig models that in turn are essential research tools for understanding the development and progression of human diseases.

3 MATERIALS AND METHODS

3.1 Materials

All chemicals were used in p.a.-quality and buffers and solutions were prepared with deionized water, aqua bidest, obtained from a Millipore device (Milli-Q®), and stored at room temperature, if not stated otherwise.

3.1.1 Apparatuses and consumables

Table 3-1: Apparatuses used

Apparatus name	Manufacturer
AccuJet pro Pipetman	Brand, Wertheim
Agarose gel electrophoresis chamber	OWL Inc., USA
Zeiss Axiovert 200 M fluorescence microscope	Carl Zeiss, Oberkochen
Cellavista High End System	Roche, Mannheim
Centrifuges 5415 D, 5417 R, 5810R	Eppendorf, Hamburg
Centrifuge Biofuge pico	Heraeus, Osterode
Centrifuge Labofuge M	Heraeus, Osterode
Centrifuge Rotanda 96	Hettich, Tuttlingen
Chyo scales	YMC Co., Japan
Eppendorf HH Mastercycler Gradient	Eppendorf, Hamburg
GeneAmp® PCR System 9700	Applied Biosystems, USA
GeneQuant Pro spectrophotometer	Amersham, UK
Gel documentation system	BioRad, Munich
GFL 3031 shaker	Hilab, Düsseldorf
Glass pipettes	Hirschmann, Eberstadt
Incubators	Memmert, Schwabach Heraeus, Osterode
Microscope DM IL	Leica, Wetzlar

MS1 minishaker	IKA Labortechnik, Staufen
Finnpipette® Multichannel pipet (300 µl)	Thermo Fisher Scientific, USA
Neubauer counting chamber	Assistent, Sondheim
Microprocessor pH meter	WTW, Weilheim
Pipettes (2 µl, 10 µl, 20 µl, 100µl, 200 µl, 1000 µl)	Eppendorf, Hamburg
Polyacrylamide gel electrophoresis chamber	BioRad, Munich
Power Pac 300 gel electrophoresis unit	
RH Basic heating plate with magnetic stirrer	IKA Labortechnik, Staufen
Severin 900 microwave	Severin, Sundern
SS35 50 ml centrifuge tubes	Eppendorf, Hamburg
Steril benches Laminair® HB2448K, HB2472	Heraeus, Osterode
Thermomixer 5436	Eppendorf, Hamburg
Thermostat Plus	Eppendorf, Hamburg
Water bath sub14	Grant, UK
WB6 water bath	Firmengruppe Preiss-Daimler, Medingen

Table 3-2: Consumables

Consumer item	Manufacturer
ABgene® 96-well PCR plates	Thermo Scientific, Ulm
Centrifuge tubes (15 ml, 50 ml)	Falcon®, Becton Dickinson, Heidelberg
6-well, 96-well F-bottom culture dishes	Greiner bio-one, Frickenhausen
60 mm, 100 mm culture dishes	Sarstedt, Nümbrecht
Cultupe sterile culture tubes	Simport, Canada
Cryotubes 1 ml	NuncTM, Denmark
Cryotubes 2 ml	Almeco, Denmark
Parafilm®M	American Can Company, USA
PCR reaction tubes (0.2 ml)	Braun, Wertheim

Pipette tips	Eppendorf, Hamburg
Pipette tipps with filter	Axygen Inc., USA
QIAtip 500	Qiagen, Hilden
SafeGrip® Latex gloves	SLG, Munich
SafeGrip® Nitril gloves	SLG, Munich
Safe-Lock reaction tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg
Serological pipettes	Greiner bio-one, Frickenhausen
Steritop GP 0,22 µm Express®plus membrane	Millipore, USA
Sterivex GP 0,22 µm	Millipore, USA

3.1.2 BAC constructs, plasmids and bacterial strains

Table 3-3: BAC and plasmid constructs used

BAC/plasmid name	Clone number	Description
248CFTRlacZ	24-5	BAC, containing *lacZ* and neokan resistance cassette
248CFTRlacZ	2-5	BAC, containing *lacZ* and neokan resistance cassette
pBSK-CFTR-lacZ	8	Containing *lacZ* and neokan resistance cassette
pBluescript II SK (-) (pBSK)		Thermo Fisher Scientific, USA
pGEM®- T Easy Vector		Promega, USA

Table 3-4: Bacterial strains

Strain	Manufacturer
DH10B	New England Biolabs, USA
TOP10	Invitrogen, Karlsruhe

3.1.3 Buffers, media and solutions

Table 3-5: Buffers and solutions used for molecular cloning protocols

Buffer/solution	Components
Chloroform-isoamylalcohol (CiA)	96 ml chloroform 4 ml isoamylalcohol Stored at 4°C protected from light
DNA loading buffer (10 ×)	10 % glycerol in aqua bidest. 1 spatula tip of bromophenolblue Add 0.5 M NaOH until colour turns blue Stored at 4°C.
DNA molecular weight standards	100 µl pUC8 Mix Marker 8 or 1 kb DNA ladder standard 100 µl 6 × loading dye 400 µl aqua bidest Stored at -20 °C
dNTPs	2 mM or 10 mM respectively dATP, dCTP, dGTP, dTTP Stored at -20 °C
Formamide loading dye (98 %)	10 ml formamide 0.5 mg bromphenolblue 0.5 mg xylene cyanol 200 µl EDTA (0.5 M)
LB medium	5 g yeast extract 10 g tryptone/peptone 2.5 g NaCl Ad 1000 ml aqua bidest pH 7.0 (adjust with 5 M NaOH) Autoclave
LB-agar plates	5 g yeast extract 10 g tryptone/peptone 5 g NaCl pH 7.0 (adjust with 5 M NaOH)

	15 g agar-agar Autoclave Cool down to 60°C Add 1 ml respective antibiotic (ampicillin 50 mg/ml, chloramphenicol 12.5 mg/ml, kanamycin 25 mg/ml) Pour into culture dishes Stored at 4°C
Lysis buffer for DNA isolation (High salt precipitation)	100 µl PK buffer (1 ×) 10 µl SDS (10 %) 4.4 µl DTT (1 M)
Phenol-chloroform-isoamylalcohol (PCiA)	25 ml phenol 25 ml CiA Stored at 4°C, protected from light
PEG-$MgCl_2$	40 % (w/v) PEG 8000 30 mM $MgCl_2$ Stored at room temperature
PK buffer (10 ×)	200 mM Tris 1 M NaCl 40 mM EDTA Stored at room temperature
Proteinase K 20 mg/ml	Stored at 4°C
Rnase A 20 mg/ml	Stored at 4°C
Sequencing buffer (5 ×)	17.5 ml 1 M Tris/HCl (pH 9.0) 125 µl 1 M $MgCl_2$ Ad 50 ml aqua bidest Stored at -20°C
Super Optimal Broth (SOC) medium	2.5 g yeast extract 10 g tryptone/peptone 0.25 g NaCl Ad 500 ml aqua bidest pH 7.0 (adjust with 5 M NaOH)

	Autoclave Ad 2 M $MgCl_2$ (final concentration 10 mM) Ad 1 M Glucose (final concentration 20 mM)
Solution A	50 mM glucose 25 mM Tris/HCl pH 8.0 10 mM EDTA/NaOH pH 8.0
Solution B	0.1 M NaOH 0.5 % (w/v) SDS Prepared freshly before use
Solution C	3 M KOAc pH 4.8 with 9 M HOAc Autoclave
STE	10 mM Tris/HCl pH 8.0 100 mM NaCl 1 mM EDTA/NaOH pH 8.0
Tbf I	30 mM KOAc pH 6.0 100 mM $CaCl_2$ 15 % (w/v) glycerol Ad 250 ml aqua bidest Autoclave Ad 1 M $MnCl_2$ (final concentration 50 mM)
Tbf II	10 mM MOPS pH 7.0 75 mM $CaCl_2$ 10 mM KCl 15 % (w/v) glycerol Ad 20 ml aqua bidest Autoclave
T-buffer	10 mM Tris Adjust to pH 8.0 with HCl
TAE (50 ×)	242 g Tris 100 ml 0.5 M EDTA (pH 8.0) 57 ml AcOH

	Ad 1000 ml aqua bidest Diluted to respective concentration prior to use.

3.1.4 Chemicals

Table 3-6: Chemicals used

Chemical name	Manufacturer
Acetic acid (glacial) (HOAc)	Merck, Darmstadt
Acrylamide-bisacrylamide (40 %)	Roth, Karlsruhe
Agar-agar	
Agarose low-melting	Thermo Fisher Scientific, USA
Agarose UltraPure™	Invitrogen, Karlsruhe
Agarose Universal	Bio&SELL, Nürnberg
Ammonium persulfate (APS)	Roth, Karlsruhe
Ampicillin	
Bromophenolblue	
Calciumchlorid ($CaCl_2$)	
Chloramphenicol	Sigma-Aldrich, Steinheim
Chloroform	Merck, Darmstadt
Dithiothreitol (DTT)	Biomol, Hamburg
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe
Ethanol (EtOH)	
Ethidiumbromide	Merck, Darmstadt
Geneticin (G418)	Invitrogen, Karlsruhe
Glucose	Roth, Karlsruhe
Glycerol	

Hydrochloric acid, 37 % (HCl)	
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	Thermo Fisher Scientific, USA

Isoamylalcohol	Roth, Karlsruhe
Kanamycin	
Magnesium chloride ($MgCl_2$)	Merck, Darmstadt
Manganese(II) chloride ($MnCl_2$)	Roth, Karlsruhe
N, N, N', N'-tetramethylethylenediamine (TEMED)	
3-(N-morpholino)propanesulfonic acid (MOPS)	
Polyethylenglycol (PEG) 8000	Roth, Karlsruhe
Peptone/Tryptone	
Phenol	
Potassium acetate (KOAc)	
Potassium chloride (KCl)	Sigma-Aldrich, Steinheim
di-Potassiumhydrogenphosphate (KH_2PO_4)	Roth, Karlsruhe
2-Propanol (iPrOH)	
Sodiumdodecylsulfate (SDS), ultrapure	
Sodium chloride (NaCl)	
Sodiumdihydrogenphophate-1-hydrate (Na_2HPO_4+2H_2O)	Merck, Darmstadt
Sodium hydroxide (NaOH)	
Sodium pyruvate	Invitrogen, Karlsruhe

Tris-(hydroxymethyl)-aminomethan (Tris)	Roth, Karlsruhe
Yeast extract	
5-Brom-4-chlor-3-indoxyl-β-Dgalactopyranosid (X-Gal)	

3.1.5 Enzymes, kits and other reagents

Table 3-7: Enzymes used for molecular cloning protocols

Enzyme name	Manufacturer
T4 DNA ligase and buffer (10x)	Thermo Fisher Scientific, USA
Taq polymerase and buffer (10x)	Agrobiogen, Hilgertshausen
Proteinase K	Roth, Karlsruhe
Restriction enzymes and buffer (10x)	Thermo Fisher Scientific, USA
Ribonuclease A (RNase A)	Roche, Mannheim

Table 3-8: Kits

Kit name	Manufacturer
CloneJET™ PCR Cloning Kit	Thermo Fisher Scientific, USA
E.Z.N.A.™ Endo-free Plasmid Maxi Kit	Omega, USA
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel, Düren
QIAEX® II Gel Extraction Kit	Qiagen, Hilden
QIAGEN Large-Construct Kit + EndoFree	

Plasmid Buffer Set	
SURVEYOR® Nuclease Kits	Transgenomic, USA

3.1.6 Other reagents

Table 3-9: Reagents used for molecular cloning protocols

Name of reagent	Manufacturer
Agarase	Thermo Fisher Scientific, USA
BigDye® terminator v3.1	Applied Biosystems, USA
DNA loading dye (6x)	Thermo Fisher Scientific, USA
dNTPs (dATP, dCTP, dGTP, dTTP)	
Gene Ruler™ 1 kb DNA Ladder	

3.1.7 Materials for cell culture

Table 3-10: Cell line used

Cell line	Cell type	Culturing medium	Splitting ratio
Niere m	Primary porcine kidney cells	DMEM, 10 % FCS	Every 2 days, 1:4- 1:5

Table 3-11: Kits and apparatuses used

Kit/Apparatus name	Manufacturer
Amaxa™ Basic Nucleofactor™ Kit for Primary Mammalian Fibroblasts	Lonza, Cologne
Nucleofector™ II	

Table 3-12: Media and solutions used for cell culture

Medium/solution	Components
Cell culture medium	DMEM with 10 % or 15 % (v/v) FCS 1 % (v/v) Non-essential amino acids (100 ×) 1 % (v/v) Sodium pyruvate (100 ×) 1 % (v/v) L-glutamine (200 mM) + Penicillin/Streptomycin (100 ×) 0.1 mM β-mercaptoethanol Stored at 4°C
Cryo medium	10 % (v/v) DMSO 90 % (v/v) FCS Prepared freshly before use on ice
PBS without Ca/Mg	8 g NaCl 0.2 g KCl 0.2 g KH_2PO_4 2.14 g Na_2HPO_4+$7H_2O$ Ad 1000 ml aqua bidest pH 7.2-7.4
Selection medium	Cell culture medium 1.2 mg/ml G418
Stop medium	10 % (v/v) FCS 90 % (v/v) DMEM
Trypsin/EDTA	PBS without Ca/Mg 0.5 % (w/v) Trypsin 0.04 % (w/v) EDTA

Table 3-13: Reagents and chemicals used for cell culture

Reagent/Chemical name	Manufacturer
Acetic acid (glacial)	Merk, Darmstadt
β-Mercaptoethanol	Sigma-Aldrich, Steinheim
Colcemid	
CollagenR	Serva, Heidelberg
Difco™ Trypsin 250	BD, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim
Dulbecco Modified Eagle Medium (DMEM)	Invitrogen, Karlsruhe
Fetal calf serum (FCS)	
Karyomax (colcemid)	
L-Glutamine (200 mM)	PAA, Austria
L-Glutamine + Penicillin/Streptomycin (100x)	
Methanol	Roth, Karlsruhe
Non-essential amino acids (100x)	Invitrogen, Karlsruhe
Vectashield Mounting Medium with DAPI	Vector Laboratories, USA

3.1.8 Oligonucleotides

Oligonucleotides were designed by hand and manufactured by Thermo Fisher Scientific, USA.

Table 3-14: Primer used for molecular cloning and screening analysis

Primer name	Primer sequence
Cs1f	5’-GTG GAG AAA GCC GCT AGA G-3’
Cs3r	5’-CCT CTT TCC TGG GCA CGT GTC CTT C-3’
Cs9f	5’-GGA ACT GGA AGC AAA TGA CAT-3’
Cs5r	5’-TTC CAA AGC TCA GCT AGA CAC-3’
scr1nr	5’-AGA ATG AGA CCC TTG TTT G-3’
neo1f	5’-CTG CTA AAG CGC ATG CTC CAG-3’
neoR-1r	5’-TCG ATC GTC CTG TAA GTC TGC-3’
scr-2lf	5’-TTG AGG CAC ATC ACT GGG TC-3’
scr-2lr	5’-ACG TTC AGA CGT AGT GTG ACG-3’
M13	5'-GGA AAC AGC TAT GAC CAT G-3'
T7	5'-GTA ATA CGA CTC ACT ATA GG-3'
pJETforward	5'-CGA CTC ACT ATA GGG AGA GCG GC-3'
pJETreverse	5'-AAG AAC ATC GAT TTT CCA TGG CAG-3'
neokanf	5'-GAC AAT AGC AGG CAT GCT G-3’
neokanR	5'-GTG GAT GTG GAA TGT GTG C-3'
lacZr	5’-GTT CGG ATA ATG CGA ACA G-3’
CFTR-3armr	5’-TAC TTG CGG CGG CAA GTC CAA GTG ATC AGT CC-3’
CFTR-5armf	5’-CTG GTT GGT ACC TTC TGT CCT CGA G TGT C-3’
neoSr	5’-GAG TCA ACT AGT CCT CAG AAG AAC TCG TCA AG-3’
neoPf	5’-CAG CTG TGC TCG ACG TTG TC-3’
neor21	5’-CCA CCA TGA TAT TCG GCA AGC AG-3’

neor22	5’-CAC GGG TAG CCA ACG CTA TGT C-3’
neof21	5’-AGA TGG ATT GCA CGC AGG TTC TC-3’
PGKr	5’-GCT GCT AAA GCG CAT GCT CCA GAC-3’
PGK41f	5’-CAG TCT GGA GCA TGC GCT TTA GCA G-3’
PGK107f	5’-CAC ACA TTC CAC ATC CAC CGG TAG-3’
3armf1	5’-GTC TGC AGA GGC CAG CAT CTT CTC CAA AC-3’
3armr3	5’-ATG GTA CCG GAA TGG CAT TTA GGT TCT TCC AAG-3’
5armf1	5’-ACG AGC TCG GTA CCT AGG TTT GTA CTC CAT TCA G-3’
5armr2	5’-TTG GAT CCG GGC TCG AGC TCC TAA TGC CA-3’
CFTR5arm-2f	5’-GAG GCA TTA ATC AGA CCA AAG-3’
CFTR5arm-3f	5’-CTC ACC TAA GCC TGA GAC TAA-3’
CFTR5arm-4f	5’-ACA CAC TAG GAT TCA GGA ATT-3’
CFTR5arm-1r	5’-CTC GGA CAC TCG AGG ACA GAA-3’
CFTR5arm-2r	5’-ATT CTG ACT CCC AAC CTT CCT-3’
CFTR5arm-3r	5’-ACA TTT CTA ACT CAA CGA TC-3’
CFTR3arm-1f	5’-TGA GCA GTT TGT CTG GAG CAT-3’
CFTR8-1f	5’-ACA ATA GCA GGA ATC ACT AGT G-3’
CFTR8-1r	5’-GGA AGT TTT TCC TGT CAT ACT-3’
pBSK1f	5’-TCA CTG ATT AAG CAT TGG TAA CTG-3’
pBSK1r	5’-CTC ACA ATT CCA CAC AAC ATA-3’
CFTR402f	5’-GGC GCC GAG AAG AGT AGG G-3’
CFTR621r	5’-TTT CCA CCC CAA ACG CAG-3’
OCTqf2	5’-CAG GTA GGT TAG GCT GAT AG-3’
OCTqr1	5’-CAA GTT CAT GAA CGG CAG AAC-3’
NGqf6	5’-GAG GCT TCA CTT GTT AAG GG-3’
NGqr4	5’-CTG AGA TCA GGG TAG ACA TAC-3’

3.1.9 Software

Table 3-15: Software used

Software name	Manufacturer
BioEdit Sequencer Alignment Editor	Ibis bioscience, USA
Double DigestTM	Thermo Fisher Scientific, USA
Finch TV Version 1.3.1	Geospiza Inc., USA
NEBcutter V2.0	New England Biolabs, USA
Cellavista SW Workstation Version 2.0.0.23	Roche, Mannheim

3.1.10 ZFNs and TALENs

ZFNs for exon 1 of the porcine *CFTR* gene were designed and manufactured by Sigma-Aldrich, USA. Each ZFN set was delivered as plasmid DNA and mRNA (pooled mRNA of both ZFNs) and stored at -80°C to avoid degeneration.

ZFNs and TALENs for exon 49 of the porcine *DMD* gene were designed and constructed by Prof. Dr. Toni Cathomen, and delivered as plasmid DNA.

Table 3-16: ZFNs designed for targeting of the *CFTR* exon 1.

Upper-case letters indicate the ZFN binding site, lower-case letters indicate the ZFN cleavage site.

ZFN set		Targeting sequence
Set 1	ZFN 1	5'- GACATCACCgCAGGTCagaga-3'
	ZFN 2	5'- gaAAAAGGGGCGAGAGGCAG-3'
Set 2	ZFN 3	5'- GACCCCGGTTCCCCCagagac-3'

	ZFN 4	5’- acCATGCAGAGGTCGCC-3’
Set 3	ZFN 5	5’- ACCCCGGTTCCCCCAgagac-3’
	ZFN 6	5’- acCATGCAGAGGTCGCC-3’

Table 3-17: ZFNs designed for targeting of the *DMD* exon 49.

Upper-case letters indicate the ZFN binding site, lower-case letters indicate the ZFN cleavage site.

ZFN name	Targeting sequence
DMDe49L	5’-GCTAAACAGccgga-3’
DMDe49R	5’-tgTGGAAGGGA-3’

Table 3-18: TALENs designed for targeting of the *DMD* exons 48 and 49.

TALEN name	Targeting sequence
TALe48	5’-TTGAAGAACAATTAAATCATCTGCTTGTGTGGCTATCTCC-3’
TALe49	5’-TAGCAGTTCAAGCTAAACAGCCGGA-TGTGGAAGGGATTTTGTCTAAAGGGCA-3’

3.2 Methods

3.2.1 Cell culture protocols

For transfection experiments a primary porcine cell line, “*Niere m*“, isolated from the kidney of a 3 months old male Landrace pig, was used. The isolation and cultivation protocols for these cells have been established by Dr. Annegret Wünsch. *Niere m* cells are a mixed cell population consisting of cells with flat, ellipsoid, fibroblast-like but also ones with rounder, epithelial-like morphology.

The suitability of this cell line for nuclear transfer and targeting has been tested by Dr. Annegret Wünsch and Dr. Mayuko Kurome.

All cell culture experiments were performed in a sterile laminar airflow cabinet. Also all media, solutions and material that were in direct contact with the cells were autoclaved or sterile filtered to avoid contamination of cell. Media and solutions were pre-warmed to 37°C in a water bath and culture dishes were pre-warmed to room temperature. Culture dishes were coated with Collagen R (0.2 mg/ml) diluted in aqua bidest. Cells were incubated at 37 °C, 5 % CO_2 and 95 % humidity.

3.2.1.1 Cultivating and passaging of cells

Porcine kidney cells were cultivated in growth cell culture medium containing 10 % FCS. Cells were usually grown up to a confluency of about 80 – 100 % in a 10 cm cell culture dish and subsequently split as following.

Niere m medium was aspirated and cells were washed with 4 ml PBS twice. To detach the cells from culture dish surface, they were incubated with 1.5 ml trypsin/EDTA (0.5 %) for approximately 3 min at 37°C until the cells got a round shape and started detaching. The reaction was stopped by with 8.5 ml stop medium and cells were transferred to a 15 ml falcon tube. To determine the amount of cells, 10 µl of cell suspension was filled into a Neubauer hemocytometer and counted. 1 x 10^6 cells were transferred to a new falcon tube and centrifuged for 5 min at 180 x g. After removing the supernatant, cell pellet was resuspended in 10 ml of fresh cell culture medium and transferred to a new 10 cm cell culture dish.

3.2.1.2 Thawing and freezing of cells

Frozen kidney cells were taken out of the liquid nitrogen tank and immediately transferred to a 37°C water bath. The thawed cell suspension was transferred into a 15 ml falcon containing 9 ml of pre-warmed stop medium. Stop medium containing thawed cells was spun down according to the protocol 3.2.1.1 and the cell pellet was resuspended in an appropriate amount of cell culture medium according to the splitting ratio and distributed to new 10 cm cell culture dishes. For each transfection experiment two 10 cm cell culture dishes were prepared, containing 1 × 10^6 cells each. The remaining cells were frozen in 1 ml pre-cooled FCS containing 10 % DMSO, placed at -80°C and transferred in liquid nitrogen for long term storage afterwards.

3.2.1.3 Transfection

For cell transfection the Amaxa Nucleofector™ Technology from Lonza was applied. This technology is based on electrical pulses and cell type specific solutions in order to transfer the foreign DNA directly into the nucleus [128].

Niere m was harvested and counted according to protocol 3.2.1.1. For transfection 0.5 x 10^6 or 1 x 10^6 cells were resuspended with 100 µl nucleofection solution of the AmaxaTM Basic NucleofectorTM Kit for Primary Mammalian Fibroblasts. In case of transfection with a ZFN only, 1 µg of ZFN was added. In case of co-transfection of DNA and ZFN 1 µg ZFN and 9 µg DNA has been added. Transfection with BAC DNA was performed with 5 µg of DNA. Cell suspension was transferred to nucleofection cuvette and after pulsing the cells according to program U-012 of the Amaxa nucleofector the cells were transferred to 6-well cell culture dish containing 5 ml cell culture medium supplemented with 15 % FCS. The transfected cells were cultivated for 48 h at 37°C for recovery, resulting in 100 % confluence. Medium exchange was performed 24 h after transfection.

3.2.1.4 Cell individualization

To obtain cell clones originated from one cell after transfection with ZFN and without using additional selection marker, it was essential to individualize the cell population 48 h after transfection. Therefore cells were harvested and counted according to protocol 3.2.1.1. The cell pellet was resuspended in an appropriate amount of cell culture medium containing 15 % FCS and distributed to 96-well dishes. An amount of 2 cells per well was seeded on cell culture plates. For a sufficient proliferation, the cell clones were cultivated for one week and the medium was exchanged every 72 h. Subsequently, cell culture plates were screened for single cell clones, which were evaluated and processed. Cell clones of good condition and a confluency of 60 – 70 % were washed with 100 µl PBS twice and detached by incubating the cell clones with 30 µl trypsin/EDTA (0.5 %) at 37°C for 4 min. The reaction was stopped with 170 µl cell culture medium containing 15 % FCS to reach a final volume of 200 µl. Each cell clone was propagated in two different wells, one for DNA isolation and the other for cryopreservation for potential SCNT. Both portions of the cell clones were harvested as described after reaching 100 % confluency. The

fraction for DNA isolation was resuspended with 170 µl stop medium after trypsinizing, transferred to a 1.5 ml tube and subsequently centrifuged for 5 min at 400 x g. The supernatant was removed and the clone pellet was frozen at -80°C. The fraction for cryopreservation was resuspended in 170 µl pre-cooled FCS containing 10 % DMSO after trypsinizing, transferred to a 1.5 ml tube and then immediately to -80°C for storage (figure 3-1).

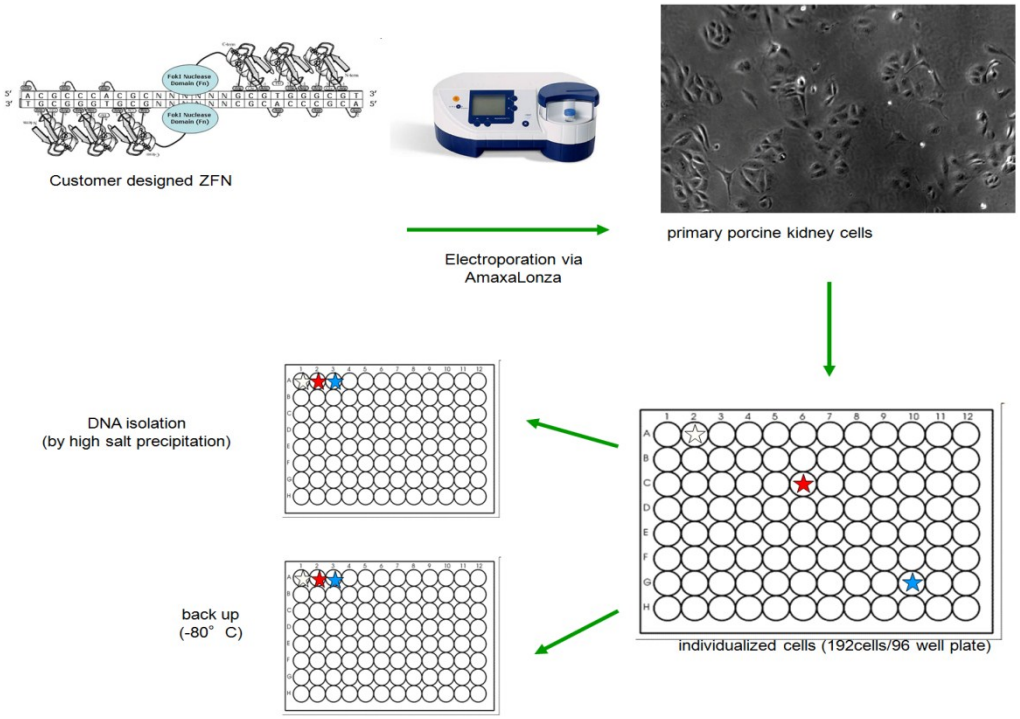

Figure 3-1: Overview over the cell culture procedure using a ZFN. Porcine kidney cells ("*Niere m*") were transfected with ZFN designed for exon 1 of the CFTR gene. Thereafter cells were seeded on 96-well dish and screened after 7 days of cultivation. Well proliferating single cell clones were splitted and stored at -80°C for different purposes.

3.2.1.5 Selection

Cells transfected with BAC DNA or other plasmid DNA carrying a neomycin resistance cassette were cultivated in selection medium containing 1.2 mg/ml geneticin (G418) to obtain single cell clones. The geneticin concentration was determined by Dr. Annegret Wünsch to eliminate *Niere m* cells, which have not integrated transfected DNA into their genome within one week. The cells were harvested and counted according to protocol 3.2.1.1 48 h after transfection and then resuspended in an appropriate amount of selection medium. Transfected cells

were mixed with non-treated wild-type *Niere m* cells and subsequently distributed to 96-well cell culture dishes. To obtain optimal cell growing conditions 500 transfected and 1500 wild-type cells were seeded per well, adding up to a final cell number of 2000 cells per well. Medium was exchanged every 72 h and after 7 days the 96-well plates were screened for G418 resistant clones. Single cell clones were cultivated and processed as described in 3.2.1.4.

3.2.1.6 Screening

For detection of single cell clones the Cellavista High End System was applied. Therefore, the selection medium was removed from the 96-well plates and 200 µl of fresh selection medium was added to each well. Subsequently, the plates were individually screened and the screening results were evaluated by the Cellavista SW Workstation controlling and evaluation software (figure 3-2).

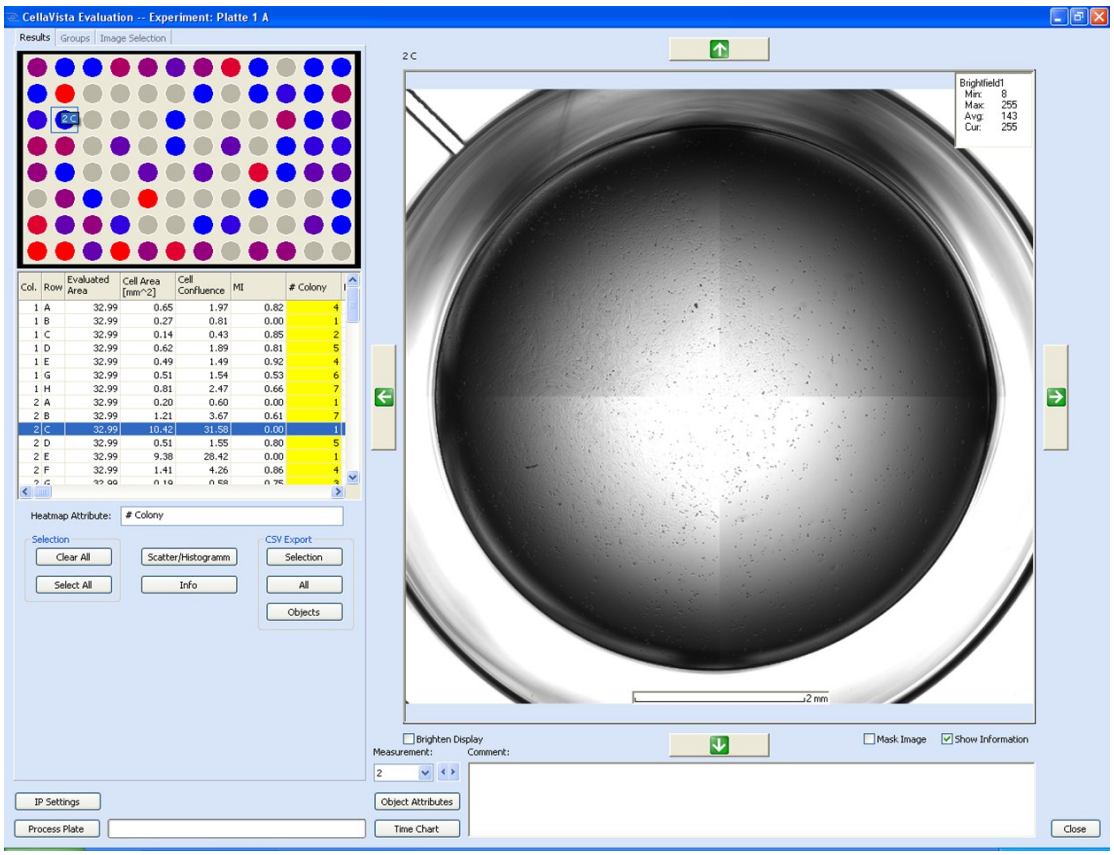

Figure 3-2: Cellavista SW Workstation software. Single cell clones on 96-well plates were screened by Cellavista High End System providing the documenting and tracking of cell clone proliferation.

3.2.1.7 Karyotype analysis

In order to analyze the karyotype of *Niere m*, a metaphase preparation had to be performed. Therefore cells were grown to a confluency of 70-90 % in a 10 cm dish and 100 µl colcemide (Karyomax; 10 µg/ml) were added to 10 ml cell culture medium. Subsequently, cells were incubated for 1 h at 37°C and then washed, trypsinized and centrifuged as described before (see protocol 3.2.1.2). The supernatant was removed and the cell pellet was resuspended in 1 ml stop medium, followed by a hypotonic treatment with 13 ml of 75 mM KCl, which were added drop wise to the cells by dropping. After incubation for 15 min at 37°C, cells were spun down again at 320 x g for 5 min and the supernatant was discarded. Thereafter, the cell pellet was resuspended in 1 ml stop medium and 10 ml ice-cold fixative containing 75 % methanol and 25 % glacial acetic acid were slowly added. Then, cell suspension was dropped onto a 45°C preheated glass slide, dried, mounted with Vectashield Mounting Medium with DAPI and sealed with a cover glass. Chromosomes were detected using an inverted epifluorescence microscope (Axiovert 200M, Zeiss) and analyzed by the ImageJ software.

3.2.2 Molecular genetic protocols

Molecular genetics protocols were used for modification of BAC DNA, cloning of pBSK-CFTR-lacZ8 and for analysis of generated single cell clones.

3.2.2.1 PCR

3.2.2.1.1 End-point PCR

The polymerase chain reaction (PCR) amplifies genetic sequences of interest, defined by specific primer sequences which were designed manually and manufactured by Thermo Fisher Scientific, USA. The reaction was performed in 0.2 ml PCR tubes at room temperature and the components were mixed to a final volume of 25 µl.

Table 3-19: Standard PCR components

Reagents	Volume [µl]
PCR buffer 10 x	2.5
dNTP (2 mM)	2.5
$MgCl_2$ (15 mM)	2.5
Taq-polymerase (5/µl)	0.2
Primer forward (10 µM)	0.5
Primer reverse (10 µM)	0.5
Template DNA	1
ddH_2O	15.3

Table 3-20: Standard PCR program

Steps	Temperature [°C]	Time [sec]	Cycles
First denaturation	95	240	1
Denaturation	95	30	35
Annealing	*	30	
Elongation	72	**	
Final elongation	72	60	1
Hold	4	∞	

Annealing temperature (*) depends on the primer sequence used for the reaction and was calculated by the 4 + 2 rule (2°C for each A and T, 4°C for each G and C, value − 5) and is indicated within the respective experiments in the results section. The elongation time (**) depends on the length of the amplicon and is calculated with 2 kb/min. Conventionally, 45 sec were used, exceptions are indicated within the respective experiments, described in the results section.

3.2.2.1.2 Colony PCR

Colony PCR is used after transformations in order to quickly screen occuring *E. coli* colonies for insertion of the desired plasmids. Therefore a reaction mix should be prepared according to protocol 3.2.2.1.1 and aliquoted to 0.2 ml PCR tubes at room

temperature. Instead of 1 µl DNA, a small amount of bacteria cells are used as a template. To do this, a single colony was touched with a fine pipette tip, dipped into the PCR reaction mix and was repeatedly pipetted up and down for better homogenization.

3.2.2.1.3 q-PCR

In genetic targeting experiments using BAC vectors, the copy number of wild-type alleles of the target locus as well as two independent reference loci was determined via q-PCR by Dr. Nikolai Klymiuk. The relative copy number of wild-type alleles at the target locus respective to the reference loci provided information whether the BAC recombined with the target site or inserted randomly.

3.2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to their length. 1 x TAE buffer was heated with 0.7 % agarose UltraPure™ or 1 % low melting agarose in a microwave to allow melting of agarose. After cooling down to 60 °C, ethidiumbromide (0.5 µg/ml) was added and the gel was poured into a gel tray attached with a comb. The chamber was filled with 1 x TAE and the comb was removed after gel polymerization. DNA samples were mixed with 1/10 volume DNA loading dye and loaded into the gel slots together with a molecular weight standard. An electric current was applied to the chamber. Voltage was applied to achieve 5 V/cm. DNA bands were visualized by UV-light and were characterized by comparing them with the molecular weight standard. If DNA fragments were used for further purposes fragments were excised under UV-light for DNA eluation.

3.2.2.3 Elution

For further processing DNA fragments were eluted from the gel, which was performed by NucleoSpin® Extract II Kit following the manufacturer's protocol. DNA was finally eluted with 30 µl elution buffer provided by the supplier. In order to determine the concentration of the eluted DNA 2 µl of the eluate was mixed with 15 µl H_2O and 2 µl DNA loading dye and loaded together with a molecular weight standard onto 0.7 % agarose gel prepared and run according to protocol 3.2.2.2.

Intensity of the bands was compared with bands of the molecular weight standard to determine the approximate amount of DNA and calculate the concentration.

For elution of DNA from a low-melting agarose gel, the desired DNA fragment was cut out of the gel, transferred into a 1.5 ml tube and placed on a heating plate at 70°C for approximately 10 min. The tube with the completely melted agarose gel was then transferred to a 42°C water bath and equilibrated for 5 min. Subsequently, 1 unit of Agarase per 100 mg of 1 % agarose was added, mixed and incubated for 30 min at 42°C. In order to hydrolyze the agarose, 0.3 M sodium acetate was added and the tube was chilled for 5 min on ice. After centrifugation at 15000 x g for 10 min the supernatant was transferred into a new 1.5 ml tube and PCiA extraction was performed for at least three times (see 3.2.2.5). DNA was precipitated by adding 3 volumes of 100 % EtOH and incubating at -20°C for 60 min. Finally, DNA was centrifuged at 15000 x g for 15 min, the supernatant was removed and the pellet was washed with 1 ml 70 % EtOH over night. The next day the pellet was again centrifuged at 15000 x g for 5 min, air dried for 6 min and resolved in 15 µl T-buffer. For quantification of the eluted DNA 2 µl of the eluate were mixed with 98 µl H_2O and 20 µl, 5 µl, 2 µl and 1 µl were loaded onto 0.7 % agarose gel. Intensity of the bands was compared with bands of the molecular weight standard to determine the approximate amount of DNA and calculate the concentration.

3.2.2.4 Digestion of DNA with restriction enzymes

This method can either be used to preparatively digest plasmid DNA for cloning or for analytical purposes, meaning to test the correctness of a plasmid. Recognition sites of restriction enzymes can be identifyed with the help of NEBcutter V2.0 software. For DNA digestion with more than one enzyme, optimal restriction conditions can be obtained from DoubleDigest™. The amount of DNA used for digestion depends on further processing, however, at least 1 µg DNA was digested with at least 1 unit restriction enzyme in 10 µl reaction mix. Restriction reaction was incubated at least 2 h at 37°C according to the amount of DNA and the optimal conditions of the used restriction enzyme.

Table 3-21: Components of an analytical and preparative digest

Components	Analytical digest	Preparative digest (for cloning)	Preparative digest (for transfection)
Buffer 10 x	2 µl	2 µl	10 µl
Enzyme 1	0.3 µl	1 µl	5 µl
Enzyme 2	0.3 µl	1 µl	5 µl
Plasmid DNA	1 µg	2-5 µg	20 µg
ddH_2O	Ad 25 µl	Ad 25 µl	Ad 100 µl

The analytical digest was mixed with 2.5 µl DNA loading dye and loaded on 0.7 % agarose gel, together with a molecular weight standard in order to determine the restriction products, which were visualized by UV-light as described in 3.2.2.2.
Sampels of a preparative digest needed to be purified by PCiA extraction before running on the gel. For the preparation of linearized DNA for transfection into porcine primary cells, digested DNA was loaded on low melting agarose gel.

3.2.2.5 PCiA extraction

After digestion it might necessary to inactivate or to remove enzymes completely from a DNA sample as the restriction enzymes would otherwise continuously cleave the DNA. For some restriction enzymes this is performed by heating of the samples to 65°C for 15 min, but not all enzymes are sensitive to heat inactivation. In this case, DNA is purified by phenol-chloroform extraction before gel separation.
The restriction reaction volume was filled up to 150 µl with ddH_2O and 100 µl PCiA were added. Then the mixture was shaken for 1 min, followed by centrifugation for 3 min at 16000 × g to separate the two phases. The upper aqueous phase containing the nucleic acids was transferred into a new 1.5 ml reaction tube without disturbing the interphase or the lower organic phase containing enzymes. 1/10 of sample volume of 3 M NaOAc and 3 volumes of EtOH 100 % (cooled to −20 °C) were added and samples were placed at −80°C for 60 min. Subsequently the samples were centrifuged at 16000 x g at 4°C for 30 min and the supernatant was removed. The DNA pellet was washed with 500 µl 70 % EtOH, centrifuged for 2.5 min and the EtOH was discarded. After air drying the DNA pellet for 6 min, it was resolved in 20 µl T-

buffer and loaded on an UltraPure™ agarose gel. The band of interest was excised and eluted according to protocol 3.2.2.3.

3.2.2.6 Ligation

For cloning DNA fragments into a vector it is necessary to ligate the parts by T4 DNA ligase. Vector and insert, which might be either a DNA fragment or PCR product, were ligated in stoichiometric amounts in a 1.5 ml tube in a total volume of 20 µl and incubated for at least 4 h at room temperature. Termination of the ligation reaction was achieved by placing the samples on 65°C thermo block for 15 min.

Table 3-22: Standard ligation components

Reagents	Volume [µl]
Vector DNA (max. 100 ng)	*
Insert DNA	**
Ligase buffer 10 x	2
T4 DNA ligase	1
ddH_2O	Ad 20 µl

Amount of insert DNA (**) was stoichiometric equally added to the reaction mix depending on the amount of vector DNA (*), which should not exceed 100 ng in total.

3.2.2.7 Heat shock transformation

In order to multiply plasmids with a high copy number and to minimize mutation events, the plasmids were transformed into a competent *E. coli* stain, TOP 10, by heat shock transformation. Therefore competent cells were thawed up on ice, 4 µl of plasmid DNA previously ligated according to protocol 3.2.2.6 was added and mixed gently. In case of a re-transformation only 1 µl of a 1/1000 dilution of a plasmid was used. After incubating the cells on ice for 20 min, they were placed in a 42°C water bath for 43 sec and immediately brought back on ice for 2 min. 1 ml of SOC medium was added to the cells and they were incubated on 37°C for 45 min for recovery. After spinning the cell down at 2300 x g for 5 min 800 µl of the

supernatant medium was removed, the cells were resuspended in the remaining medium and plated on LB agar plates containing an appropriate antibiotic (ampicillin 50 µg/ml, kanamycin 25 µg/ml and chloramphenicol 12.5 µg/ml). For cloning of PCR fragments into blue/white selectable plasmid (e.g. pGEM T-easy) plates were additionally supplemented with 40 µl IPTG (100 mM) and 40 µl X-Gal (20 mg/ml in Dimethylformamide) for differentiation of positive (insert containing) and negative (emply) plasmid colonies. After incubating the plates at 37°C over night the bacteria colonies were counted and plates were wrapped with Parafilm®M and stored at 4°C until further processing.

3.2.2.8 Preparation of heat shock competent cells

A 5 ml overnight culture of the *E. coli*-stain TOP10 was inoculated and 1 ml was transferred into 250 ml TYM medium the next day. Bacteria were grown to an OD_{600} value of 0.7 -0.8, distributed to 6x SS35 centrifugation tubes and incubated in ice-water for 10 min. After centrifugation with 1300 x g at 4°C for 10 min, the pellets were resuspended in 12 ml pre-cooled TbfI solution each carefully avoiding air bubbles and distributed on 2 x SS35 centrifugation tubes. The cell solution was left in ice-water for 10 min again and centrifuged as described above. The supernatant was discarded, and pellets were carefully resuspended in 4.8 ml TbfII solution each. A volume of 100 µl cell suspension per tube was distributed on an appropriate amount of pre-cooled 1.5 ml reaction tubes placed on ice. The tubes were transferred to -80°C for long term storage.

3.2.2.9 DNA isolation

Depending on the type of the DNA, different protocols were used for isolation. In any case, the concentration of the isolated DNA was measured with the GeneQuant Pro spectrometer at a wave length of 260 nm by adding 5 µl DNA to 95 µl aqua bidest (1:20 dilution) and the samples were stored at -20°C (plasmids) or 4°C (genomic DNA).

3.2.2.9.1 Isolation of genomic DNA

DNA from primary cell clones grown in 96-well cell culture plates was isolated via high salt precipitation protocol. Lysis buffer for DNA isolation by high salt precipitation was freshly prepared, 100 µl were added to the cell pellet and mixed thoroughly. The samples were incubated at 60°C for 1 h and 2 µl proteinase K were added, followed by incubation at 60°C for another 60 min. After that, 30 µl of 4.5 M NaCl were added, samples were placed on ice immediately and subsequently centrifuged at 16100 × g for 20 min. The supernatant was transferred into a new reaction tube and the DNA was precipitated with 90 µl 2-propanol. After centrifugation at 16100 × g for 20 min, supernatant was removed and the DNA pellet was washed with 200 µl 70 % EtOH overnight. Samples were centrifuged the other day at 16100 × g for 20 min, the supernatant was discarded and the DNA pellets were air dried for 6 min. DNA was resolved in 30 µl T-buffer and left at 60°C for 2 - 6 h.

3.2.2.9.2 Isolation of plasmid DNA

Preparation of plasmid

To isolate plasmid DNA 5 ml LB medium containing the appropriate antibiotic (ampicillin 50 µg/ml, kanamycin 25 µg/ml and chloramphenicol 12.5 µg/ml) were inoculated with bacteria colony carrying the desired plasmids and grown with shaking at 37°C overnight. For long-time storage of certain plasmids glycerol stocks were made by mixing 900 µl 60 % glycerol with 300 µl overnight bacterial culture, which were stored at −80°C. For the preparation of plasmids, overnight cultures were centrifuged at 1300 × g for 10 min and the supernatant was removed. Pellets were resuspended in 750 µl STE buffer, transferred into a new 1.5 ml reaction tube and centrifuged again at 4500 × g. Bacteria pellets were resuspended thoroughly in 200 µl solution A and 400 µl of solution B was added. The samples were mixed gently by inverting the tubes several times and placed on ice for 5 min. Lysis reaction was neutralized with 300 µl of solution C. After gently mixing, sample tubes were placed on ice for another 3 min for precipitation of proteins and cell debris and then centrifuged for 10 min at 16100 × g. The supernatants were transferred into new 1.5 ml reaction tubes, 4 µl RNase A was added to each tube, which were then incubated

at 37°C for 45 min. Afterwards a PCiA extraction was performed as described in 3.2.2.5 to remove RNase and other protein contaminations. Following that, DNA was precipitated with 650 µl 2-propanol and pelleted by centrifugation at 16100 × g for 10 min. The supernatant was discarded and the DNA pellet was washed with 700 µl 70 % EtOH overnight. In order to remove the EtOH samples were centrifuged at 16100 × g for 5 min, supernatant was discarded and DNA pellet was air dried for 6 min. DNA was resolved in 55 µl T-buffer and incubated at 55°C for 1 h.

Endotoxin-free preparation of plasmid DNA or BAC DNA

For transfection of DNA into porcine primary cells, 200 ml LB medium containing an appropriate antibiotic (ampicillin 50 µg/ml or kanamycin 25 µg/ml) were inoculated with a bacterial colony containing the desired plasmid or BAC, and incubated with shaking overnight at 37°C.

Plasmid DNA was isolated endotoxin-free using the E.Z.N.A.^{TM} Endo-free Plasmid Maxi Kit following the manufacturer's protocol.

BAC DNA was isolated using the Large-Construct Kit following the manufacturer's protocol. To remove any endotoxins, the protocol was slightly modified. In brief, BAC was isolated from *E.coli* by alkaline lysis and precipitated with iPrOH to reduce the volume. BAC was resolved in 9.5 ml of xx buffer and linearized DNA was digested with 200 µl ATP-dependend exonuclease and 300 µl 100 mM ATP at 37°C for 60 min. Then, 850 µl buffer ER was added and the DNA was incubated at 4°C for 30 min for endotoxin removal. For final purification, 10 ml of Buffer QS was added and the sample was loaded on pre-equilibrated QIA-tip 500 columns, following the manufacturer's protocol again.

3.2.2.10 PEG precipitation

For a successful sequencing of plasmids it is necessary to purify them additionally via precipitation with polyethylene glycol (PEG) precipitation. 20 µl PEG-$MgCl_2$ and 20 µl aqua bidest were mixed with 20 µl plasmid and incubated for 10 min at room temperature. The mixture was centrifuged for 20 min at 16100 × g and the supernatant was discarded. Then the DNA pellet was washed with 100 µl 70 % EtOH

overnight and centrifuged for 2 min at 16100 × g afterwards. The supernatant was removed and the DNA pellet air died for 6 min and resolved in 20 µl T-buffer.

3.2.2.11 Sequencing

In order to determine the exact sequence of analysed DNA, samples were sequenced by capillary sequencing at the Helmholtz centre, Munich. For sequencing plasmid DNA was precipitated with PEG according to protocol 3.2.2.10 and PCR products were purified via NucleoSpin® Extract II Kit following the manufacture's protocol. In order to obtain an appropriate amount of DNA for the sequencing reaction, the following formula was used for calculation:

DNA amount (ng) = the length of sequence, which is to be sequenced (bp)⁄ 100 × 1.5

Plasmid DNA was diluted with aqua bidest to the desired concentration and 1 µl was used for sequencing reaction, whereas purified PCR products were used directly. In case of plasmid DNA, fragments ligated into a pGEM vector were sequenced using pGEM specific primers T7 and M13, however, for other vectors specific primer were designed. PCR products were sequenced using the same primer as has been used for PCR reaction of this amplicon.
Sequencing reaction mix was prepared in 0.2 ml reaction tubes at room temperature with a final volume of 10 ml.

Table 3-23: Standard sequencing components

Reagents	Volume [µl]
sequencing buffer 5 x	4
BigDye chemical	1
Primer (10 µM)	1
Template	1
ddH2O	3

Table 3-24: Standard sequencing program

Steps	Temperature [°C]	Time [sec]	Cycles
First denaturation	95	60	1
Denaturation	95	5	40
Annealing	50	10	
Elongation	60	240	
Hold	4	∞	

After sequencing reaction the samples were purified using 2.5 µl 125 mM EDTA and 30 µl 100 % EtOH (cooled to -20°C) for each reaction tube. The tubes were left on ice for 15 min and then centrifuged at 16000 x g at 4°C for 30 min. After removing the supernatant the DNA pellet was washed with 50 µl 70 % EtOH. The next day samples were centrifuged again for 5 min on 16100 × g, EtOH was discarded and samples were air dried for 6 min. DNA was resolved in 30 µl aqua bidest, transferred to a sequencing plate (ABgene® 96-well PCR plates) and stored at -20°C before transportation to the Helmholtz centre. The results were analysed with the help FinchTV Version 1.3.1 and BioEdit software.

3.2.2.12 Next generation sequencing

Porcine primary cells transfected with ZFNs and TALENs, respectively, designed to target the porcine *DMD* gene were analyzed for introduced mutations at the targeting site by next generation sequencing. Cells were incubated after transfection for 48 h at 37°C and harvested. Genomic DNA was isolated from a mixed cell population for each cell culture experiment via high salt precipitation and the targeting site of ZFNs and TALENs was amplified. The produced amplicons were sequenced in Gene Center Munich on the Illumina Genome Analyzer IIx.

*3.2.2.13 Cel*I assay

Single cell clones were screened for ZFN-mediated mutations via *Cel*I assay. ZFN targeting site of single cell clones and wild-type DNA was amplified by end-point PCR (see 3.2.2.1.1) and 10 µl were used for the mutation detection assay. Therefor the SURVEYOR® Nuclease Kit was applied following the manufacturer's protocol. The

obtained DNA fragments were mixed with 1 µl DNA loading dye and loaded on 0.7 % agarose gel, together with a molecular weight standard for separation. Finally, the results were visualized by UV-light as described in 3.2.2.2.

3.2.2.14 Single-strand conformation polymorphism (SSCP)

ZFN transfected single cell clones were screened for induced mutation at the ZFN cleavage site via SSCP. Polyacrylamide gel electrophoresis was applied to separate single-stranded DNA according to their conformation. For a 12 % gel the following components were mixed:

Table 3-25: Polyacrylamide gel components

Components	Volume (ml)
40 % Acrylamide-bisacrylamide	6
10 x TAE	1
Glycerol	2
ddH_2O	11

80 µl of 10 % APS and 32 µl of TEMED were added to the solution and poured immediately between two glass plates fixed in a gel caster. An appropriate comb according to the sample number was attached. After polymerization the glass plates together with the gel were placed in an electrophoresis chamber and filled up with 0.5 x TAE and the comb was removed. Voltage was applied to achieve 10 V/cm in order to prerun the gel for 45 min.

The ZFN target site of the cell clones was amplified b end-point PCR (see 3.2.2.1.1) and 4 µl of the produced PCR product were mixed with 12 µl of formamide loading dye. The samples were heated at 95°C for 10 min and immediately placed at -20°C for further 10 min. Subsequently, the samples were loaded into the gel slots together with an appropriate molecular weight standard and run for at least 1 h. DNA was stained with ethidiumbromide (0.5 µg/ml) and visualized by UV-light.

4 RESULTS

In preliminary experiments, primary pig cells were examined for their suitability to genomic modifications by designed nucleases. For all experiments a primary cell culture, *Niere m*, was used. The cells were isolated by Dr. Annegret Wünsch from the kidney of a three months old Landrace boar and showed a typical heterogeneous morphology. After passaging this mixed population several times, spindle-shaped cells dominated and resulted in fibroblast-like cultures at passage 13 (P13; figure 4-1). The proliferative potential was evaluated until passage 29 and we did not observe any morphological signs of senescence. Moreover, long-term experience of our lab showed that *Niere m* cells are suitable for SCNT after 10 passages. Morphology and karyotype results of this work are consistent with results generated by Anne Richter later on during her Ph.D. thesis.

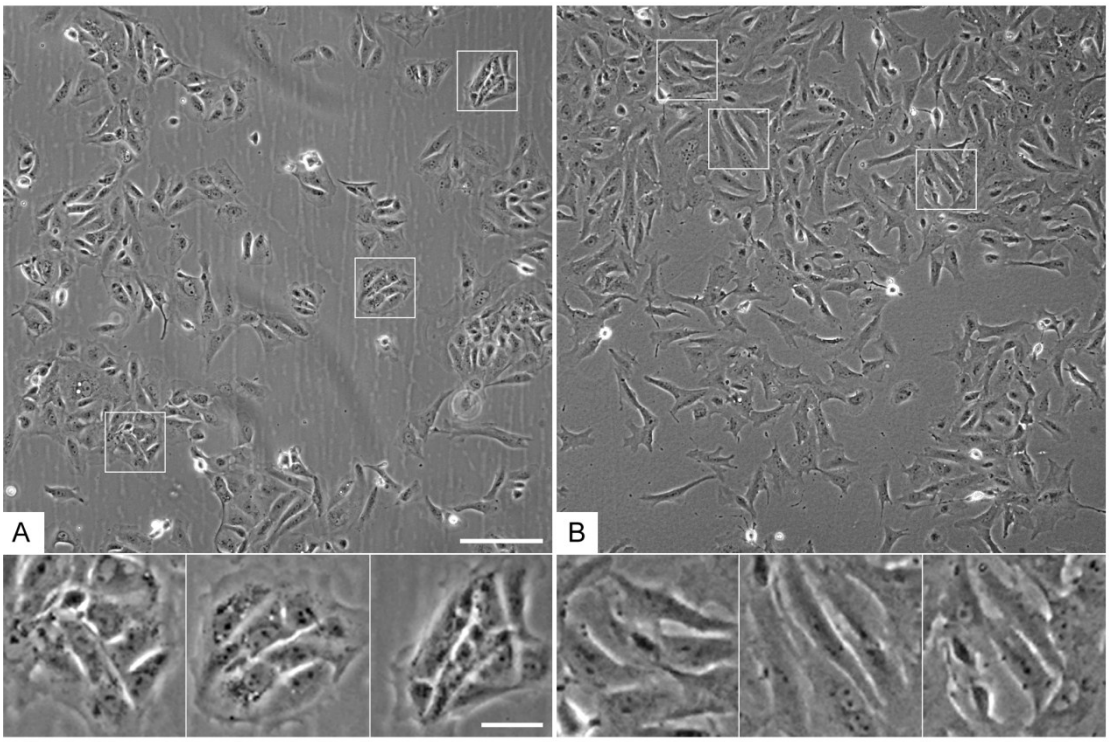

Figure 4-1: Morphology of *Niere m*. Passage 2 (P2) of porcine kidney cells show mixed morphology of (A) rounder endothelial-like cells and (B) fibroblasts-like morphology in P6. The insets show magnifications of the indicated areas (scale bar: 200 µm and 50 µm, respectively).

Niere m cells showed high proliferation potential in cell culture and the karyotype has been determined to evaluate the genetic stability of the cell line. 155 *Niere m* cells blocked in metaphases by colcemid were screened using an inverted epifluorescence microscope. 100 were found to be spread sufficiently and counting of chromosomes revealed that 80 % of the cells carried the expected karyotype of 2n=38, XY (figure 4-2).

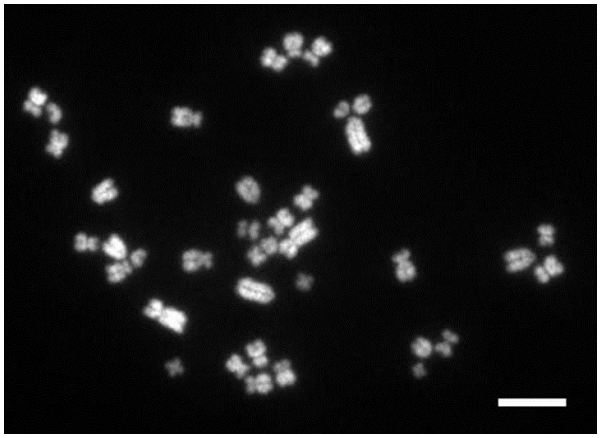

Figure 4-2: Karyotype analysis. Chromosomes of porcine kidney cells (P3) in metaphase stage spread and stained on a slide for determination of the karyotype of these cells, showing a normal karyotype of 2n=38,XY (scale bar: 10 µm).

The general transfection efficiency of *Niere m* cells was determined by transfecting 0.5 x 10^6 cells with 1 µg of a *GFP* encoding plasmid. Transfected cells were visualized by an inverted epifluorescence microscope 24 h after transfection and revealed a transfection efficiency of 60 to 70 % (figure 4-3).

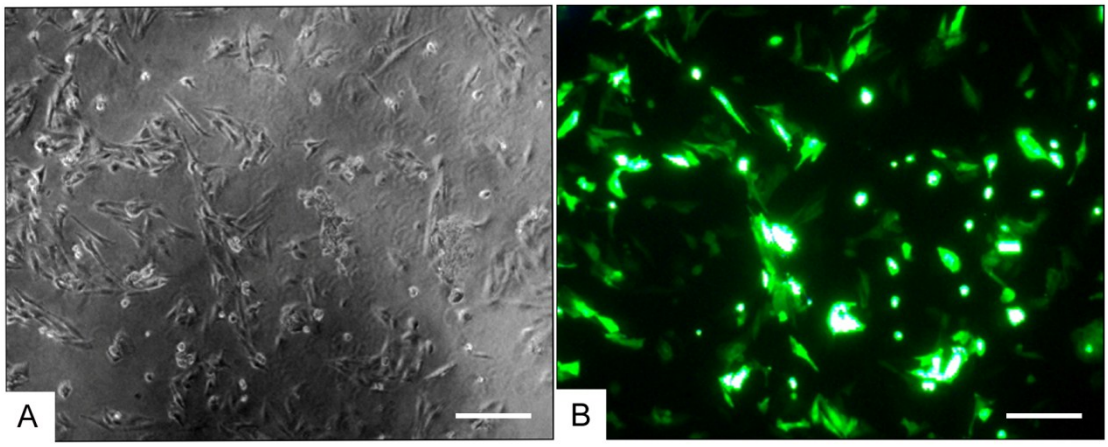

Figure 4-3: Determination of transfection efficiency of porcine kidney cells. (A) *Niere m* transfected with GFP acquired with transmission light and (B) with a fluorescence filter. Visual evaluation results in an efficiency rate of approximately 60 – 70 % (exposure time: 516.4 ms, scale bar: 200 µm).

Site-directed mutagenesis via NHEJ induced by nuclease-mediated DSBs does not involve integration of exogenous DNA and thus genetically modified cells cannot be identified by antibiotic selection. For identification of cell clones with induced mutations, cells have to be separated, cultured independently and characterized prior to SCNT. To achieve a sufficient number of cells for genomic analysis each clone has to undergo at least 15 cell divisions. Although the *Niere m* cells did not show any signs of senescence at passage 15, their cultivation as individual clones might impair their proliferation and survival capability.

Thus, the potential of *Niere m* cells to grow as discrete cell clones was tested by dilution assays in 96-well cell culture dishes. Seeding 192 cells per 96-well plate resulted in a sufficient number of single cell clones, but a high number of wells containing more than one clone was observed. Plates with 96 cells showed only 10 wells, containing more than one clone, but a lower number of defined single cell clones was observed as well (table 4-1). Consequently, 192 cells per 96-well plate were used for further experiments.

Table 4-1: Overview of single clones obtained after seeding the cells with different dilutions.

Cells/ 96well	Number of plates	Single clones	Multi clones
96	2	35	10
192	2	61	32

4.1 Evaluation of ZFN efficiency

To give opportunity to both, the disruption of the *CFTR* gene by non-functional mutations due to NHEJ as well as the introduction of a defined sequence modification by homologous recombination at the target site with a DNA vector after DSB, we selected ZFN sets to target the exon 1 of the *CFTR* gene (figure 4-4). This exon contains 134 bp 5'UTR transcriptional start site and 52 bp of *CFTR* coding region. Three ZFN pairs were obtained from the supplier (Sigma-Aldrich) all of them cleaving the *CFTR* locus within the 5' UTR, 98 bp, 1 bp and 1 bp upstream of the transcriptional start codon ATG (figure 4-5).

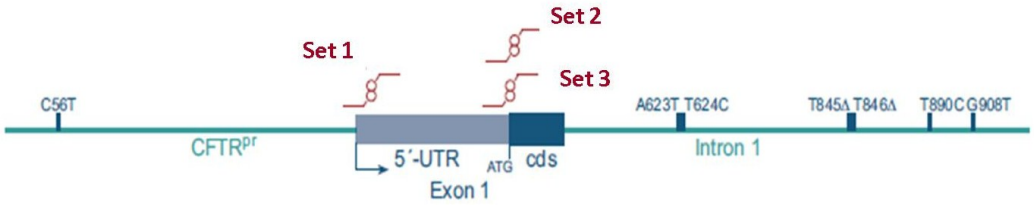

Figure 4-4: ZFN binding sites.

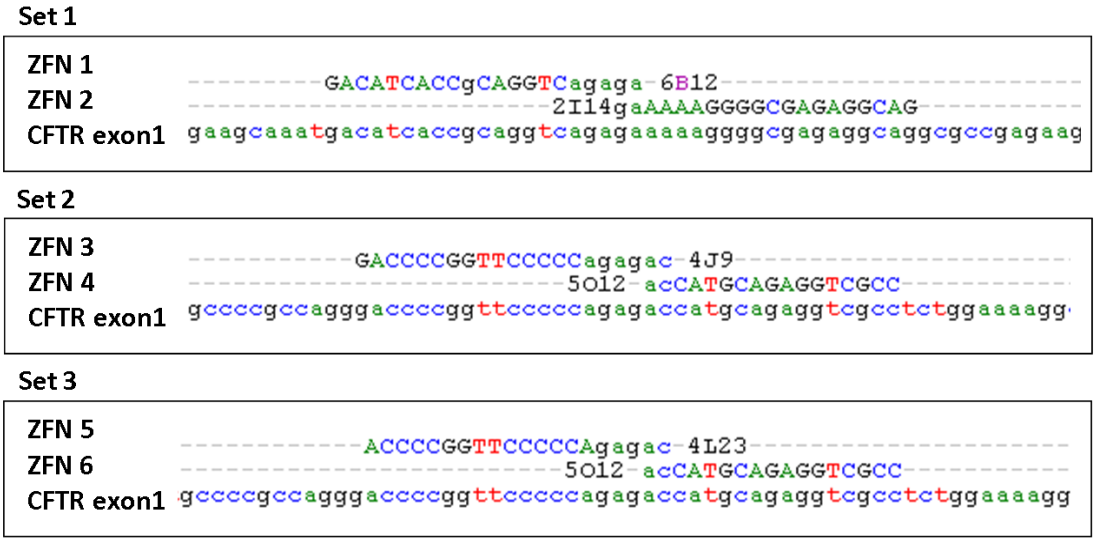

Figure 4-5: Binding and cleavage sites of CFTR ZFNs. Although targeting sites of ZFN sets 2 and 3 differ only in one base pair, ZFN set 2 shows a higher cleavage activity.

For each ZFN pair, plasmid DNA and mRNA encoding one ZFN were tested. In addition, *Niere m* cells were incubated at 30°C or 37°C after transfection of 0.5 x 10^6 cells with 1 µg nucleic acid. The cells were plated onto 6 cm dishes and recovered for 48 h. DNA was isolated from splitted cells and the target locus was amplified by PCR and cloned into a destination vector (figure 4-6).

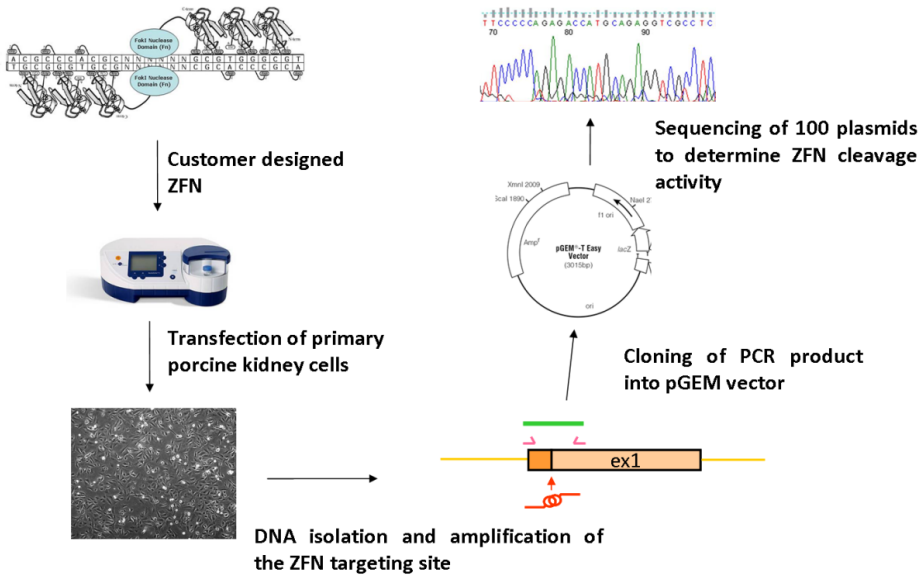

Figure 4-6: Examination of ZFN cleavage activity. *Niere m* cells were transfected with different ZFN pairs and cells were incubated at 30°C and 37°C, respectively. Cells were harvested for DNA isolation and amplification of the ZFN binding site. The obtained PCR products were cloned into the pGEM T-easy vector and transformed into TOP10 competent cells. For each ZFN set

approximately 100 bacterial colonies were picked for colony PCR and the resulting amplicons were sequenced in order to determine DNA alteration at the ZFN targeting site. Targeting sites were amplified using primer pair Cs1f/Cs3r for ZFN sets 1 (annealing temperature: 62°C), and Cs9f/Cs5r for ZFN set 2 and 3 (annealing temperature: 58°C).

100 clones were sequenced for each experiment. Analysis of the sequences revealed that deletions of 2-70 bp were dominant whereas insertions and single nucleotide polymorphisms were detected with a lower frequency. The latter might also occur from PCR-derived misincorporation of dNTPs. However, the probability for this event is low as the likelihood of a PCR mistake within a region of 10 bp, corresponding to the cleavage site, after 35 cycles is below 1 %, based on an assumed error rate of 10^{-6} for the *Taq* polymerase. Regarding the different parameters examined, NHEJ occurred more often at 30°C (table 4-2) than at 37°C (table 4-3). For the other examined features the findings were less consistent. While transfection experiments using ZFN plasmids recovered highest efficiency of ZFN set 2 at both temperatures, mRNA transfection was significantly more efficient in some cases, but did not reveal any mutations in others. This was particularly true for mRNA of ZFN set 2, which failed to produce any modifications in repeated attempts at 30°C, probably due to mRNA degeneration. Consequently, this mRNA was not used for further experiments.

Table 4-2: Cleavage efficiency of CFTR ZFN pairs at 30°C.

	ZFN set 1	ZFN set 1	ZFN set 2	ZFN set 2	ZFN set 3	ZFN set 3
Temperature	30°C	30°C	30°C	30°C	30°C	30°C
Incubation time	48 h	48 h	48 h	48 h	48 h	48 h
DNA	Plasmid	mRNA	Plasmid	mRNA	Plasmid	mRNA
Colony number	100	100	105	103	100	101
Colonies positive for a mutation	4	38	18	0	3	11
Efficiency	4 %	38 %	17 %	0 %	3 %	11 %

Table 4-3: Cleavage activity rate of CFTR ZFN pairs at 37°C (n.d. = not determined).

	ZFN set 1	ZFN set 1	ZFN set 2	ZFN set 2	ZFN set 3	ZFN set 3
Temperature	37°C	37°C	37°C	37°C	37°C	37°C
Incubation time	48 h	48 h	48 h	48 h	48 h	48 h
DNA	Plasmid	mRNA	Plasmid	mRNA	Plasmid	mRNA
Colony number	100	96	100	n.d.	100	101
Colonies positive for a mutation	5	7	11	n.d.	4	0
Efficiency	5 %	7 %	11 %	n.d.	4 %	0 %

4.2 Screening of ZFN-mediated mutations

Several methods are available to screen the generated single cell clones for ZFN-mediated mutations at the target site. The most commonly used is an assay based on the heterodimerzation of DNA strands and their cleavage by the enzyme *Cel*I [147]. After amplification of the target locus by end-point PCR, the PCR products were again denaturated and re-hybridized, forming either homodimers of fully complementary DNA or heterodimer, occurring from the hybridization of DNA fragments descending from different alleles and resulting in imperfect double helix. These mismatches are recognized and cleaved by *Cel*I. Although various efforts were made in preliminary experiments to optimize the specificity of the *Cel*I enzyme, the method remained unsuitable for the targeting locus (figure 4-7).

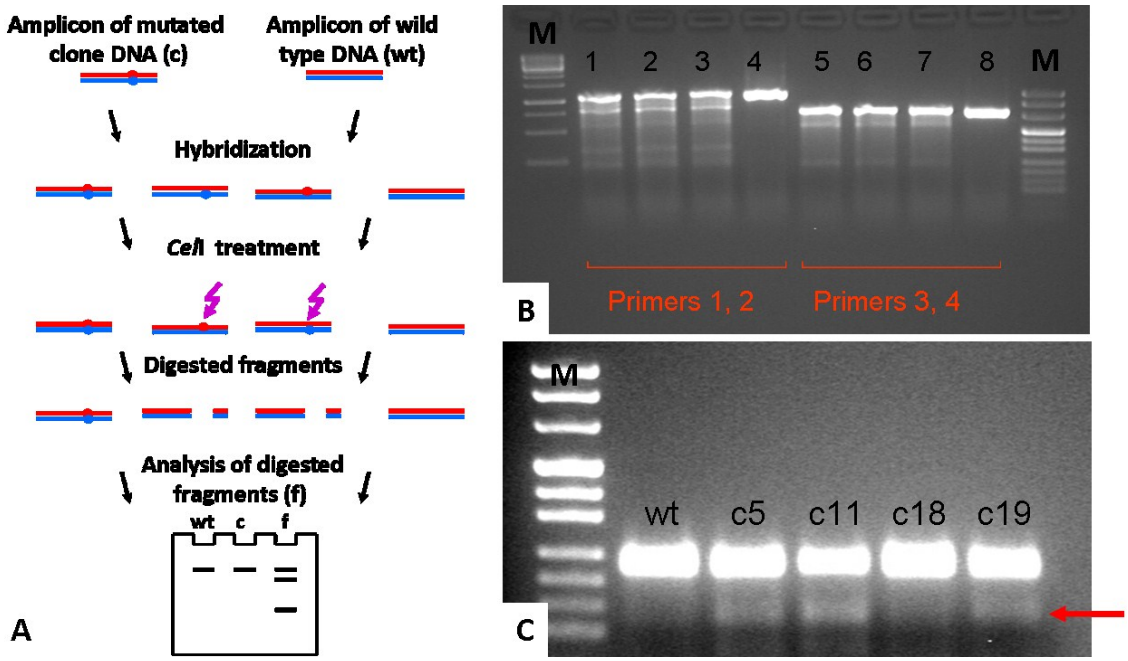

Figure 4-7: *Cel*I assay. (A) Hybrid DNA fragments of clone DNA carrying a mutation on the target site and wild-type DNA form mismatches which are recognized and cleaved by *Cel*I. (B) Optimization experiments for screening porcine *CFTR* locus. Lanes 1-3 and 5-6 show amplified ZFN target site of wild-type *Niere m* cells with addition of $MgCl_2$ in different concentrations (0.15 mM, 0.25 mM and 1.5 mM) digested by *Cel*I, resulting in unspecific cleavage. Lanes 4 and 8 show the untreated PCR product (M: molecular weight standard, wt= wild-type). (C) Screening results of single cell clones, performed by *Cel*I assay (digested fragments are indicated by the arrow). The clones were previously confirmed carrying a mutation at the ZFN target site, but cell clone c18 was not detected by *Cel*I possibly, whereas a mutation was indicated by sequencing analysis.

A further technique for detection of induced mutations is the single strand conformation polymorphism (SSCP) assay [148]. Again the target locus is amplified by PCR, but different alleles are detected by their differences in mobility after denaturation and formation of secondary structures of single DNA strands. In repeated screening experiments PCR products of generated single cell clones were denatured and run on a polyacrylamide gel, but the distinct differentiation of targeted and wild-type cell clones remained critical (figure 4-8).

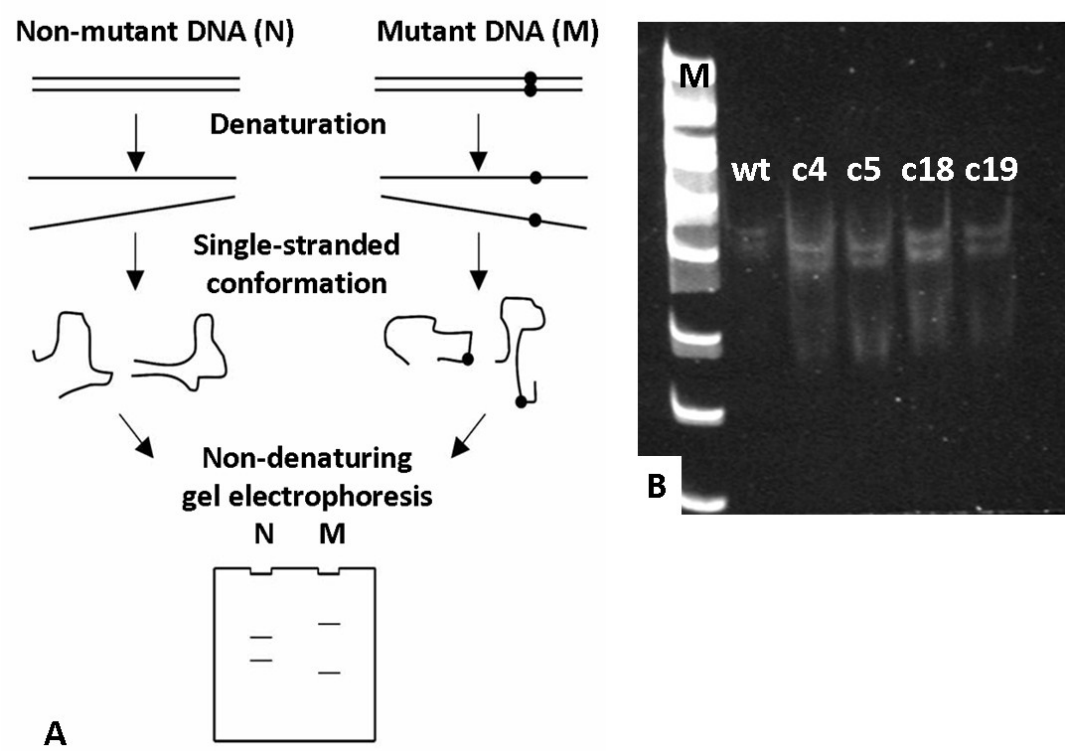

Figure 4-8: Single strand conformation polymorphism. (A) The denatured PCR products form different conformations depending on their DNA sequence and therefore, they show different mobility on a polyacrylamide gel. (B) DNA from clones with proven mutations were compared to DNA from wild-type (wt) DNA (M=molecular weight standard).

The third evaluated method was the sequencing of amplified ZFN targeting site, either directly or after cloning into plasmids. This represents a robust method that is easily transferable to different targeting sites. As both, *Cel*I digest and SSCP revealed the necessity for specific optimization for each target site, sequencing remained the preferred screening method. Therefore, it was decided to screen the generated single cell clones by sequencing, providing a reliable method not only to detect induced mutations, but also to characterize them.

4.3 NHEJ-mediated gene targeting

The high rates of ZFN-induced mutations suggest that disruption of both wild-type alleles might be achievable in one experiment although, at an expected frequency lower than 10 %. The preliminarily confirmed capability of *Niere m* cells to proliferate as single cell clones suggests that transfection of cells with ZFN, subsequent growth as discrete clones and the characterization of defined clones would give rise to the identification of clones with defined biallelic mutations prior to SCNT.

0.5 x 10^6 cells were transfected with 1 µg of ZFN set 2 plasmid DNA and incubated in 6 cm cell culture dishes for 48 h (EPO191010) and 72 h (EPO240211) at 30°C. Subsequently, cells were harvested and seeded on 96-well cell culture dishes at a number of 192 cells per 96-well plate (figure 4-9).

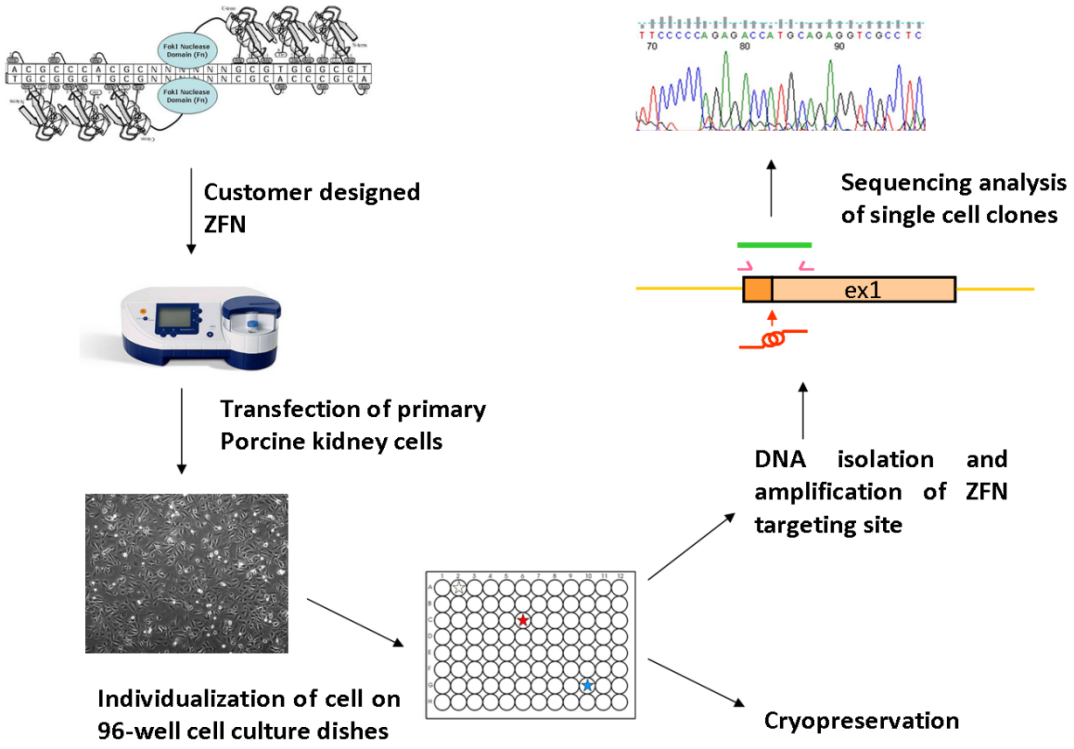

Figure 4-9: Generation of single cell clones. *Niere m* cells were transfected with ZFNs and after 48 h or 72 h distributed on 96-well cell culture dishes with a dilution of 192 cells per plate. Proliferating cell clones were splitted and harvested in two portions for cryopreservation and DNA isolation. ZFN targeting site of each cell clone was amplified for sequencing analysis (ex1= exon 1).

In total 4800 cells were seeded on 25 96-well plates for EPO191010 and 2880 on 15 96-well cell culture dishes for EPO240211. After an incubation time of one week, 385 cell clones were splitted for EPO191010 and 267 of them were harvested for screening. In this initial experiment no cell clones were used for cryopreservation. In EPO240211, 211 single cell clones were splitted after one week and 134 were harvested for DNA analysis and cryopreservation, respectively. Thus, a total of 401 single cell clones was obtained from both electroporation experiments and out of these, 325 were used to isolate genomic DNA via high salt precipitation. PCR products of the target locus were amplified and sequenced, revealing that 74 (23 %) clones carried a mutation at the ZFN cleavage site (table 4-4). Various different mutations were detected at the ZFN cleavage site (figure 4-10 and 4-11)

Table 4-4: ZFN application to generate single cell clones.

ZFN set 2	**EPO191010**	**EPO240211**
Cell passage	P6	P3
DNA	Plasmid	Plasmid
Temperature	30°C	30°C
Incubation time	48h	72h
Single cell clones	385	211
Harvested cell clones	267 (70 %)	134 (64 %)
Sequenced cell clones	191	134
Clones positive for a mutation	49 (26 %)	25 (19 %)
Biallelic targeted cell clones	4 (2 %)	10 (7 %)

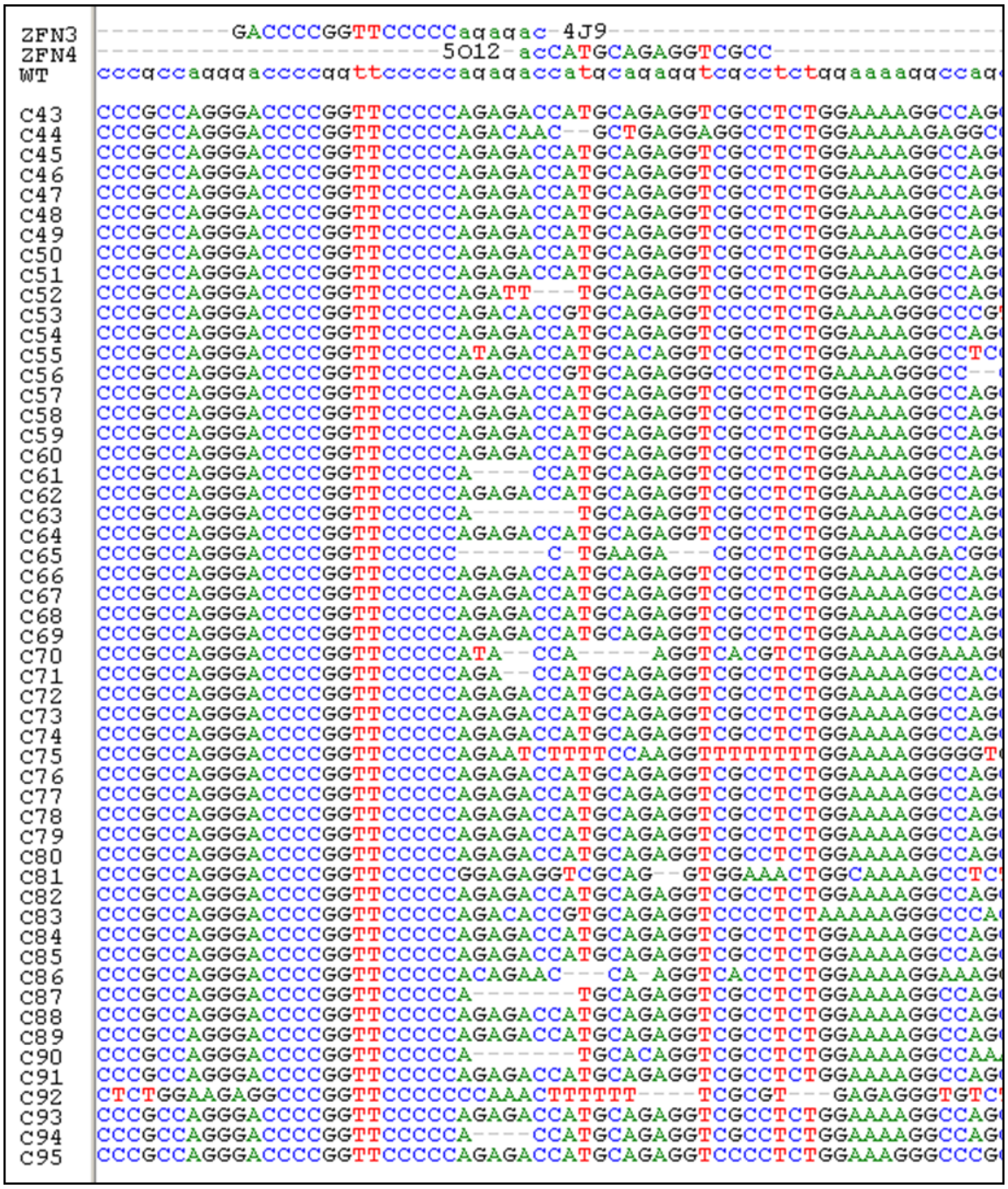

Figure 4-10: Mutations after transfection with ZFN set 2. The type of the introduced mutation is not predictable, resulting in a variety of different sequence alterations.

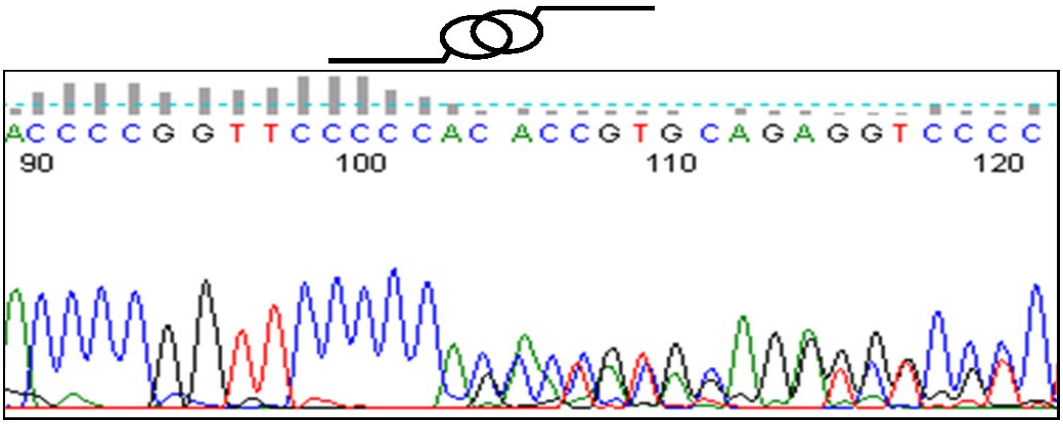

Figure 4-11: Electropherogramm of single cell clone C70. Sequenced clone showed a mixed signal due to mutation, introduced by ZFN-triggered NHEJ.

Electropherogram analysis of the clone sequences was used to spot candidate clones that might carry mutations on both alleles. For 14 of these clones the PCR product was ligated into the pGEM T-easy vector and 10 plasmids were sequenced for each clone. All 14 cell clones revealed that both CFTR alleles carried distinct mutations (figure 4-12). In all cases, a maximum of 2 distinct alleles was found, indicating that ZFNs are active immediately after transfection, but activity declines before the cells divide. The type and frequency of the mutations corresponded to, and thus confirmed, the mutations found in mixed populations.

Although the cleavage site of ZFN pair 2 is located only 1 bp upstream of the *CFTR* start codon, in this experiment we only identified 2 of the biallelic targeted cell clones (i.e. 0.6 % of screened clones) with a disrupted ATG. Both clones however carried still one allele with an intact start codon and consequently, are not capable of the production of *CFTR*-null pigs, lacking both functional alleles.

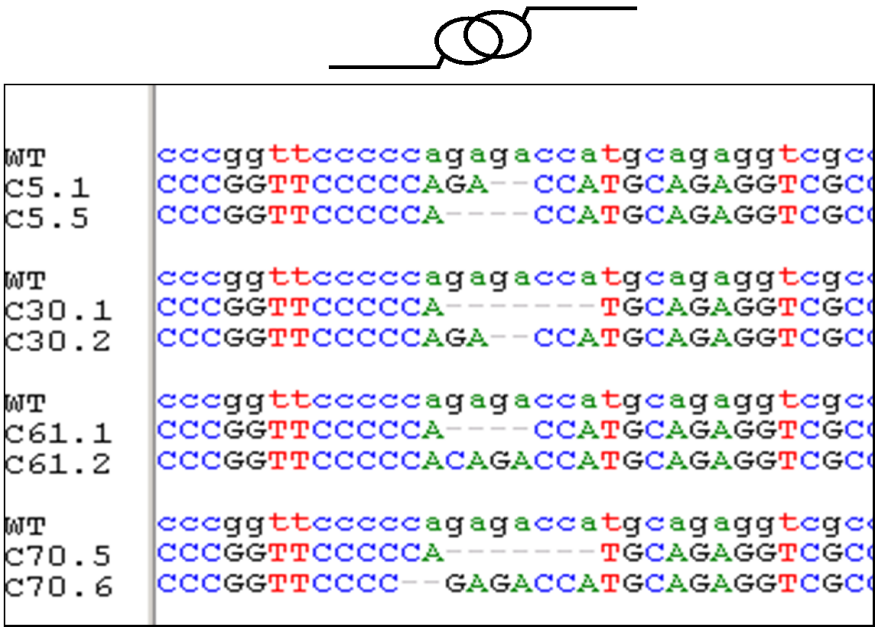

Figure 4-12: Biallelic targeted single cell clones. Cloning and sequencing of candidate clones showed modifications on both alleles of the *CFTR* gene.

However, the finding that (i) customer designed ZFNs resulted in reproducible modifications of the target site and (ii) the high efficiency of NHEJ facilitated the generation of biallelic gene alterations in considerable numbers give evidence that nuclease based technologies are suitable for genomic modifications in pig primary cells. The fact that we did not achieve null-alleles of *CFTR* was caused by the ZFN binding site selection due to initially intended usage of ZFN for both, NHEJ-induced introduction of mutations and HR-based gene targeting.

4.4 HR-mediated gene targeting

In a second experiment, we combined ZFN sets with modification vectors to introduce a reporter gene into the porcine *CFTR* locus. A BAC vector was constructed as described previously [67] with the exception that the *lacZ* gene was placed downstream of the start codon in order to replace exon 1 of the *CFTR* gene rather than a STOP box as described in the publication. The BAC vector, CFTR-248-lacZ BAC 24-5 (designed and constructed by Katrin Krähe and Dr. Nikolai Klymiuk), was used directly providing homologous arms of 84 kb and 88 kb, respectively, and a smaller, truncated vector was created as the BAC construct was modified by shortening the

homology arms to 1685 bp and 1190 bp, respectively. The smaller vector (figure 4-13) was generated by digestion of the BAC vector with *Avr*II/*BstB*I to obtain a 8606 bp DNA fragment consisting of a 5' homology arm (1685 bp long), the *lacZ* gene, a neomycin resistance cassette together with a phosphoglycerate kinase (PGK) promoter providing transcription in any transfected cell type [32] and a polyandenylation termination signal, as well as a 1190 bp long 3' homology arm (figure 4-13). Subsequently, the DNA fragments were co-ligated with a *Sac*I/*Xba*I adapter into a *Sac*I/*Cla*I digested pBSK vector. A plasmid carrying the truncated vector was analyzed by sequencing to affirm the correct sequence composition and to determine possible alterations in the exogenous *lacZ* genes and the neomycin resistance cassette. Thus, both vectors shared the *lacZ* reporter gene as well as a neomycin resistance cassette for modification of the *CFTR* gene, but differed by the length of their homology arms. The targeting vectors were prepared free of endotoxins prior using for transfection experiments in the cell culture. The BAC vector was linearized with *Asc*I and the truncated vector was excised from the plasmid backbone with the enzymes *Avr*II/*Sac*II.

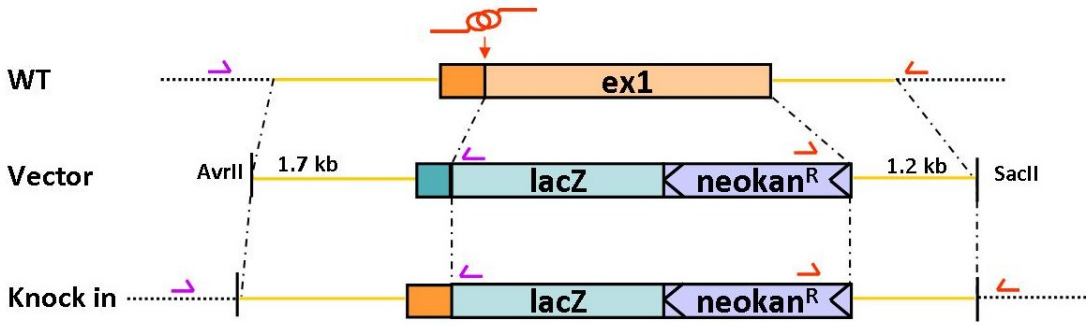

Figure 4-13: CFTR-lacZ-8 for replacement of exon 1 of the *CFTR* gene. Constructed plasmid consists of *lacZ* and a neomycin resistance cassette flanked by homologous arms and linearized by *Avr*II and *Sac*II for transfection of *Niere m* cells. Primer, indicated in purple and red, can only produce an amplicon when the vector is recombined correctly with the targeting site.

4.4.1 Truncated vector

Two electroporation experiments were performed using 1 µg of ZFN set 1 in combination with 9 µg of the linearized vector for transfection of 0.5 x 10^6 *Niere m* cells. To provide a sufficient rate of homologous recombination between the *CFTR* locus and a targeting vector, *Niere m* cells were incubated at 37°C for 48 h after electroporation for recovery (table 4-5).

Table 4-5: Transfection experiments using pBSK-CFTR-lacZ-8 and ZFN set 1 for co-transfection.

Nucleofection	Cell passage	Incubation temperature/time	DNA concentration	DNA (µg)	ZFN set 1 DNA/mRNA (µg)
EPO040611	P2	37°C/48 h	1140 ng/µl	2	n.d./2
EPO050711	P3	37°C/48 h	1140 ng/µl	8,9	0.5/2

Then, cells were distributed on 96-well cell culture dishes, mixed with untreated porcine kidney cells in a ratio of 1:4 for better cell proliferation. For positive selection of clones with an integrated vector, cells were grown in selection cell culture medium containing 1.2 µg/ml G418 and 15 % FCS and screened for single cell clones after one week. For EPO040611 32 single cell clones were detected and 13 were harvested for analysis. Co-transfection of targeting DNA and ZFN plasmid DNA of EPO050711 resulted in 133 single cell clones and 58 of them were obtained for screening. After transfection with ZFN mRNA, 302 single cell clones were recovered and 152 showed well proliferation (table 4-6).

Table 4-6: Single cell clones generated after transfection with ZFN set 1 and pBSK-CFTR-lacZ-8.

Experiment	EPO040611	EPO050711	
DNA	**Plasmid**	**Plasmid**	**mRNA**
Single cell clones	32	133	302
Harvested cell clones	13	58	152
Analyzed cell clones	13	58	152
clones positive for a knock-in	0	0	4 (fragmentary)

Clones were splitted 1:2 on two different plated to be harvested, partly for DNA analysis and partly for cryopreservation for optional SCNT. For both experiments genomic DNA was isolated from the single cell clones and end-point PCR was performed using the screening primer neof1/scr1nr, amplifying a 1397 bp long amplicon, if the vector has been correctly copied into the target site (figure 4-13). Isolated DNA was also used for PCR with control primer Cs1f/Cs3r, amplifying a 363 bp fragment, in order to determine the DNA quality. No PCR products were obtained from single cell clones using screening primer, indicating that the transfected targeting vector has not been copied into the targeting site. However, amplification of the targeting site using the control primer revealed unexpected PCR fragments of 1025 bp in four single cell clones generated using ZFN set 1 mRNA. PCR product of the four single cell clones was cloned into the pGEM vector and colony PCR was performed with 10 bacterial colonies for each cell clone after transformation. Subsequently, the obtained PCR products were sequenced, revealing the insertion of DNA fragments occurring from the vector. In all four cases, the insert represents a 659-661 bp fragment of the PGK promoter of the neomycin resistance cassette (figure 4-14). The observed insertion of the fragment occurred rather from DNA capture during the NHEJ repair procedure that by HR-based insertion. The reason why in all four cell clones a similar fragment was inserted was not further investigated. However, it was evident that additional genetic modification had taken place in other parts of the genome, since the clones were resistant to G418, suggesting that the cell clones carried a complete neomycin resistance cassette.

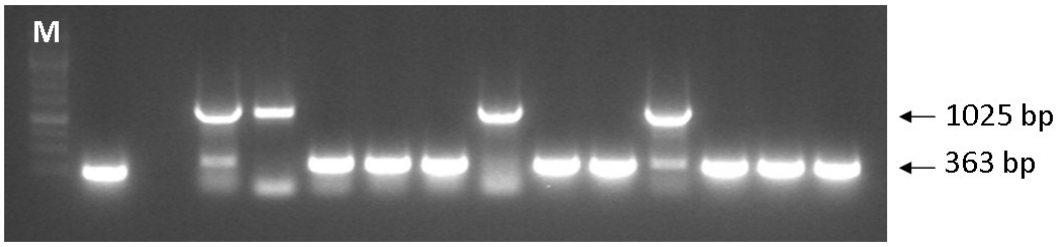

Figure 4-14: Screening of generated clones by PCR using primer Cs1f/Cs3r. Clones with no targeted knock-in show a 363 bp band, four single cell clones resulted in a 1025 bp band, indicating a fragmentary insertion of 659-661 bp (M: molecular weight standard).

4.4.2 BAC vector

As reviewed, the efficiency of HR-based gene targeting can be increased by using vectors with long homologous arms [67]. This strategy was successfully used in our lab to modify the *CFTR* locus by BAC vectors. Targeting efficiency of 1.2-2.9 % has been achieved in our lab in previous projects. In this experiment we aimed to increase the frequency of HR events by ZFN-mediated DSB. Targeting efficiency of the CFTR-248-lacZ 24-5 BAC was compared with the efficiency of HR-based targeting when BAC was co-transfected with ZFN.

0.5 x 10^6 *Niere m* cells were transfected with 3.4 µg of CFTR-248-lacZ BAC DNA (lot 1) and incubated for 48 h (table 4-7). Afterwards, the cells were seeded on 96-well cell culture dishes, mixed with untreated *Niere m* cells in a ratio of 1:4, and grown in selection cell culture medium for one week before screening for single cell clones. In nucleofection experiment EPO141111, 32 single cell clones were detected and 17 of them harvested for further analysis and cryopreservation (table 4-7). For experiments EPO281111 A and B, DNA from two different preparations (DNA lot 1 and 2) has been used, resulting in 111 single cell clones for EPO281111 A, 81 of which were harvested. No clones could be obtained from nucleofection EPO281111 B, probably due to a possible BAC DNA degradation during or after the preparation (table 4-7).

Table 4-7: Electroporation experiments using BAC CFTR-248-lacZ 24-5 (n.d. = not determined).

Nucleofection	EPO141111	EPO281111 A	EPO281111 B
Cell passage	P4	P3	P3
Incubation temperature/time	37°C/48 h	37°C/48 h	37°C/48 h
DNA lot	1	1	2
DNA concentration	340 ng/µl	340 ng/µl	275 ng/µl
DNA (µg)	3.4	3.4	2.75
Single cell clones	32	111	0
Harvested cell clones	17	81	n.d.
Analyzed cell clones	17	81	n.d.
Cell clones with a targeted *lacZ* knock-in	1	0	n.d.

The generated cell clones were screened using "loss-of-native-allele" assay via q-PCR, which allows a relative comparison between the amplified copy numbers of the target locus with two reference loci (figure 4-15). Correctly targeted single cell clones carry an HR-mediated insertion of the BAC transgene at the target site, replacing exon 1 of the *CFTR* gene either on one or on both alleles. A primer pair (CFTR402f/CFTR621r) was designed for amplification of the wild-type *CFTR* target locus. In addition, primer pairs OCTqf2/OCTqr1 for amplification of the *OCT4* gene and NGqf6/NGqr4 for the *NANOG* gene were designed, in order to use the amplicons of these two genes as references. For BAC which integrates off-target - randomly - into the genome remain both *CFTR* alleles intact and the copy numbers of the produced amplicons of *CFTR*, *OCT4* and *NANOG* gene will result in a ratio of 2:2 (OCT4/CFTR or NANOG/CFTR) as would occur in wild-type cells. In case the *CFTR* gene is targeted correctly on one of the two alleles (heterozygous targeting), the ratio of OCT4/CFTR or NANOG/CFTR changes to 2:1. Cell clones carrying a transgene

on both *CFTR* exon 1 alleles (homozygous targeting) show a ratio of 2:0 (OCT4/CFTR or NANOG/CFTR), since the wild-type target locus is replaced on both alleles and is not detectable by the PCR. A standard curve was obtained by serial dilutions of DNA from un-transfected *Niere m* cells varying from 25-10000 copies per sample. The copy numbers of standards and clones have been determined in duplicates.

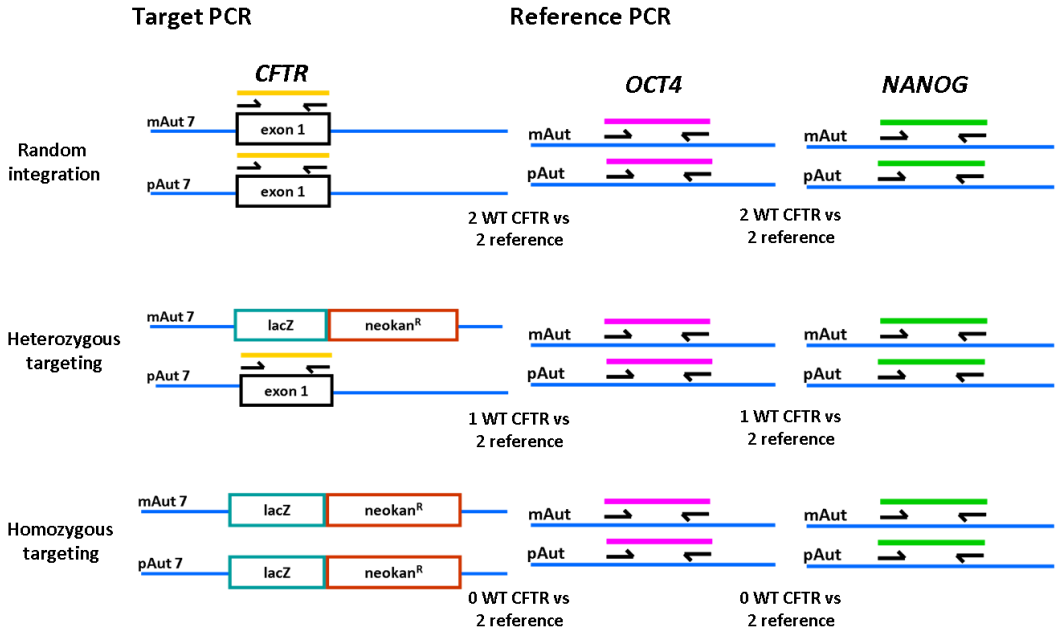

Figure 4-15: q-PCR screening method. The copy number of the target locus was compared with the copy number of two reference genes, *OCT4* and *NANOG.* mAut 7: maternal autosomal chromosome 7, pAut 7: paternal autosomal chromosome 7, WT: wild-type.

Genomic DNA from the produced 98 cell clones was isolated via high salt precipitation for q-PCR screening. For each cell clone copy number values were generated for the *CFTR*, *OCT4* and *NANOG* loci. Clones showing copy numbers lower than 30 at the reference site were excluded from further analysis. For the other clones relative copy number of OCT4/CFTR and NANOG/CFTR were calculated. Based on the assumption of the approximate targeting rate of 1-3 %, as was found in other targeting experiments in our lab, one would expect the mean value of the relative copy number ratio within a given set of clones to tend towards 1.0. The rarely occurring clones with heterozygous HR were identified as outliners within the majority of clones that did not undergo HR.

We determined the mean relative copy number (mvc) as well as their standard deviation (sd) within a given set of clones and defined an exclusion limit (el) as following:

el = mvc ± 2 * sd

Clones with both the OCT4/CFTR and NANOG/CFTR relative copy numbers above the exclusion limit were examined in repeated assays. Of the 98 clones generated from transfection of *Niere m* cells with the CFTR-248-lacZ 24-5 BAC 1 clone (L2) was identified as outliner and confirmed in a second assay. The calculated targeting efficiency of 1 % was in the expected range of other BAC targeting experiments (figure 4-16).

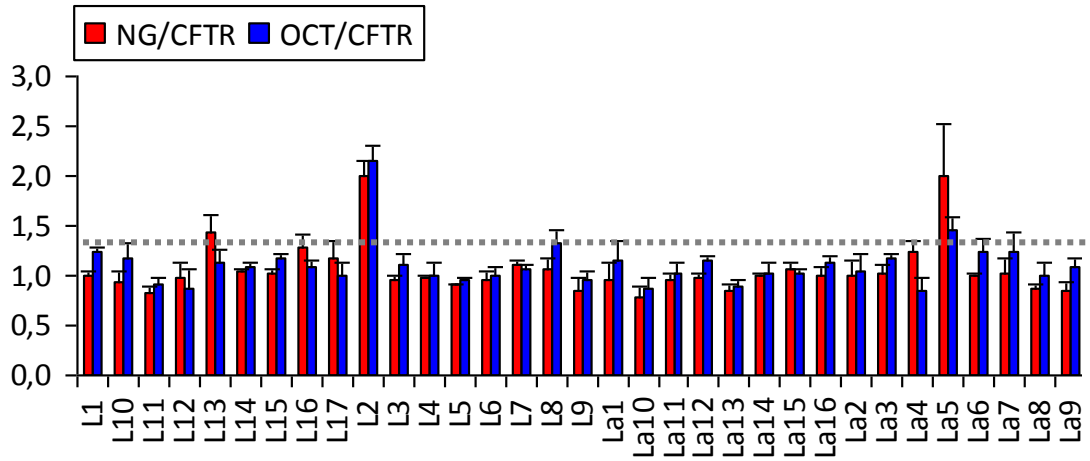

Figure 4-16: NANOG/CFTR and OCT4/CFTR copy number ratios. L1-L17 represent cell clones of EPO141111 and La1-La16 are cell clones of EPO281111 A. Cell clone L2 was targeted correctly on one allele of the *CFTR* gene. Cell clone La5 is not considered as targeted in repeated experiments. Dotted line indicates the mv=1.3 which represent the elimination limit.

Large homologous sequences are known to promote HR events. In order to determine the effect of BAC vectors with large homology arms on the frequency of HR events, we co-transfected the CFTR-248-lacZ 24-5 BAC with ZFN set 2. *Niere m* cells were transfected with, 3.4 µg BAC DNA (lot 1) for EPO291211 and for EPO250112 and EPO260112 1 µg (lot 3) was used for each experiment. After electroporation, cells were incubated for 48 h at 37°C and distributed on 96-well cell

culture dishes. Cells of EPO250112 experiment were seeded in two different dilutions. Transfected *Niere m* cells were mixed with untreated cells in a ratio of 1:4 (EPO250112 A) and 1:2 (EPO250112 B). After one week incubation with selection medium plates were screened for single cell clones. Overall, 75 cell clones were harvested for DNA analysis and cryopreservation (table 4-8).

Table 4-8: Electroporation experiments using BAC CFTR-248-lacZ 24-5 DNA and ZFN set 2.

Nucleofection	EPO291211	EPO250112 A	EPO250112 B	EPO260112
Cell passage	P4	P5	P5	P5
Incubation Temperature/time	37°C/48 H	37°C/48 H	37°C/48 H	37°C/48 H
DNA lot	1	3	3	3
DNA concentration	340 ng/µl	660 ng/µl	660 ng/µl	660 ng/µl
DNA (µg)	3.4	1	1	1
Single cell clones	27	53	40	8
Harvested cell clones	16	22	31	6
Analyzed cell clones	16	22	31	6
Heterozygote targeting	6 (38 %)	8 (36 %)	15 (48 %)	3 (50 %)
Homozygote targeting	4 (25 %)	0	5 (16 %)	1 (16 %)

Q-PCR screening was performed as described above, but the identification of outliners was impaired by the finding that only a minority of clones revealed a copy number ratio around one. Instead, three ranges of relative copy numbers were defined, those clones with a copy number ratio around 1, those with a copy number ratio around 2 and those with a copy number above 5, representing the $CFTR^{WT/WT}$, $CFTR^{lacZ/WT}$ and $CFTR^{lacZ/lacZ}$ alleles. For $CFTR^{WT/WT}$ and $CFTR^{lacZ/WT}$ mvc and sd were calculated and following el was determined:

el = mvc ± 2*sd

The copy number ratio of NANOG/CFTR and OCT4/CFTR should be infinite in the case of homozygous targeting. However, due to unspecific amplification of a genomic fragment in the absence of the CFTR template, this value becomes finite and ranges between 6.7 and 106.1 (figure 4-17). All clones were clearly assigned to one of the tree ranges for both, the NANOG/CFTR and OCT4/CFTR relative copy numbers. Of the 75 clones examined, 32 were heterozygously targeted whereas 10 showed HR events at both alleles (figure 4-18), indicating a targeting rate of 56 % which was considered higher than the rate of NHEJ determined for ZFN set 2 at 37°C (11 %).

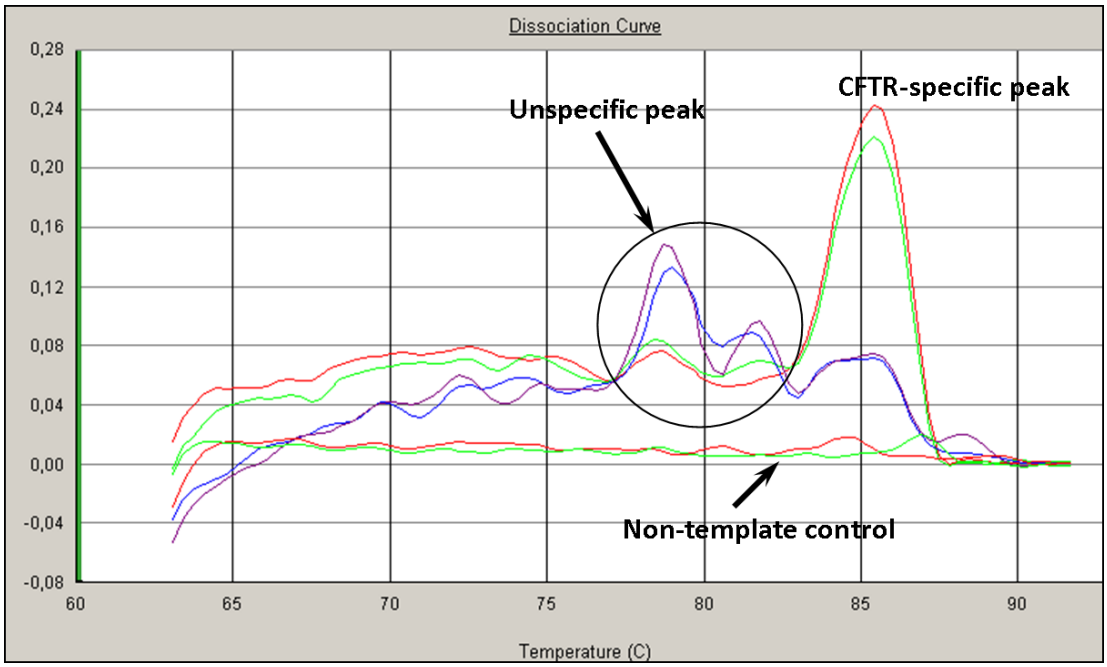

Figure 4-17: Dissociation curve of CFTR q-PCR screening. Primers binding specifically to the CFTR locus show a distinct high peak. Unspecifically binding of primer pairs in absence of the CFTR locus, as is the case for homozygously targeted cell clones, shows a different, lower peak (highlighted) and can be clearly distinguished from control samples containing no DNA template at all.

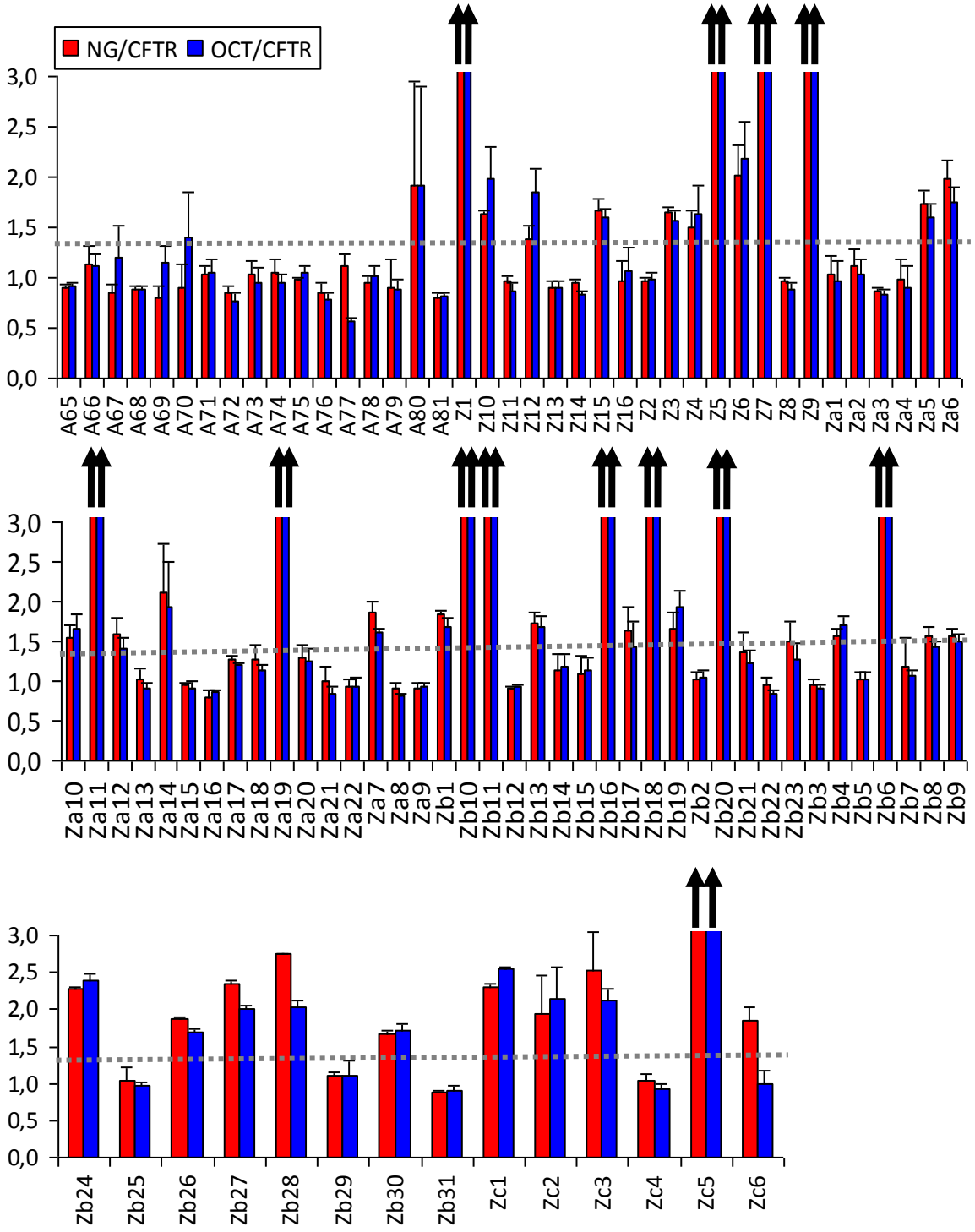

Figure 4-18: q-PCR analysis of cell clones. Single cell clones A65-A81 have been generated in EPO281111 using the BAC vector alone. Cell clone A80 is not considered as correctly targeted in repeated experiments. The other cell clones resulted from experiments with combination of BAC vector and ZFN. Cell clones (Z1-Z16) of EPO291211 are showing heterozygous (Z3, Z4, Z6, Z10, Z12, Z15) and homozygous (Z1, Z5, Z7, Z9) targeting. Heterozygous and homozygous targeting was also found in cell clones of EPO 250112 A, B (Za1-Za22 and Zb1-Zb31) and EPO260112 (Zc1-Zc6), showing mv of > 1.3 (indicated by the dotted line, back arrows indicate a higher mv as shown by the diagram).

4.5 TALENs

Even though zinc-finger-based nucleases were shown to be effective in the experiments described above, the relatively rare occurrence of suitable binding sites in mammalian genomes (approximately every 350 bp) limits their usage. This was exemplified by our study, where the intended application of ZFN for both, NHEJ and HR, at the porcine *CFTR* gene was not possible. Thus, nucleases with alternative DNA binding domains such as TALENs, which are assumed to have suitable binding sites every 35 bp, are of interest. However, while ZFN have been optimized for years and are available from various sources, TALEN technology is still in its infancy. To date, publications about gene targeting experiments using TALENs in porcine cells are not available. Therefore, the suitability for transfection and targeting of porcine primary cells and the cytotoxic potential of these nucleases needed to be investigated. We examined the application of TALENs for another porcine gene, the X-linked *DMD*. Two TALENs as well as one ZFN were designed and constructed by Prof. Dr. Toni Cathomen to introduce mutations into exon 48 or 49 of the *DMD* gene (figure 4-19).

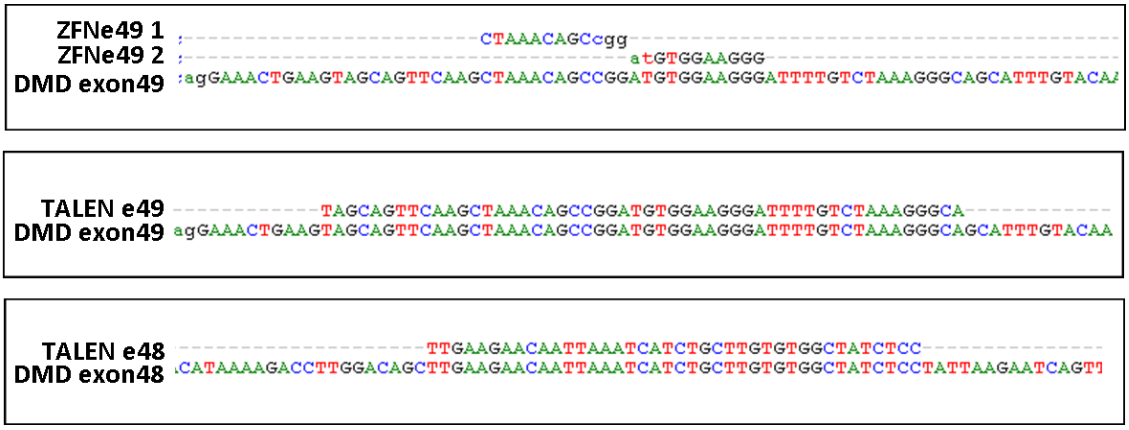

Figure 4-19: Binding and cleavage sites of DMD ZFN and TALENs. For sequencing analysis targeting sites were amplified with primer ex49ZFN2f/ex49ZFN3r for TALEN and ZFN of exon 49 and ex48TAL2f/ex48TAL2r for TALEN of exon 48 (annealing temperature: 60°C for both primer pairs).

In contrast to the *CFTR*-specific ZFNs that were purchased from a commercial supplier, nucleases for *DMD* gene were designed based on publically available databases for ZFNs and TALENs. All nucleases were delivered as plasmids and transfected into *Niere m* cells after an endotoxin-free preparation.

0.5 x 10^6 *Niere m* cells were transfected with 1 µg TALEN or ZFN DNA and incubated on 6 cm cell culture dishes for 48 h at 37°C (figure 4-20). After transfection differences in viability and proliferation capability were observed. Although, an equal number of *Niere m* cells has been used for each transfection experiment, 24 h after transfection the cells transfected with TALNe49 showed a notably lower cell confluency as cell of the other two experiments due to a possible cytotoxic effect of TALENe49. Subsequently, the cells were harvested and genomic DNA was isolated from a mixed cell population for each experiment via high salt precipitation, according to procedure described for the initial characterization of ZFNs.

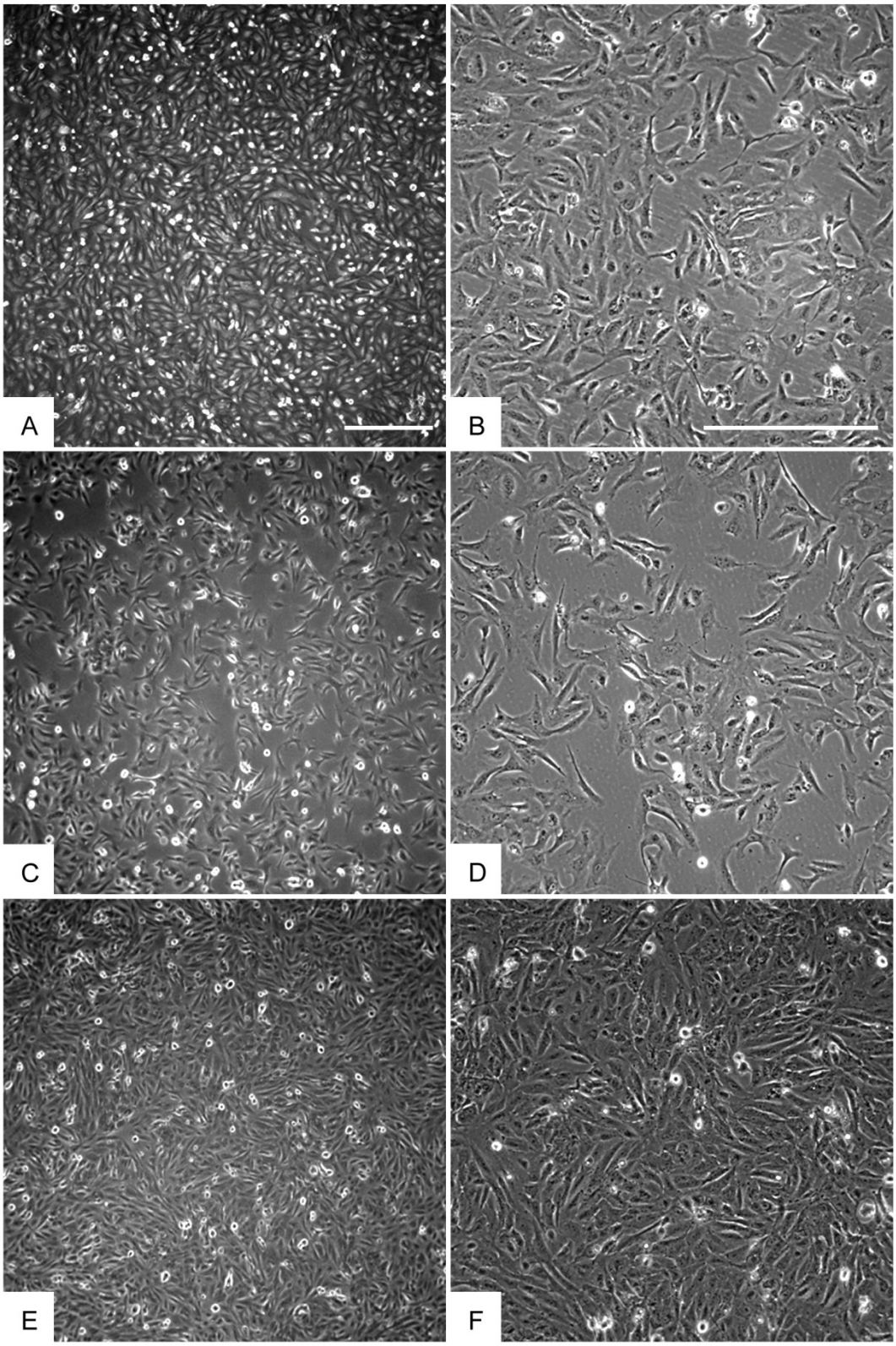

Figure 4-20: *Niere m* cells transfected with nucleases for porcine *DMD* gene. (A, B) Porcine kidney cells transfected with TALENe48, (C, D) TALENe49 and (E, F) with ZFNe49 and incubated for 48 h at 37°C. Cells showed different proliferation potentials 24 h after transfection, indicating nuclease activity (scale bars: 200 µm).

However, instead of cloning PCR products and sequencing plasmids, here we followed an alternative strategy using deep sequencing technology that enables the high throughput analysis of nucleic acids. The limitation of an expected read length of approximately 80 bp was overcome by the analysis of two overlapping PCR products for each nuclease binding site. The PCR products were designed to contain the forward primer of the amplicon 40 bp upstream of the binding site and the reverse primer of the other amplicon 40 bp downstream of the binding site. The PCR amplicons were 279 bp to 355 bp long due to technical reasons. The potential restriction of introduced mutations larger than 300 bp was taken into account, since initial characterization of ZFNs (see above) revealed only gaps of 2 bp to 70 bp resulting from NHEJ based cleavage site repair.

For all three nucleases, at least 45,000 reads were analyzed for each PCR product and results showed deletions and insertions at the targeting site or at least in closest proximity to it for both, TALENs and ZFN (table 4-9).

For TALENe48 overall 48 sequence modifications, 34 deletions and 14 insertions were detected (table 4-10).

Table 4-9: Sequencing analysis overview of TALENs and ZFN designed for the *DMD* gene (indels = insertions and deletions).

	TALENe48	TALENe49	ZFNe49
Reads for amplicon1	80000	53000	60000
Reads for amplicon2	77000	78000	45000
Insertions	14	1	254
Deletions	34	125	118
Indels	48 (<0.1 %)	126 (<0.1 %)	372 (0.35 %)

In contrast to the ZFN-mediated mutations observed before, cells transfected with TALENe48 showed mutations of only 1 bp length and the mutations were spread widely over the targeting sequence with no preference for the cleavage site (figure 4-21). TALENe49 also showed a very low cleavage activity. In total, 126 sequence alterations were detected, revealing 125 deletions and 1 insertion. Again, all mutations had a length of 1 bp and were spread widely around the targeting site

(figure 4-22). In contrast to the introduced deletions no other point-mutations have been found, indicating that the detected alterations rather occurred from TALEN-mediated DSBs than from an error of the polymerase during the PCR procedure.
Analysis of ZFNe49 PCR products recovered 118 deletion of mostly 1bp to 5 bp, but longer deletions of 31 bp were a rarer event (table 4-11). In addition, 254 insertions were detected at the targeting site, showing in most cases duplications of 5 bp. The ZFN-mediated mutations were accumulatively located, as observed before for *CFTR*-specific ZFNs, at the ZFN cleavage site or very close to it (figure 4-23) and no mutations were found outside of this segment with one exception, indicating that the detected point mutations here rather resulted from ZFN cleavage than simply due to a PCR error.

Table 4-10: Sequencing results of TALENe48 and TALENe49 transfection.

		TALENe48	TALENe49
Deletions	**Length**	1	1
	Frequency	34	124
Insertions	**Length**	1	1
	Frequency	14	1

Table 4-11: Results of ZFNe49 transfection.

Deletions		Insertions	
Length (bp)	**Frequency**	**Length (bp)**	**Frequency**
31	13	6	2
25	2	5	121
15	1	4	45
13	6	3	24
8	6	2	37
6	3	1	25
5	14		
3	29		
1	44		

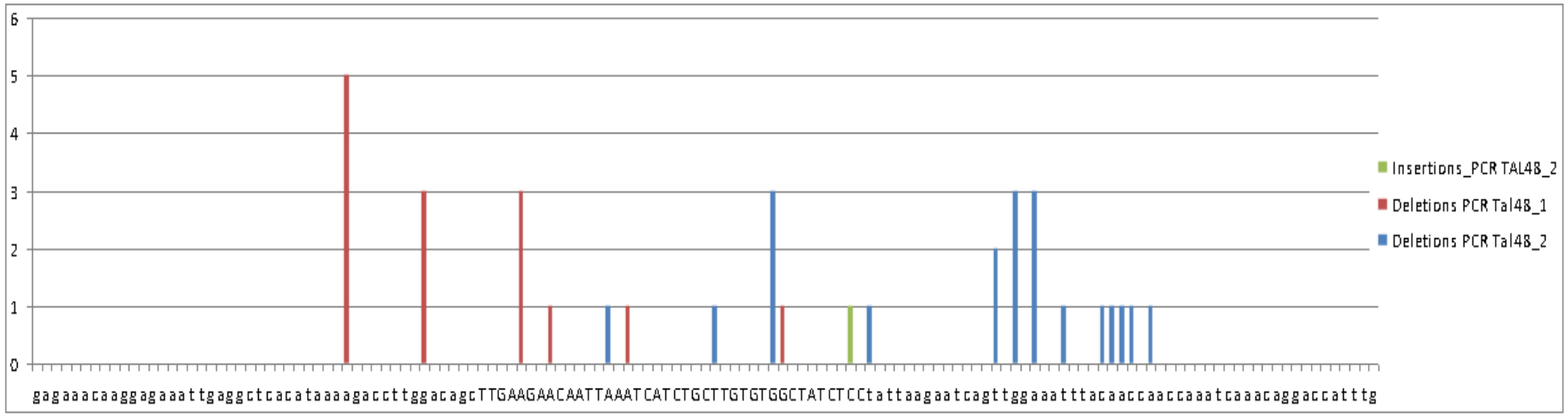

Figure 4-21: Mutations mediated by TALENe48. Sequencing analysis revealed deletions and insertion of 1 bp, widely spread over the targeting site (capital letters).

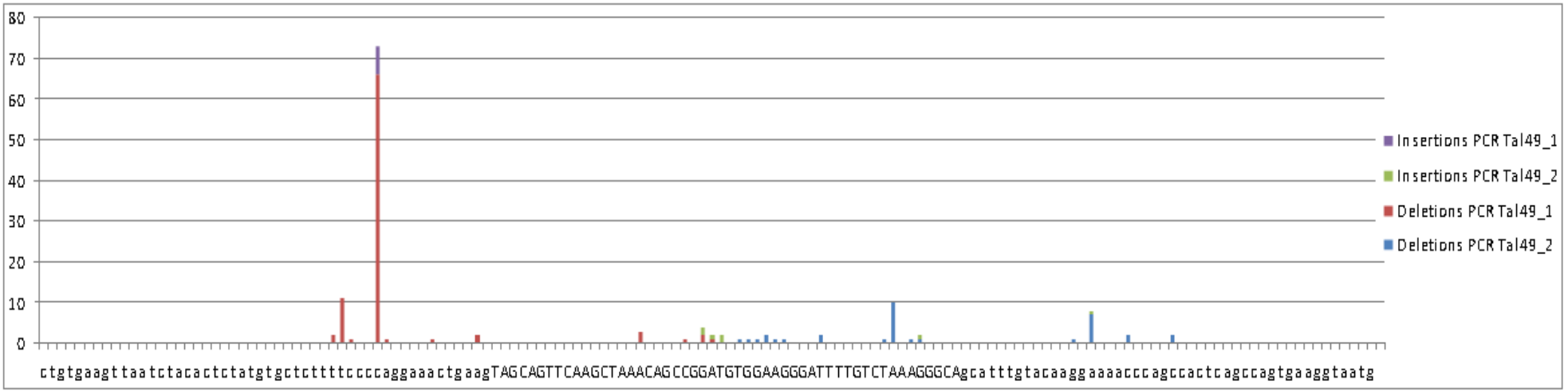

Figure 4-22: Sequencing results for TALENe49. Only a few deletions of 1 bp were found for TALENe49. Again, the sequence alterations were detected rarely direct at the targeting site (capital letters).

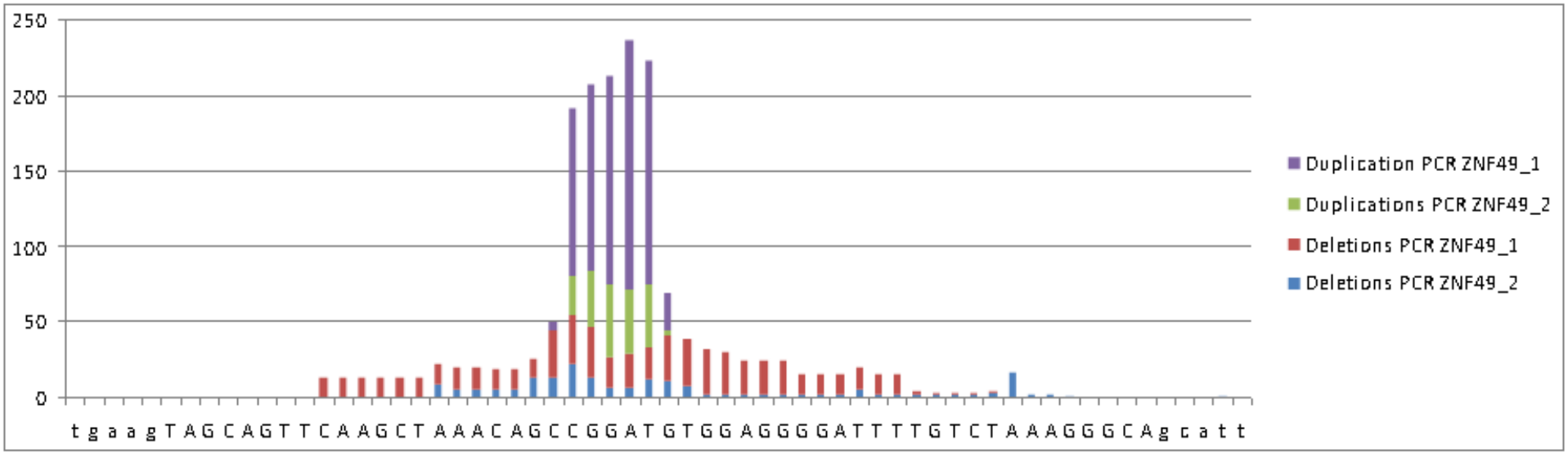

Figure 4-23: Distribution of ZFNe49-mediated mutations at the targeting site. As expected sequence alterations occurred directly at the cleavage site (capital letters) and also very close to it. Number of deletions and duplication for amplicon 1 and 2 are different due to the unequal number of reads sequenced for each amplicon.

5 DISCUSSION

A variety of applications in biomedicine and biotechnology, including the generation of transgenic animals depend on the capability to alter DNA sequences stably and in a site-specific manner. Until recently, gene targeting has been conventionally achieved by homologous recombination of host genome with DNA-based vectors [55, 67]. Despite several pig models having been established by different gene targeting strategies [5, 55, 67], modifications of the porcine genome remain a time consuming and complex procedure. Hence, alternative targeting tools providing rapid and reliable genetic modification are required. The novel technology of designed nucleases offers a powerful tool and enlarges the options to modify the porcine genome in a specific manner. However, the overall suitability of these technologies still needs to be proven in porcine primary cells. This study aimed at the evaluation of designed nucleases for modification of the porcine genome and presents strategies to improve the targeting efficiency. Nucleases were tested for NHEJ- or HR-introduced modification on autosomes and sex chromosomes. In addition to establishment of reproducible targeting and screening protocols, a *lacZ*-reporter for the *CFTR* gene was generated. Moreover, the gained data suggest that the usage of nucleases in combination with DNA-based vectors provides an efficient tool to examine the cellular repair machinery in mammalian cells.

Zinc finger nuclease, which consists of a specific DNA binding domain linked to a *Fok*I endonuclease for DNA cleavage, is one of the promising artificial molecules used for gene targeting [84]. Theoretically, they can be designed to target every possible DNA locus due to the binding domain, which is formed by variable zinc finger motifs [88]. Web-based tools as the ZiFiT software and others allow a rapid selection of suitable ZFN binding sites for many different target genes and modular assembly or other techniques, enabling a quick construction of ZFNs [95]. Although, publicly available platforms exist, providing archives of engineered zinc-finger arrays for ZFN design, the construction of novel ZFNs for new targeting loci remains time consuming and requires the employment of extensive tests [94].

In this project we used two different sources of ZFN. ZFN sets for the *CFTR* gene were designed by a commercial supplier (Sigma-Aldrich) and carried a DNA binding domain of 5 and 6 ZFs, respectively, each of them specifically recognize and bind to 3 nucleotides. Similarly constructed ZFNs for modification of the porcine genome were published in two studies. In both publications an exogenous *eGFP* gene was targeted *in vitro* and *in vivo* by commercial ZFNs obtained from the same supplier and consisted of 5 and 6 ZFs, respectively [75, 146]. In another publication, reporting a biallelic modification of the porcine *GGTA1* gene and a consequent knock-out of this gene, the ZFNs were constructed by the group itself, however these nucleases also carried a DNA binding domain of 5 ZFs [5]. Thus, the specificity of ZFN binding is ensured by a length of at least 30 bp. This is considerably above a limit of 17 bp that guaranties the uniqueness of a defined sequence in mammalian genomes, at least by statistical means and thus, lowers the risk of off-target binding and cleavage [89]. In contrast, the *DMD*-specific ZFNs were designed by the publically available database and did only provide binding to 18 bp. The shorter binding length of the *DMD*-specific ZFN pair (3 ZFs for each ZFN) might have resulted in a lower affinity to the targeting site and thus, may have caused the poor efficiency of *DMD* modification.

The *CFTR*-specific ZFNs were designed for exon 1 as an introduced mutation in this exon would subsequently lead to a frame shift of the transcript, preventing the production of a truncated protein [149]. Statistically, a suitable binding site for ZFN can be found every 500 bp [94, 108]. Therefore, no ZFN binding sites were found for the encoding region of *CFTR* exon 1, providing a sufficient cleavage activity for targeting experiments. Anyway, the detected binding sites were located very close to the start codon, expecting to be still capable to mediate mutations that affect the coding region. Three ZFN sets showing the highest cleavage activity were delivered, enabling the defined modification of *CFTR* via NHEJ and HR.

Examination of ZFN targeting efficiency was performed at two different temperatures as it had been described previously, that optimal temperature for a high cleavage activity of ZFN was 30°C [150]. The mechanism how the low temperature affects the targeting is unclear, but one explanation might be that the

low temperature is retarding the protein activity necessary for a correct DNA repair and thus, more DSBs are repaired in an incorrect manner. Since the porcine primary cells require a constant temperature of 37°C for an optimal proliferation, the differences in cell proliferation and ZFN activity were investigated at 30°C and 37°C. Altogether, the ZFN designed for the *CFTR* gene showed a lower activity at 37°C than at 30°C. Other than reported [150], the target cell line *Niere m* showed no notable stagnation of proliferation when incubated at 30°C, although the observed number of apoptotic cells after transfection seemed to be slightly increased at the lower cultivation temperature. Interestingly, ZFN pairs 2 and 3 for the CFTR gene were composed of one common ZFN (5O12) and one ZFN (4J9 and 4L23, respectively) that differed by only 1 bp in the binding sequence. The efficiency of the ZFNs, however, differed significantly and underlines the importance of appropriate ZFN design.
Data recovered that ZFN sets delivered as mRNA had shown a particularly strong decrease of activity when incubated at 37°C possibly caused by a quicker degradation at the higher temperature, but for sets 1 and 3 delivered as plasmid DNA cleavage activity remained similar at both temperatures. No target site alterations were detected in three independent experiments using the mRNA of ZFN set 2 probably caused by degradation of this mRNA. However, the activity of ZFN set 2, transfected as plasmid DNA, was still high at both tested temperatures, suggesting the application of this set for further targeting experiments.

Several mutation detection methods were tested in order to identify a reliable and rapid protocol. Although, amplification and cloning of the ZFN targeting sites for colony PCR and the subsequent sequencing analysis are time consuming, this procedure allows picturing the general cleavage efficiency of each ZFN under standard conditions in a mixed cell population without longsome generation of single cell clones and an individual examination of each clone. The commonly used *Cel*I assay for mutation detection [147, 151] was unsuitable for the CFTR targeting segment due to unspecific cleavage of the *Cel*I enzyme in this locus and extensive optimization efforts would be needed for application of this method. Further mutation detection assays like single strand conformation polymorphism [152] also

required optimization for each targeting locus, like optimal amplicon size, polyacrylamide gel concentration and a matching denaturation protocol.

For targeting of the *CFTR* gene ZFN set 2 was used as this ZFN pair cleaves the DNA 3 bp upstream of the start codon and the probability that mutations at the cleavage site affect the ATG codon is high. Furthermore, initial experiments demonstrated the high cleavage activity of this ZFN set at both temperatures. Despite the high efficiency rate of ZFN set 1 mRNA, the cleavage site of this ZFN is 98 bp upstream of the start codon and initial experiments revealed deletion of maximum 70 bp at the targeting site, making set 1 unsuitable for disruption of the transcriptional start codon via NHEJ. Targeting efficiencies of up to 20 % were reported previously [99] for *in vitro* experiments and up to 15 % for targeting of porcine cells [75]. Direct comparison recovers that targeting rate of 26 % and 19 % obtained in our experiments is consistent with their results. However, the biallelic targeting rate of 2 % and 4 % is lower than reported [99, 146] and only 2 of the biallelicly targeted single cell clones showed a deletion of the ATG start codon. The data suggests that in case the ZFN targeting site is located outside of the coding region the induced mutations not always generate a functional gene knock-out due to the unpredictability of introduced mutation. A disruption of the transcriptional reading frame is possible, but assumes the generation of a large number of clones to obtain a single cell clone with the desired mutation on both alleles, e. g. a deletion large enough to eliminate the start codon. ZFN targeting sites located within the coding region facilitate a reading frame shift as deletions of only a few base pairs lead to a modification of the transcript. Therefore, a further targeting strategy was applied for generation *CFTR* knock-out animals based on the ZFN technology.

In contrast to the site-directed, but undefined mutations generated by NHEJ after ZFN induced DSBs, combination of ZFN with a DNA vector provides the opportunity to introduced mutations not simply at a defined site, but also in a defined manner. Various data have been published, reporting ZFN-mediated and HR-based gene targeting in transformed cell lines [94, 99, 153], but also in ESCs and iPSCs [101] as well as in mouse primary fibroblasts [8]. In the latter, a previously inserted, mutated

eGFP transgene was corrected via ZFN-mediated homologous recombination, resulting in a targeting efficiency of approximately 2 %. Generally, the targeting vector used for ZFN-mediated targeting consists of a transgene flanked by short homologous arms of 500-2000 bp [101, 154], requiring in some cases isogenic DNA [56], but not in all [101]. In order to facilitate screening, a selection system is needed, e.g. a *GFP* reporter gene [8, 154] or a selection cassette.

Here we aimed at the HR-mediated introduction of a *lacZ* reporter gene at the porcine *CFTR* locus. In order to study the role of *CFTR* in selected tissues or the embryonic development in pigs, transgenic animals carrying a reporter *lacZ* gene driven by the *CFTR* promoter need to be established. The activation and the expression level of the *CFTR* gene can then be analyzed in different embryonic stages by detecting the *lacZ* expression via X-Gal staining as was demonstrated in mice models [155, 156]. Two targeting vectors, a BAC vector with homology arms of > 80 kb and a truncated vector with homology arms of < 1700 bp, were designed for HR-based targeting experiments. Both vectors carried a modification of approximately 5700 bp, containing a *lacZ* gene as well as a neomycin selection cassette. Electroporation experiments using the smaller targeting vector in combination with ZFN set 1 plasmid DNA or mRNA showed no HR events, although a cleavage had taken place at the targeting site, illustrated by NHEJ events with parts of the targeting construct in 4 of 152 cell clones. One possible explanation might be that the homology arms (1685 bp and 1190 bp) of the targeting construct were too short. Successful HR-mediated gene targeting has already been shown with homology arms < 1000 bp and in special cases as little as 50 bp as well [74, 101, 153]. However, it was reported that the length of homology arms depend on the length of the embedded transgene, since the copying process of large transgenes requires more stability by larger homology arms, assuming that an insert of 5700 bp require larger homology arms than 1200 bp as used for the experiment [74]. In addition, the successfully used vectors mentioned in the above publications carried homology arms that were placed directly left and right of the ZFN cleavage site. In contrast the used *lacZ* targeting construct carried a 3' homology arm, homologous with a sequence 3500 bp downstream of the ZFN targeting site, which might have constricted the homologous recombination. The distance of the 5' homology arm

(98 bp) might not have interfered with HR as it was reported before that efficient recombination takes place within 400 bp surrounding the induced DSB [86].
In contrast, to the devastating experience of combining ZFN with a small targeting vector, showing no HR events at the target site, the usage of modified BAC was more fruitful. These vectors were successfully used for modification of mouse ESCs [65] and porcine primary fibroblasts as well [67]. In our lab we have demonstrated HR-mediated gene targeting of porcine genes with BAC vectors alone and achieved a targeting efficiency of 1.2 % to 2.9 % for *CFTR*, *GGTA* and *DMD* genes. In this study, the transfection of pig cells with a *lacZ* containing BAC resulted in a targeting efficiency of a similar rate (1 %). The combination of the BAC with a ZFN pair, however, boosted the targeting efficiency by more than an order of magnitude (56 %).

Although, ZFN combined with AAV targeting vectors was reported previously [157, 158], achieving targeting efficiencies of up to 65 % in human cell lines, both groups showed targeting of an *eGFP* transgene. In other reports, ZFN were also co-transfected with integrase-defective lentiviral vectors [159] for targeting of transformed cell lines and human ESCs, achieving efficiencies of up to 50 %, but targeting rates for primary cells remained low [8]. Combination of ZFN-induced DSBs and HR-mediated gene targeting using a BAC targeting vector as donor DNA has not been reported to date and was applied in this work for the first time. The increased HR in the presence of ZFN induced DSBs is in accordance with others [99, 153, 154], demonstrating that DSBs are of crucial importance for HR-based gene targeting.
The impressive rates of HR, however, depended on proper optimization of the transfection procedure and critical balance of cells and nucleofected DNA. In the initial experiments with 1 µg of different ZFNs more than 200 single cell clones were obtained, depending on the amount of transfected ZFN encoding DNA, electroporations delivered considerably different numbers and viability of cells.
Experiments with BAC DNA were more critical, as a decreased number of single cell clones was obtained from these transfections. This might be explained by the large size of the BAC vector of approximately 200 kb. The uptake of large amount of

exogenous DNA might be precarious for primary cells and consequently, reduce their viability.

Co-transfection experiments with ZFN and BAC DNA showed an even more pronounced decrease in the number of single cell clones and minor changes in the ratio of ZFN/vector or DNA/cells resulted in complete failure of the experiment. These results suggest that the following protocol represents the capacity limit of nucleofection. On the other hand, the huge size of the BAC vector requires high amount of DNA due to stoichiometric reasons.

A striking finding of this study was the extraordinary rate of HR (56 %) compared to NHEJ (11 %), using ZFN pair 2 at 37°C. This might be explained by the dominant role of HR in DSBs repair, which would be in contrast to a previous report [71]. This hypothesis might explain the well-known aspect of ZFN that their potential for mutagenesis is higher at 30°c than at 37°C [150]. Taking into account a considerable proportion of HR in DSBs as well as the impaired activity of the cellular recombination machinery at 30°C, it is obvious that ZFN activity is not higher at the lower temperature but the preference of the two pathways, HR and NHEJ, to repair a DSB shifted towards the latter. In addition, the high rate of HR was achieved with an approximate transfection efficiency of 60-70 %, indicating that the recombination event at the target site is extremely efficient, as it required the entry of both ZFN plasmid and the BAC vector. It seems that both events, ZFN-mediated DSBs as well as BAC-mediated homologous recombination, work at a level of almost 100 %, meaning that every cell transfected with both, ZFN and BAC, will certainly be correctly targeted.

In conclusion, our ZFN data revealed their efficacy for genetic modification via NHEJ and HR in primary pig cells and the experiments gave insights into the repair mechanisms after DNA DSBs. However, the rare occurrence of ZFN binding sites in mammalian genome limits their usage. Therefore, TALENs might be alternative nuclease tools.

TALENs were reported to be less cytotoxic [112] and be available for a broader application due to a higher frequency of targeting sites [109]. Furthermore, improved protocols enabled the design of TALENs with a promising targeting

efficiency for a variety of genes [109, 160, 161]. TALENs and a ZFN pair were designed to target the porcine *DMD* gene to evaluate their efficiency for genetic modification in porcine cells.

The aim was the generation of a large animal model for Duchenne muscular dystrophy (DMD) and - in later experiments - the transformation of the severe DMD phenotype to a milder BMD phenotype by exon skipping. Thus, only exons encoding an integer number of amino acids were valuable targets for our approach.

Exon 48 and 49 of *DMD* were selected as target loci with TALENs designed for both exons and a ZFN only for exon 49. TALENs and ZFN for the porcine *DMD* gene were not obtained from a commercial supplier, but designed using publicly available platforms and protocols [4, 70, 95, 109]. Therefore, the constructed nucleases were not extensively pre-evaluated, but tested directly by transfecting porcine primary cells. After transfection, differences in cell proliferation and viability were observed. TALE nucleases have been used for targeting of human transformed cell lines and for human ESCs and iPSCs as well, achieving promising targeting efficiencies in NHEJ- and HR-based targeting experiments [162, 163]. However, targeting of primary cells was not reported to date. Transfection of porcine primary kidney cells with TALENs and ZFN for the porcine *DMD* gene resulted in marginal targeting efficiencies approximately 100-fold lower than reported for other cell types [162, 163] or than demonstrated in this project for the *CFTR* locus. A general sensitivity of porcine primary cells was excluded due to the contra directory findings for the *CFTR*-specific ZFNs. However, the Gaussian-like distribution of mutations around the cleavage site of ZFN was in contrast to the distribution of TALEN-induced mutations, showing no order at all and suggesting that on the one hand the binding affinity of the TALEN DNA binding domain might be not efficient enough to provide a stable dimerization of the *Fok*I domain and thus, a cleavage of the DNA. On the other hand it might be more critical to target a gene that is located on the X-chromosome, like *DMD*, than on an autosomal chromosome, like *CFTR*, as the repair mechanisms of sex chromosomes in male cells are not completely understood. Generally, the repair of DSBs on sex chromosomes in male cells via HR is not possible as no sister chromatid is available. Therefore, DSBs might be repaired by alternative repair mechanisms,

like microhomology-mediated end joining or other end joining pathways that are less error-prone [31].

The poor efficiency of TALENs, even in comparison to the DMD-specific ZFNs, indicates the necessity of further optimizations to develop functional TALENs, e.g. the length of the DNA binding domain or the length of the spacer between the two domains [112].

The proper construction of designed nucleases remains a challenge, since the publically available platforms are mostly optimized for species like plants (soybean, rice and maize), yeast, nematode, fruit fly, zebrafish, mouse, rat and human, but there is no platform providing optimized ZFN arrays or TALEN binding sites for the pig genome [70]. This makes it difficult to identify suitable targeting sites in the porcine genome or to evaluate the chosen ones for off-target cleavage. Although the TALEN and ZFN binding sites can be investigated for binding affinity to other sequences than the target locus, the generated nucleases cannot be tested for their suitability to target the porcine genome and therefore, the constructed nucleases might show unexpected off-target cleavage.

Different aspects can be addressed in future experiments in order to complement the results of this project.

The characterized *Niere m* single cell clones carrying the *lacZ* reporter gene at the *CFTR* targeting site need to be tested for their suitability for SCNT, examining the capability of the targeted cell clones to generate viable animals after SCNT. *LacZ*-reporter animals reflect an invaluable model system to address a variety of questions according *CFTR* gene expression and regulation in different embryonic stages. Combined with the previously established *CFTR* knock-out pigs, it might give an insight into compensatory mechanisms of cells caused by the lack of the CFTR.

Further, the combination of two targeting strategies, i.e. ZFN and BAC targeting vector, resulted in unexpected high targeting efficiencies. However, the combination of BAC vector and ZFN should be tested for further loci as well, e.g. the *DMD* gene. Targeting of the porcine *DMD* gene, using ZFN/TALEN with BAC constructs, can help to examine whether the results obtained from the *CFTR* targeting are representative for other targeting loci as well. It needs to be investigated whether the low targeting

efficiency of ZFN/TALEN for *DMD* can be increased by the addition of a BAC vector and whether the observed targeting of an X-chromosome linked gene - like *DMD* - is more critical than a gene on an autosome. In addition, the combination of ZFN and BAC vector might provide an interesting tool for the examination of the cellular DNA repair machinery.

Designed nucleases are a promising tool for targeting the porcine genome. When designed and constructed thoroughly, ZFNs can target desired loci via either non-homologous end joining or homologous recombination. For the latter donor DNA containing large homologous regions, e.g. BAC vectors, triggers the recombination event with a particular high frequency. The newly developed TALENs represent a potential targeting strategy, but for application for the porcine genome this technology still requires further investigations.

6 SUMMARY

Modification of the porcine genome using nuclease-based targeting tools

Artificial nucleases, consisting of a DNA binding domain and a DNA cleavage domain, have the ability to specifically bind defined DNA loci and introduce double-strand breaks (DSBs) in order to activate the cellular damage response. The cellular repair system eliminates the DSBs by either non-homologous end joining (NHEJ) or homologous recombination (HR). Both repair pathways can be used to introduce desired DNA alterations into the host genome. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) represent designed nuclease-based targeting tools for the introduction of DSBs at genomic target sites. The general suitability of both techniques to target various cell types *in vitro* and *in vivo* has already been shown in previous studies. Several genetically modified animals have been generated using these nucleases, demonstrating their potential for the generation of animal models crucially needed. As animal models are essential implements for understanding of gene functions, study of human diseases and development or improvement of therapeutic treatments, the engineering or evaluation of new gene targeting tools for the generation of these animals are important.

The aim of this work was the modification of the porcine cystic fibrosis transmembrane conductance regulator (*CFTR*) gene using ZFNs which in turn promotes NHEJ and HR. To achieve this, three ZFN sets were obtained, targeting exon 1 of the *CFTR* gene. In preliminary experiments of this work, the targeting efficiency has been determined at different temperatures (30°C and 37°C) by transfecting primary porcine kidney cells (*Niere m*) either with ZFN-encoding plasmid DNA or mRNA. ZFN set 2 was found to result in the highest cleavage efficiency at both temperatures and thus, was used for further experiments. As the future purpose will be the generation of *CFTR* knock-out pigs, single cell-derived clones were produced after transfection with ZFN and subsequent screening of these clones resulted in a targeting efficiency of up to 26 %. 4 % of these targeted clones carried a mutation on even both alleles. The binding sites of *CFTR*-specific ZFNs are

located not within the coding region, but 1 bp upstream of the transcriptional start codon (ATG). Therefore, only 2 cell clones showed a disrupted ATG and generation of functional knock-out at the *CFTR* gene was ultimately not possible using the NHEJ pathway.

In order to target the gene via HR, two targeting vectors, both carrying a β-galactosidase (*lacZ*) reporter gene and a neomycin resistance cassette, were designed for a co-transfection with ZFN. The bacterial artificial chromosome (BAC) vector contained homology arms of over 80 kb, while the truncated vector carried homology arms of 1200-1700 bp. Transfection experiments using the truncated vector did not show HR events at the ZFN cleavage site. In contrast, targeting efficiencies of the BAC vector were unexpectedly high. In total, 56 % of the generated single cell clones carried the introduced *lacZ* gene and the resistance cassette at the target site. Furthermore, 13 % of these cell clones were homozygously targeted, demonstrating the high potential of these experiment settings.

Targeting experiments with TALENs that were designed to bind exon 48 and 49 of the porcine dystrophin (*DMD*) gene resulted in a particular low targeting efficiency (<1 %). Deep sequencing of the generated single cell clones revealed induced mutations of 1 bp, which were not located at the targeting site as was observed in ZFN experiments, but were spread widely all over the TALEN binding site. These data suggested a low binding affinity and cleavage activity of the constructed TALENs. However, as the *DMD* gene is located at the X-chromosome, alternative repair systems might be involved in the repair of induced DSBs, resulting in a low frequency of mutations at the target site and further experiment would be necessary to address this aspect more closely.

7 ZUSAMMENFASSUNG

Modifikation des Schweinegenoms mit Hilfe Nuklease-basierenden Targetings

Künstliche Nukleasen, die aus einer DNA bindenden Domäne und einer DNA schneidenden Domäne bestehen, besitzen die Fähigkeit festgelegte DNA-Loci spezifisch zu binden und Doppelstrangbrüche (DSBs) einzufügen, um dadurch die zelluläre Antwort auf DNA-Schäden zu aktivieren. Das zelluläre Reparatursystem beseitigt den DSB entweder mittels der nicht-homologen Endverknüpfung (NHEJ) oder mittels homologer Rekombination (HR). Beide Reparaturwege können benutz werden, um gewünschten DNA-Veränderungen in das Zielgenom einzuführen. Zinkfingernukleasen (ZFN) und Transcription Activator-like Effectors Nukleasen (TALEN) repräsentieren konstruierte Nuklease-basierende Targetingmethoden zur Einführung von DSB an jedem beliebigen Zielort im Genom. Beide Technologien haben ihre prinzipielle Fähigkeit zum Gentrageting in verschiedenen Zelltypen *in vivo* und *in vitro* in vorherigen Studien bereits gezeigt. Mehrere genetisch modifizierte Tiere wurden mittels dieser Nukleasen erstellt, was ihr Potenzial in der Entwicklung von Tiermodelle beweist. Da Tiermodelle essentielle Hilfsmittel für das Verständnis von Genfunktionen, die Untersuchung menschlicher Krankheiten und die Entwicklung oder Optimierung von therapeutischen Behandlungen sind, ist die Entwicklung und Evaluation neuer Targeting-Tools für die Erstellung dieser Tiere wichtig.

Das Ziel dieser Arbeit war es, das Cystic Fibrosis Transmembrane Conductance Regulator- (*CFTR*) Gen im Schwein mit Hilfe von ZFN und die daraus resultierenden NHEJ und HR zu verändern. Um dies zu erreichen, wurden drei ZFN Sets erstellt, welche jeweils Exon 1 des *CFTR*-Gens binden. In vorbereitenden Experimenten wurde die Targetingeffizienz bei unterschiedlichen Temperaturen (30°C und 37°C) bestimmt, indem primäre Schweinenierenzellen (*Niere m*) sowohl mit ZFN-kodierender Plasmid-DNA als auch mRNA transfiziert wurden. Der Befund zeigte, dass ZFN Set 2 die höchste Effizienz bei beiden Temperaturen aufwies, weshalb dieses für weitere Experimente verwendet wurde. Da das über diese Arbeit hinausführende Ziel die Erstellung eines *CFTR* knock-out Schweins ist, wurden nach der Transfektion mit ZFN Einzelzellklone hergestellt und diese wiederum einzeln auf

Modifikationen untersucht, was eine Targetingeffizienz von bis zu 26 % ergab. 4 % der getargeteten Klone trugen eine Mutation auf beiden Allelen. Die Bindungsstellen der *CFTR*-spezifischen ZFNs befanden sich nicht in der kodierenden Region, sondern 1 bp vor dem Transkriptionsstartcodon (ATG). Daher zeigten nur 2 Zellklone ein zerstörtes ATG und die Erstellung von einem funktionalen Knock-out des *CFTR*-Gens mittels NHEJ war daher nicht möglich.

Um das Gen mittels HR zu targeten, wurden zwei Targetingvektoren, die beide ein β-galactosidase- (*lacZ*) Gen und eine Neomycin-Resistenzkassette tragen, konstruiert und mit ZFN co-transfiziert. Der bakteriellen artifiziellen Chromosom-(BAC) Vektor enthielt homologe Arme von über 80 kb, während der trunkierte Vektor homologe Arme von 1200-1700 bp trug. Transfektionsexperimente mit dem trunkierten Vektor ergaben keine homologe Rekombination an der ZFN-Schnittstelle. Dagegen war die Targetingeffizienz des BAC-Vektors unerwartet hoch. Insgesamt trugen 56 % der generierten Einzelzellklone das eingefügte *lacZ*-Gen und die Resistenzkassette an der ZFN-Bindungsstelle. Desweiteren waren 13 % der Zellklone homozygot getargeted und zeigten damit das große Potential dieses Experimentaufbaus.

Targetingexperimente mit TALENs, die konsturiert waren, um an das Exon 48 und 49 des Schweinedystrophingens (*DMD*) zu binden, ergaben eine besonders niedrige Targetingeffizienz (<1 %). Hochdurchsatzsequenzierung der generierten Einzelzellkone zeigte eingeführte Mutationen von 1 bp, die nicht an der TALEN-Bindungsstelle lokalisiert waren, wie es in ZFN-Experimenten beobachtet wurde, sondern über die TALEN-Bindungsstelle verteilt waren. Die Daten legen eine niedrige Bindungsaffinität und Spaltungsaktivität der erstellten TALENs nahe. Da sich das *DMD*-Gen jedoch auf dem X-Chromosom befindet, könnten alternative Reparatursysteme an der Reparatur der eingefügten DSBs beteiligt sein, die eine niedrige Mutationsfrequenz verursachen. Allerdings sind noch weitere Experimente notwendig, um diesen Aspekt näher zu beleuchten.

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