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AA	amino acid
AAV	adeno-associated virus
ACTB	β-actin
A. dest.	deionized water
AHXR	acute humoral xenograft rejection
amp	ampicillin
ATP	adenosine tri-phosphate
AVR	acute vascular rejection
BAC	bacterial artificial chromosome
bGH	bovine growth hormone
bla	blasticidin
bp	base pairs
bsr®	blasticidin resistance cassette
cAMP	cyclic adenosine mono-phosphate
CACCs	calcium-activated chloride channels
$CaCl_2$	calcium chloride
CD59	protectin
CF	cystic fibrosis
CFMDB	cystic fibrosis mutation data base
CFTR	cystic fibrosis transmembrane conductance regulator
$CHCl_3$	chloroform
CiA	chloroform isoamylalcohol
c-myc	myelocytomatosis viral oncogene
cn	copy number

cnr	copy number ratio
ΔF508	deletion of the phenylalanine 508 in *CFTR*
DMEM	Dulbecco modified Eagle medium
DMSO	dimethylsulfoxide
dNTPs	deoxyribonucleotides
DTT	dithiothreitol
DSB	double strand break
dsRed2	discosoma sp. red
DT-A	diphtheria toxin A
eBFP	enhanced blue fluorescent protein
EDTA	ethylene diamine tetra acetic acid
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anemia virus
EPO	electroporation (nucleofection)
ESCs	embryonic stem cells
EST	expressed sequence tag
ET	embryo transfer
EtOH	ethanol
F-factor	fertility factor
FISH	fluorescent *in-situ* hybridization
FLP	flippase
FRT	flippase recognition target
G418	geneticin
GGTA1	α-1,3-galactosyltransferase
HAR	hyperacute rejection

HCl	hydrochloric acid
hDAF	human decay accelerating factor
hGH	human growth hormone
HIV-1	human immunodeficiency virus type I
HOAc	acetic acid
HPRT	hypoxanthine phosphoribosyltransferase
HR	homologous recombination
iAmOH	isoamylalcohol
iPrOH	isopropanol
IPTG	isopropyl-beta-D-thiogalactopyranoside
IRES	internal ribosomal entry site
IVM	*in-vitro* matured
kan	kanamycin
kb	kilobases
KOAc	potassium acetate
lacZ	β-galactosidase
lepR	leptin receptor gene
LOWA	loss of wild-type allele
loxP	locus of cross-over in P1
mESCs	murine embryonic stem cells
µg	microgram
µl	microliter
$MgCl_2$	magnesium chloride
min	minutes
ml	milliliter

MLV	murine leukemia virus
$MnCl_2$	manganese chloride
MV	mean value
n-3	omega-3
NT	nuclear transfer
NaCl	sodium chloride
NaOH	sodium hydroxide
$neokan^R$	neomycin/kanamycin resistance cassette
neo^R	neomycine resistance
NHEJ	nonhomologous end joining
NTC	no template control
o/N	overnight
PAC	P1 artificial chromosome
PCiA	phenol chloroform isoamylalcohol
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PERV	porcine endogenous retrovirus
pFF	porcine fetal fibroblast
PGK	phosphoglycerate kinase
PhOH	phenol
pKC	porcine kidney cells
PMI	pronuclear microinjection
PNS	positive/negative selection
polyA (or pA)	polyadenylation site
qPCR	quantitative real-time PCR

R-domain	regulatory domain
RFLP	restriction fragment length polymorphism
RNase A	ribonuclease A
RT	room temperature
RVD	repeat variable di-residue
SA	splice acceptor
SCNT	somatic cell nuclear transfer
2 SD	two times standard deviation
SDS	sodium dodecyl sulfate
sec	seconds
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMGT	sperm-mediated gene transfer
SNP	single nucleotide polymorphism
SV40	Simian virus 40
TALE	transcription activator-like effector
TALENs	transcription activator-like effector nucleases
tk	thymidine kinase gene
TRIS	tris-(hydroxymethyl)-aminomethan
UNG	uracil-N-glycosylase
X-Gal	5-bromo-4chlor-3-indoxyl-β-D-galactopyranoside
XT	xenotransplantation
YAC	yeast artificial chromosome
ZFN	zinc finger nuclease
Zn	zinc

1 INTRODUCTION

As the initial focus, to modify livestock for agricultural purpose, such as growth performance, feed efficiency and body composition (Pursel, 1998), lactation performance (Zuelke, 1998), reproduction, disease resistance and immune responsiveness (Muller *et al.*, 1998) does not meet the expectations, large animals came in a tighter focus for alternative areas. Especially the pig, due to indications like physique, the ability to standardize the environmental situation (housing, feeding, and sanitation standard), the well established reproductive technology and advanced techniques of genetic modification of the porcine genome, represents an ideal model organism for both, human diseases and xenotransplantation (Aigner *et al.*, 2010). Several different technologies to produce transgenic animals, primarily developed in the mouse, such as pronuclear microinjection (PMI) (Gordon *et al.*, 1980), sperm-mediated gene transfer (SMGT) (Lavitrano *et al.*, 1989) or viral gene transfer (Jaenisch *et al.*, 1976), were later on adapted to livestock (Brem *et al.*, 1985; Hofmann *et al.*, 2003; Kurome *et al.*, 2006). In mice the main disadvantages arising from those methods, reported as random, partially multicopy integration of the transgene, insertional mutagenesis, positional effects, oncogene activation, low integration efficiencies or offspring mosaicism (Wheeler, 2003) have been partially bypassed with the establishment of murine embryonic stem cells (ESCs) (Evans *et al.*, 1981; Martin, 1981) and the development of strategies to genetically modify them (Kuehn *et al.*, 1987). In order to circumvent the lack of porcine ESCs, an alternative method, termed somatic cell nuclear transfer (SCNT), has become an indispensable tool to generate large animal models from genetically modified somatic cells (Campbell *et al.*, 1996; Wilmut *et al.*, 1997). Different strategies to engineer primary somatic donor cells by the introduction of DNA or RNA have been developed, achieved by viral or non-viral, in turn subdivided in physical and chemical methods (Kobayashi *et al.*, 2005). Viral transgenesis is most frequently performed using retroviral, especially lentiviral, or adeno-associated viruses (AAV) (Park, 2007). Non-viral DNA delivery methods are grouped in chemical (Azzam *et al.*, 2004) and physical (Magin-Lachmann *et al.*, 2004) systems. The most common methods among them are reported to be lipofection (Felgner *et al.*, 1987), electroporation (Neumann *et al.*, 1982) and nucleofection (Martinet *et al.*, 2003) as an advanced method of electroporation. With SCNT the possibility to produce even tailored porcine animal models arose. Site directed mutagenesis is achieved by homologous recombination (HR) of constructed targeting vectors with the target locus. It is reported that the frequency of targeted HR events is much lower compared to random integrations of the vector construct throughout the genome in most mammalian cell lines, necessitating strategies to increase the frequency of HR events in somatic cell gene targeting (Wang *et al.*, 2003). Several parameters obviously influence the frequency of HR events, such as a positive correlation of the efficiency with the increased length of the homologous regions of the targeting vector (Hasty *et al.*, 1991; Deng *et al.*, 1992), the need of an isogenic vector construction (te Riele *et al.*, 1992) and locus dependency of the absolute targeting efficiency (Wang and Zhou, 2003). Additionally, enrichment of the targeted clones by positive and negative marker selection (Izant *et al.*, 1985; Mansour *et al.*, 1988), promoter- or polyadenylation-trap experiments or artificially introduced double strand breaks (DSB) by site-directed nucleases inducing repair mechanisms, can increase the targeting efficiency remarkably (Karreman, 1998).

Different targeted porcine animal models, including a disease model for cystic fibrosis targeting the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene (Rogers *et al.*, 2008) and animals containing targeted deletion of the *GGTA1* (α-1,3-galactosyltransferase) gene, making them interesting as organ sources for the xenotransplantation research (Lai *et al.*, 2002), have already been developed. The aim of this doctoral thesis is to demonstrate whether the establishment of two different porcine animal models (*CFTR* and *GGTA1*), utilizing the extended homologous regions provided by the respective bacterial artificial chromosome (BAC)-based targeting vectors to increase the targeting efficiency in primary porcine kidney cells as nuclear donor cells for SCNT, is feasible.

2 OVERVIEW OF LITERATURE

2.1 Transgenic large animals

2.1.1 Background

Ever since domestic animals played an important role in maintaining human wellbeing, as they provide basic materials like food and clothing. As a matter of fact, one tried to improve phenotypical amenities by classical breeding, although these processes act very slowly and the alteration of many genes often happens in an unregulated manner (Laible *et al.*, 2009). In farm animals originally a special focus lies on the optimization of production characteristics like growth performance, feed efficiency and body composition (Pursel, 1998), lactation performance (Zuelke, 1998), reproduction, disease resistance and immune responsiveness (Muller and Brem, 1998). The benefit of transgenic farm animals for their agricultural use is on the decline due to decreasing demand and insufficient implementation of scientific issues. In this context, one point of interest to improve the conversion of feed to body weight gain in comparison to sibling controls was examined by the generation of transgenic pigs, carrying structural genes for human or bovine growth hormone (hGH or bGH) ligated to a mouse metallothionein-I (MT) promoter, driven by the idea to mimic the mouse model, in which the introduction of a growth hormone gene markedly increased the growth rate and final size of the animals (Palmiter *et al.*, 1982). Though, the resulting persistent excess of hGH and bGH led to health effects in modified pigs like lameness, lethargy, gastric ulcers and anestrous gilts (Pursel *et al.*, 1990). Attempts to improve transgenic livestock resistance to viral infection were also not promising (Muller *et al.*, 1992). Nowadays, the Western civilization aspires towards incomparable health awareness, opening an additional niche for transgenic farm animals bringing up the term ‘functional food’. Transgenic pigs were generated, which express a humanized *Caenorhabditis elegans* gene, fat-1, encoding an omega-3 (*n*-3) fatty acid desaturase. The hfat-1 transgenic pigs produce high levels of n-3 fatty acids, usually mainly contained in fish-oil, which are known to improve heart function and help to reduce the risks for heart disease (Dai *et al.*, 2002).

Especially the pig is reflecting many beneficial properties used as large animal model for selected human diseases and as a source for donor organs in xenotransplantation (Platt, 1998; Bendixen *et al.*, 2010). With its domestication, about 9000 years ago, accompanied by the rise of agriculture, the pig underwent rapid evolution all over the world through artificial phenotypical selection, as for example resulting in a decreased skeletal size (Giuffra *et al.*, 2000). Due to the long history of the pig meeting agricultural demands also the sanitation standards, housing and feeding are well established to date. Its reproductive characteristics are favorable to other large animals reflecting a relatively short gestation period of around 114 days, an early sexual maturity with six to eight months of age and large litter sizes ranging from eight to twelve piglets. With the theoretical potential of three deliveries per year, also not depending on season, one single sow is able to produce 24-36 pigs per year, compared to two to four in general in sheep (Newman *et al.*, 1995) and one to two in cattle (reviewed in Wolf *et al.*, 2000). Similarities in size, physiology, anatomy, metabolism, pathology, organ development and disease progression to human, even without any genetic alterations, make the pig an interesting model organism, being established in reasonable periods and with acceptable costs (Lunney, 2007). Furthermore, the ethical issues concerning a porcine model organism are not that critical, at least compared to primate animal models, keeping in mind that in 2010 almost 59 million pigs were slaughtered for meat production in Germany, anticipating that these numbers are going to increase with every year (evaluated by Statistisches Bundesamt Deutschland, 2010). The importance of the pig as model organism was underlined in 2006, when the pig whole genome sequencing project has been launched and was initiated by the Swine Genome Sequencing Consortium (SGSC) (Archibald *et al.*, 2010). Evidently, the porcine genome, comprised of 18 autosomes and 2 sex chromosomes, exhibits tight similarity in size and also complexity to the human genome. Unlimited access to a wide range of porcine genomic and expressed sequence tag (EST) sequences, extensive trait loci, linkage and physical maps, single nucleotide polymorphisms (SNPs) and expression data via GeneBank and other databases have been provided over the years (Chen *et al.*, 2007). An additional benefit is the possibility to genetically modify the porcine genome quite effectively to reflect particular human diseases or enable xenotransplantation (reviewed in Aigner *et al.*, 2010). Taken all those facts together led to the generation of several different porcine disease models, including cardiovascular diseases (Hao *et al.*, 2006), obesity or hypertension (Dyson *et al.*, 2006), diabetes (Renner *et al.*, 2010), alcoholism (Wallock-Montelius *et al.*, 2007), skin physiology (Simon *et al.*, 2000), lipoprotein metabolism (Ginsberg *et al.*, 1997), intestinal function (Domeneghini *et al.*, 2006), nutrition (Mitchell, 2007), injury and repair (Winter, 2006), neurodegenerative diseases (Uchida *et al.*, 2001; Kragh *et al.*, 2009), retinitis pigmentosa (Ross *et al.*, 2012) and cystic fibrosis (Rogers *et al.*, 2008; Klymiuk *et al.*, 2011) and contributes to manifest the idea to swap pigs for non-human primates as donor animals in xenotransplantation (Klymiuk *et al.*, 2010). In conclusion, its body composition, the possibility of the environmental standardization (housing, feeding, and sanitation standard), the well established reproductive technology and advanced techniques of genetic modification of the porcine genome combine prerequisites pointing the pig out as an ideal model organism for both, human diseases and xenotransplantation.

2.1.2 General methods for the establishment of transgenic pigs

One of the major advantages of pigs as animal models is the ability to modify the porcine genome by several different techniques of genetic engineering. In 1971 Brackett and co-workers first were able to demonstrate that mammalian spermatozoa show an ability to act as shuttle vectors for foreign DNA, also described as sperm mediated gene transfer (SMGT) (Brackett *et al.*, 1971). The technique was described with high efficiency in the mouse (Lavitrano *et al.*, 1989), subsequently successfully adapted for use in farm animals, such as for the generation of human decay accelerating factor (hDAF) transgenic pigs (Lavitrano *et al.*, 1999), as well as pigs multitransgenic for three reporter genes: enhanced green and blue fluorescence protein (eGFP and eBFP) and red fluorescent protein (DsRed2) (Webster *et al.*, 2005). Since the integration sites are unknown and the number of inserts may fluctuate between individual embryos and experiments (Habermann *et al.*, 2007) up to now, SMGT cannot be considered as a routine and effective application to mammalian livestock species. Viral transgenesis aims at the introduction of foreign DNA into preimplantation embryos by using virus shuttles. Lentiviruses, as members of the family *Retroviridae*, are characterized by their ability to reversely transcribe their viral genome into double stranded DNA by an enzyme called reverse transcriptase and subsequently integrate it into the host genome as a so called provirus (Guo *et al.*, 2008). This retroviral infection method was first described to generate transgenic mice by Jaenisch and colleagues in 1976. The lentiviral gene transfer was successfully adapted using human immunodeficiency virus (HIV-1) based vectors (Hofmann *et al.*, 2006) or an equine infectious anaemia virus (EIAV) (Whitelaw *et al.*, 2004) vector to establish eGFP transgenic pigs. The major advantage of this method is the relatively low technical effort of presenting a virus to embryos in various developmental stages. In contrast, the application of this system may be influenced by its capacity limitation (< 10 kb DNA) (Pfeifer, 2004). Finally, in 1980 Gordon and colleagues were able to show, that DNA injected into the pronuclei of single-cell embryos is incorporated, expressed and transmitted to the offspring of transgenic mice. (Gordon *et al.*, 1980). The same method, also known as pronuclear microinjection (PMI) has been used to generate transgenic livestock (Brem and Springmann K, 1985; Hammer *et al.*, 1985; Klose *et al.*, 2005), mainly for agricultural and reproductive purpose (Galli *et al.*, 2008). However, in this method the integration pattern is reported to be mosaic, the DNA integration efficiency is often very low (< 1%), making it also quite costly especially for large animal approaches, and the technique is restricted to additive gene transfer (Wolf *et al.*, 2000). In mice, those disadvantages have been partially bypassed with the development of murine embryonic stem cells (ESCs) (Evans and Kaufman, 1981; Martin, 1981) and the establishment of strategies for their modification (Kuehn *et al.*, 1987; Ramirez-Solis *et al.*, 1995). Due to the lack of porcine ESCs, somatic cell nuclear transfer (SCNT) has become the leading tool for generating animals from genetically engineered somatic cells.

2.1.3 Somatic cell nuclear transfer

Initial nuclear transfer experiments using blastomeres of 16-cell-embryos as nuclear donors resulted in the generation of cloned sheep (Willadsen, 1986). In 1996, Campbell and co-workers showed that an expansion of ovine embryonic cells before using them for nuclear transfer is possible in cell culture (Campbell *et al.*, 1996). The first successful approach via SCNT using not only embryonic but adult somatic cells as nuclear donor cells was Dolly the sheep (Wilmut *et al.*, 1997), followed by many more species resulting in live birth with the most prominent among them like mice (Wakayama *et al.*, 1998), cattle (Zakhartchenko *et al.*, 1999), goats (Baguisi *et al.*, 1999), pigs (Betthauser *et al.*, 2000; Polejaeva *et al.*, 2000) and rabbits (Challah-Jacques *et al.*, 2003).

In brief, donor nuclei obtained from various tissues are transferred to the cytoplasm of a zygote or a metaphase II oocyte, from which its genetic material previously had been removed (reviewed in Wolf *et al.*, 1998). After membrane fusion by an electric current and activation, either chemically or by an electric pulse, cells are allowed to reprogram using medium that arrests the cell cycle, and cultured *in vitro* (Niemann *et al.*, 2003). This technology bears several advantageous characteristics, such as the possibility to pre-select donor cells concerning gender or transgene expression. Even a single cell can be expanded and used for SCNT. Additionally, the production of mosaic animals is out of the question, since a cloned transgenic animal originates from a single, stably transfected cell (Aigner *et al.*, 2010). Though, only a small number of cloned embryos are able to develop normally as a result of unpredictable epigenetic reprogramming impacts (Hochedlinger *et al.*, 2003). Initial nuclear transfer experiments were performed to clone animals by the use of embryonic or later of somatic cells, but to introduce modifications into the genome of the individual of interest, somatic primary cells have to be genetically engineered. This opens the opportunity to introduce a transgene of interest into a mature cell, by viral or non-viral, in turn distinguishable between physical and chemical methods.

2.1.3.1 Genetic engineering of primary somatic cells

SCNT as a tool for the generation of transgenic animal models necessitates the genetic modification of the somatic primary donor cells through the introduction of DNA or RNA by different ways. Viral transgenesis mainly includes the use of retroviral vectors and adeno-associated viruses (AAV). So far, two types of retroviral vectors have been established to generate transgenic animals: prototypic retroviruses such as murine leukemia virus (MLV) and lentiviruses such as human immunodeficiency virus (HIV). One main difference between those two groups of retroviruses is the ability of the lentivirus genome to be directly transported to the nucleus, enabling the transduction of non-dividing cells as well. They offer a large spectrum of different host cells to be infected at different cycle stages, subsequently used for the generation of transgenic animals (Lois *et al.*, 2002; Pfeifer *et al.*, 2002). Also transgenic pigs could be generated with high efficiency by lentiviral vectors (Hofmann *et al.*, 2003; Whitelaw *et al.*, 2004). AAVs, described as single stranded DNA viruses, enable site-directed mutagenesis of the host genome. It was reported that adeno-associated virus-mediated gene targeting has been used to deliver targeting vectors to cell lines and primary cells (Inoue *et al.*, 1999; Porteus *et al.*, 2003). Up to now porcine disease models for cystic fibrosis were generated by Rogers and co-workers using AAVs in concert with SCNT (Rogers *et al.*, 2008). Viral vectors represent a powerful transduction tool, but also exhibit several drawbacks such as their immunogenicity, oncogenic properties, vector inactivation and the need for a relatively large-scale environment for their production, which leads to additional expenditure for the improvement of non-viral gene transfer systems.

Non-viral DNA delivery methods are subdivided in chemical systems, such as lipofection, calcium phosphate precipitation, cationic polymers or molecular conjugates (Azzam and Domb, 2004; Kulkarni *et al.*, 2006) and physical systems, such as gene guns, also known as particle bombardment technique, microinjection, electroporation, sonoporation, laser assisted delivery and magnetofection, (Magin-Lachmann *et al.*, 2004; Andre *et al.*, 2010). Out of these two groups, lipofection and electroporation are the most common techniques used for the transfection of primary cells. In the late 1970s the possibility of a passive encapsulation of DNA into liposomes, preventing its degradation by plasma nucleases, was reported (Hoffman *et al.*, 1978). Felgner and colleagues described the use of cationic liposomes as efficient carriers for intracellular DNA delivery, in which the transfection is mediated by a spontaneous electrostatic interaction between the liposome (positive) and the DNA (negative), resulting in an efficient condensation of the nucleic acids. The liposome/DNA complex (net positive charge) associates with the cell surface (negative charge). Fusogenic properties, accumulated during cationic liposome formulation, enable fusion and/or destabilization of the plasma membrane, alleviating the intracellular release of the complexed DNA (Felgner *et al.*, 1987). Advantages of lipofection over viral transduction are simplicity of production, low toxicity and low immunogenicity (Iversen *et al.*, 2005). Electroporation means the reversible permeabilization of the cell membrane to exogenous DNA in the surrounding media by an exposure of the cell to an electrical field, increasing the efficacy of gene transfer with proliferating cells. Adherent cells have to be detached from their substratum, resulting in unwanted physiological effects undermining cell viability (Neumann *et al.*, 1982). Nucleofection, as advancement of the physical electroporation, is favorable for the transfection of primary cells and mammalian cells, so far considered to be difficult or even impossible to transfect, due to the fact that the substrate is transferred directly into the nucleus, independent from cell division accompanied by nuclear envelope breakdown (Hamm *et al.*, 2002; Maasho *et al.*, 2004). It represents a combination of electrical parameters with cell type specific reagents. Optimal nucleofection conditions are not depending on the transfected substrate but on the cell type to be transfected. This means, as a main advantage, that identical conditions are used for the nucleofection of DNA including BACs, RNA, siRNAs or other biologically active molecules (Maurisse *et al.*, 2010). Comparing the efficiencies of those non-viral DNA delivery methods, of course also depending on cell-type and transfection conditions, stated nucleofection prior to electroporation, followed by lipofection. Nevertheless, cytotoxicity is reported to be higher with nucleofection compared to lipofection (Yanez *et al.*, 1999; Iversen *et al.*, 2005; Jacobsen *et al.*, 2006).

2.1.3.2 Type of modification

The development of SCNT and the different possibilities of introducing DNA into primary cells or preimplantation embryos push the generation of large animal models. The objective of transgenic technology to generate animals exhibiting stable integration of foreign DNA in their germline is achieved, depending on the requirements, by additive or targeted gene transfer. Mice produced by injection of retroviral Simian virus 40 (SV40) DNA were the first animals carrying experimentally inserted genes, although germline transmission, later on shown by transduction of mouse embryos with a Moloney leukemia virus (Jaenisch, 1976) was not achieved with this mouse model (Jaenisch *et al.*, 1974). Viral transduction was also adapted for the production of transgenic pigs (Hofmann *et al.*, 2003; Whitelaw *et al.*, 2004), although the first transgenic pigs were produced by PMI (Brem and Springmann K, 1985; Hammer *et al.*, 1985). In general, the efficiency of PMI is very low, also accompanied by effects of random integration, such as insertional inactivation, varying expression levels due to positional effects or mosaicism of the founder (reviewed in Wolf *et al.*, 2000). By then, genetic modification of domestic animals depend on coincidental events for incorporating exogenous DNA randomly into the genome regarding both, number of integrated copies and location of their integration. Therefore, it is not possible to accurately predict the phenotype of the resulting animals, nor a specific inactivation or modification of genes is feasible (Piedrahita *et al.*, 1999). In 1985, the first ‘planned’ modification of the human *β-globin* gene in murine erythroleukemia cells was achieved (Smithies *et al.*, 1985). Since then, the most examples for targeted modifications of the genome represent an inactivation of specific genes. New findings like selectable markers, enrichment protocols and site-directed recombination systems open a broad range of modification possibilities for specific genes, starting with single base-pair substitutions, conditional and tissue-specific inactivation and gene replacement (Cohen-Tannoudji *et al.*, 1998). The site-directed modification of animal genomes is accomplished either by homologous recombination (HR) events or by non-homologous end joining (NHEJ) using site-directed nucleases (Zinc finger nucleases; ZFNs and transcription activator-like effector nucleases; TALENs) as a tool for the introduction of double-strand breaks (DSBs). In general, HR can be described as a fundamental, regenerative process within living organisms, mostly occurring very rare, requiring a complex set of reactions and extensive homology. HR usually occurs in mammals during the meiotic cleavage of germ cells or can be described as a process driven by the attempt of the endogenous DNA repair machinery to mend DSBs. Furthermore, this mechanism opens the possibility to introduce defined modifications (replacements or deletions) into the genome (Ellis *et al.*, 1989; Court *et al.*, 2002). The regions of homology, necessary for HR, are DNA stretches shared by the two molecules supposed to recombine. Those regions are freely selectable, making it possible to specifically alter any position on a target molecule (Muyrers *et al.*, 1999). For the most cells an up to 1000-fold higher frequency of random (nonhomologous) integrations, mediated by NHEJ occurs (Merrihew *et al.*, 1996; Sargent *et al.*, 1997). Therefore, several strategies to improve the ratio of targeted integrants compared to random integrants came in a tighter focus.

2.2 Increasing the targeting efficiency: vectors for gene targeting

The introduction of defined mutations necessitates the construction of targeting vectors, containing the engineered DNA, as well as DNA sequences up- and downstream of the modification cassette homologous to the target locus. Those stretches of homology enable the tailored integration of the vector construct into the locus of interest by HR. Nevertheless, the frequency of targeted HR events is much lower compared to random integrations of the vector construct throughout the genome in most mammalian cell lines (beyond mouse ESCs), which can necessitate screening thousands of clones representing a very time- and cost-intensive method to find a biallelic targeted gene knock-out (Colosimo *et al.*, 2000; Vasquez *et al.*, 2001). Accumulated evidence indicates that mouse embryonic stem cells exhibit a higher recombinogenic potential compared to somatic cells, whereas the absolute targeting efficiency in mESCs varies from 1×10^{-5} to 1×10^{-6} per electroporated cell compared to a two orders of magnitude lower frequency in somatic cells, for example 2.8×10^{-7} to 2.75×10^{-7} in sheep (Denning *et al.*, 2001). Apparently, this makes it important to trace strategies to increase the frequency of HR events in somatic cell gene targeting (Wang and Zhou, 2003). Comparing experiences using mouse embryonic stem cells for gene targeting emerged several parameters influencing the frequency of HR events. It was stated that an increase of the HR frequency is correlated with the length of the homologous regions of the used targeting vector, mediated by the use of large vector vehicles, such as yeast artificial chromosomes (YACs), P1 artificial chromosomes (PACs) or bacterial artificial chromosomes (BACs) (Hasty *et al.*, 1991; Deng and Capecchi, 1992). Base pair heterologies between vector and target DNA negatively influence the frequency of HR, favoring an isogenic vector construction (te Riele *et al.*, 1992). Additionally, enrichment of the clones by positive and negative marker selection (Izant and Weintraub, 1985; Mansour *et al.*, 1988) optionally combined with Cre/loxP or FLP/FRT systems (Baer *et al.*, 2001) and promoter- or polyadenylation-trap experiments can increase the targeting efficiency remarkably (Karreman, 1998).

2.2.1 Positive and/or negative selection

Targeting vectors generally are classified in two groups: replacement and insertion vectors. The replacement vector is linearized, resulting in a colinearity of the vector sequence with the target sequence. Mediated by the flanking homologous regions a double crossover facilitates the replacement of the chromosomal DNA by the vector sequence. In contrast, the insertion vector is linearized within the homologous region, resulting in a duplication of the genomic sequence after HR. Both vector types were tested targeting *hprt* with the result that the insertion vector targeted up to ninefold more frequently than the replacement vector, with equal length of the homologous sequence (Hasty *et al.*, 1991). Nevertheless, the most widely used targeting vectors are replacement vectors, based on a previous report that the recombination frequencies were comparable to those using insertion vectors (Thomas *et al.*, 1987). Additionally, positive/negative selection is possible using this kind of vector (Mansour *et al.*, 1988). The most common positive selection marker is the aminoglycoside phosphotransferase gene, providing resistance to antibiotics such as kanamycin, geneticin (G418) and neomycin (neo^R). In addition, genes encoding for the hygromycin B phosphotransferase, xanthine-guanine phosphoribosyl transferase, blasticidin-S deaminase or puromycin-N-acetyl-transferase have been applied as well. Most frequently a cassette containing neo^R under the control of a strong promoter, such as the phosphoglycerate kinase (PGK) promoter, is used (Santerre *et al.*, 1984; von Melchner *et al.*, 1992; Ramirez-Solis *et al.*, 1995; Cheah *et al.*, 2001). Superiorly, selection cassettes providing the possibility to be removed by site-specific recombinases, such as Cre, FLPe or ΦC31 (Voigt *et al.*, 2008), after successful integration, are favorable (Birling *et al.*, 2009; Tuntufye *et al.*, 2011). Otherwise the selection cassette might interfere with the expression of surrounding genes, as an example reported by Fiering and co-workers in 1995. A targeted deletion of the 5'-DNase hypersensitive site 2 of the locus control region of the β-globin locus, subsequently replaced by a PGK-neo^R cassette resulted in a markedly reduced globin expression, leading to fetal death of homozygous mutant mice. FLP-mediated excision of the selection cassette restored viability and maintained normal globin expression (Fiering *et al.*, 1995). Positively selected cells reflect the integration of the desired transgene, but not if random or site-directed integration took place. A combined strategy termed 'positive/negative' selection (PNS) was developed in the group of Capecchi to enrich clones which undergone HR, therefore being targeted. A thymidine kinase (*tk*) gene from the herpes simplex virus was inserted at one end of the linearized targeting construct, already carrying a positive selection marker. After treatment with a toxic nucleoside analogue (e.g. gancyclovir) randomly transfected cells were eliminated, whereas correctly targeted cells, that have undergone HR, have lost the *tk* gene. Typically, those strategies result in 3- to 10-fold targeting enrichment (Mansour *et al.*, 1988; Karreman, 1998). The diphteria toxin A (DT-A) fragment and immunotoxin-mediated cell killing have successfully been applied as negative selection (Yagi *et al.*, 1990; Kobayashi *et al.*, 1996).

2.2.2 Gene trapping

Gene trapping is described as a type of insertional mutagenesis through vectors, which render to simultaneously disrupt and report the expression of the endogenous gene. First generation vectors were used to trap actively transcribed genes in undifferentiated ESCs and are subdivided, according to their integration area, in promoter trap and gene trap vectors. The promoter trap strategy enriches intragenic integration events by the use of vectors containing a promoterless selectable marker (e.g. neo^R or β-geo; a fusion of neomycin phosphotransferase and β-galactosidase), which have to be integrated into an exon of a transcriptionally active locus, capturing the promoter of the target cell, to be selectable for neomycin or *lacZ* staining. Though, silent gene loci are missed by this strategy (Skarnes *et al.*, 1992; Friedrich *et al.*, 1993). Gene trap vectors are able to integrate into intronic sequences. At the 5'-end of the reporter gene they carry a splice acceptor (SA) site, which enables the vector to be spliced to the endogenous gene resulting in a fusion transcript. Improving this strategy led to the introduction of an internal ribosomal re-entry site (IRES) between the SA and the reporter gene. On that account, the reporter gene can be translated even without being fused to the trapped gene (Lako *et al.*, 2000). Second generation vectors trap silent loci. Those vectors still carry a promoterless reporter gene with a 5'-SA site, but the antibiotic resistance gene is controlled by a constitutive promoter. As a result, the reporter gene expression is still regulated by the endogenous promoter, whereas the antibiotic selection is independent of the trapped gene (Niwa *et al.*, 1993). Hanson and colleagues reported that a promoterless vector system targeting the *c-myc* gene in a rat fibroblast cell line led to a 5000 to 10000 fold targeting increase (Hanson *et al.*, 1995). Targeted knock-out of the α-1,3-galactosyltransferase gene (*GGTA1)* by a promoterless vector in porcine fibroblasts resulted in an increase in the targeting efficiency compared to PNS (Harrison *et al.*, 2002). An additional strategy to select for intragenic vector integration, applied in mESCs, is indicated by polyadenylation (poly A) trap, in which the mRNA of a selectable marker gene lacking a poly A signal in a gene trap vector is only stabilized when the vector gets a cellular poly A signal (Salminen *et al.*, 1998).

2.2.3 BAC vectors

Beside the possibility to increase the frequency of HR events by selection systems and gene trapping, the increase of the homology regions of the targeting vector represents a promising alternative (Hasty *et al.*, 1991). Facing this task, large vector vehicles for targeting approaches, such as YACs, PACs and BACs came into a tighter focus. Previous studies turned towards the use of modified BACs as targeting vectors for the transfection of murine ESCs (Testa *et al.*, 2003; Yang *et al.*, 2003; Testa *et al.*, 2004; Yang *et al.*, 2005), later on also for human ESCs (Song *et al.*, 2010). Barakat and co-workers developed a new BAC-targeting strategy in mice. The use of a RFLP (restriction fragment length polymorphism) present in genetically polymorphic ES hybrid cell lines, generated by crossing C57BL/6 female mice with Cast/Ei male mice, provide a convenient readout for proper targeting. *Rnf12*, encoding a nuclear factor involved in X chromosome inactivation, was used as the target gene of interest to evaluate the new targeting method. As a result, this strategy is feasible for the introduction of genetic alterations in murine ESCs via BAC-targeting cassettes coupled with a reliable readout method based on allele specific PCR (Barakat *et al.*, 2011).

The BAC system is based on the F (fertility)-factor known from *E. coli* and its replication is strictly controlled (Mori *et al.*, 1986). Usually, BACs are maintained in one or two copies in the bacterial host and are, in contrast to YACs, resistant to mechanical shearing. Additionally, as an advantage in time and costs, it is possible to use conventional plasmid purification protocols for their isolation (Yang *et al.*, 1997; Warming *et al.*, 2005). Compared to conventional targeting vectors, able to carry up to 20 kb genomic inserts, BACs are described as circular molecules that are capable to carry large, regularly 200 to 300 kb, genomic regions of interest (Shizuya *et al.*, 1992). The stability and the large insert capacity opens a broader application range of BACs, such as tools for high-resolution physical mapping, making them the cloning system of choice for constructing physical maps of the human, mouse and pig genome sequencing projects (Anderson *et al.*, 2000; Chung *et al.*, 2004; Humphray *et al.*, 2007). In conclusion, the application area of BACs ranges from the establishment of long-range physical maps to positional cloning disease genes to whole-genome sequencing, as BAC libraries were used for the human genome project (Lander *et al.*, 2001). They are taken as a source of substrates in shotgun sequencing projects, enabling the setup of an end sequence database (Mahairas *et al.*, 1999). Additionally, overlapping clone sets, contigs, are generated by restriction fingerprints (Marra *et al.*, 1997). Those sequence contigs, containing scaffolding information, are mapped to a localized genomic region after a direct genomic shotgun sequencing approach (Hoskins *et al.*, 2000). Finally, BACs are used to increase the HR frequency in targeting approaches using ESCs to establish various mouse models (Valenzuela *et al.*, 2003; Yang and Gong, 2005; Barakat *et al.*, 2011). Effective targeting efficacies of up to 28% have been observed (Yang and Seed, 2003).

Furthermore, to enable the use of BACs in gene targeting approaches, novel recombination tools using bacterial enzymes have been established. Those facilitate the modification of any DNA region of interest, allowing the introduction of desired mutations into BACs independently of restriction sites for cloning (Wang *et al.*, 2009). The conventional method of construct design, using restriction enzymes and DNA ligase to cut and rejoin DNA, is often limited by the availability of the appropriate restriction enzyme sites for the respective cloning steps. This problem appears in the vector construct as well as in the genomic DNA quite frequently, since the vector and the genomic target site contain hundreds of kilobases, as present in BACs. Additionally, the PCR amplification of DNA fragments for cloning, prevalently used in traditional approaches, proved to be relatively fault-prone also limited by the length of the desired amplificate (Zhang *et al.*, 1998; Muyrers *et al.*, 1999; Copeland *et al.*, 2001; Muyrers *et al.*, 2001). In *E. coli*, the endogenous recombination mechanisms are initiated by the cooperation between RecA (strand invasion protein) and RecBCD (exonuclease) and need linear dsDNA to start the recombination machinery (Murphy, 1998). An alternative recombineering approach was developed independent of RecA, instead using phage-derived, functionally and operationally equivalent, protein pairs: RecE/RecT from the Rac phage or Redα/Redβ from λ-phage. This recombinogenic engineering strategy was also termed ET recombination (ET cloning) (Zhang *et al.*, 1998), λ-mediated recombination (Yu *et al.*, 2000) or GET recombination (Nefedov *et al.*, 2000). The interaction between RecE/Redα, encoding 5‘-3‘ exonucleases, and RecT/Redβ, representing DNA annealing proteins, is necessary for the initiation of HR (Kolodner *et al.*, 1994). The need of using linear targeting DNAs in ET recombination requires the endogenous

RecBCD endonuclease activity to be absent or silenced (Murphy, 1991). It was reported that in ET cloning only short homology regions of 35-60 nucleotides are sufficient for HR events, simply amplified by oligonucleotide synthesis. Combining those regions of homology with a selectable gene, there is even no need for the construction of targeting plasmids, because linear PCR products can be used (Narayanan *et al.*, 1999). In comparison to RecA approaches, it was noted that the recombination efficiencies using the RecE/RecT and Redα/Redβ protein pairs are higher, indicated by 80% correctly modified candidates. The identification of correct clones can be performed by employing antibiotically selectable genes, or even more simply, by conventional PCR screening (Muyrers *et al.*, 2000). Those strategies, of course, open the way to easily modify any DNA of interest. Moreover, it represents a time-saving and easy-to-handle methodology to modify BACs, subsequently used as targeting vectors to establish animal models.

However, there is still one important point to be kept in mind. Targeting vectors in their simplest form consist of a gene for drug selection flanked by homologous arms enabling HR in the target sequence. If BAC vectors are used for targeting approaches, conventional screening methods such as long-range PCR, utilizing one primer outside of the construct in conjugation with one primer present on the selectable marker (Lay *et al.*, 1998), or Southern blotting, which usually identifies correctly targeted clones and permits the verification of a single-copy insertion of the construct, are not feasible. To bypass these problems alternative screening methods are necessary. These include: (i) to permit Southern blotting, one short arm on the targeting construct has to be designed (Testa *et al.*, 2003), (ii) to detect the number as well as the chromosomal localization of integrated BAC sequences, fluorescent in-situ hybridization (FISH) is used (Yang and Seed, 2003) and (iii) via quantitative real-time PCR it is possible to screen for the loss of sequences, deleted in the targeting construct (Valenzuela *et al.*, 2003) also described as the ‘loss of wild-type allele’ (LOWA) assay (Frendewey *et al.*, 2010).

2.2.4 Site-directed nucleases

DNA double strand breaks (DSB) in mammalian cells can be repaired via two distinct mechanisms: (i) homologous recombination (HR) and (ii) nonhomologous end-joining (NHEJ). Both pathways are highly conserved from yeast to vertebrates. However, HR plays a major role in any DSB repair in yeast, whereas in vertebrates NHEJ contributes more frequently to DSB repair, resulting in an imperfect repair process often leading to changes to the DNA sequence at the site of DSB (Sonoda *et al.*, 2006). In mammalian systems, as represented in Chinese hamster ovary cells, a 9:13 ratio between HR and NHEJ has been reported (Santiago *et al.*, 2008). An additional possibility to increase the targeting efficiency is represented by the volitional introduction of artificial DSBs at a desired locus to be targeted by site-directed nucleases, inducing HR.

Initial experiments in mouse ESCs, using the homing nuclease I-SceI to induce DSB, stimulates the gene targeting at a selectable *neo* locus enormously (at least 50-fold). But due to the fact that a specific recognition sequence for I-SceI has to be introduced to the target locus to facilitate its function, other nuclease types were favored for gene targeting approaches (Smih *et al.*, 1995; Taghian *et al.*, 1997). Zinc-finger nucleases, first reported in 1996 by Kim and colleagues (Kim *et al.*, 1996), are described as hybrid molecules composed of a specifically designed polymeric zinc finger domain, recognizing the desired DNA target sequence, and a FokI nuclease cleaving domain (Carroll, 2011). This newly developed technology enables the design of Zn-finger proteins theoretically recognizing any 18 bp target sequence, which is long enough to address a unique target within the mammalian genome (Mani *et al.*, 2005; Remy *et al.*, 2010). The endonuclease activity of FokI is dependent of DNA binding, which is only achieved by dimerization of the FokI domain. During cleavage of double-stranded DNA, a pair of hybrids, consisting of a Zn-finger and nuclease domain bind simultaneously to the DNA. The subsequent DNA scission by the dimeric FokI nuclease occurs without any site-specificity. A transient expression of ZFNs in cells leads to a site-specific DSB in the endogenous target gene. This DSB is most likely repaired via NHEJ, creating not predetermined mutations that might cause gene disruptions (Santiago *et al.*, 2008; Katada *et al.*, 2009). Additionally, it was recently reported by Olsen and colleagues that the formation of site-specific DSB by ZFN increases the rate of HR between a specific genomic target and a donor plasmid (Yan *et al.*, 2009) opening the possibility of gene correction indicating one more step towards gene therapy (Olsen *et al.*, 2010).

Recently, several groups described possibilities to engineer DNA-binding specificities based upon transcription activator-like effector (TALE) proteins from *Xanthomonas* plant pathogens (Moscou *et al.*, 2009; Boch *et al.*, 2010). Within their structure, a central repeat domain is required for DNA recognition. This domain consists of repeat units with 33-35 amino acids each specifying one target base. Two critical, adjacent amino acids within one repeat, called ‘repeat variable di-residue’ (RVD), mediate the base preference of each unit. Due to the appearance of the preferred binding site of the TALE and its specific RVD, it is possible to predict some kind of code, in which each repeat is specifying its targeted base (Boch *et al.*, 2009). These investigations led to the establishment of TALE-nuclease chimeras (TALENs) as site-specific endonucleases for selective genome cleavage (Bogdanove *et al.*, 2011). Those truncated TALE variants linked to the catalytic domain of FokI, were able to modify the endogenous human genes *NTF3* and *CCR5*, indicating that TALEN architectures are able to efficiently modify the genome of mammalian cells at an endogenous locus by NHEJ (Miller *et al.*, 2011).

2.3 Site-directed modification of two different porcine loci

The techniques for targeted modifications of desired genes were established in the mouse during the 1980s (reviewed in Capecchi, 1989), including gene knock-outs in specific tissues (Chapman *et al.*, 1999) and single-base pair introductions into specific genes (Dickinson *et al.*, 2000). It was possible to target endogenous genes by HR in pluripotent ESCs. After reimplantation into the early embryo, chimeric animals have been generated, some of which exhibited germline transmission of the modification, subsequently used to generate mouse strains carrying the knock-out allele by breeding. Until now this technology is still not transferable to livestock due to the lack of germ line-competent ESCs (Stice *et al.*, 1998) or other pluripotent stem cells. In the first transgenic livestock, produced by SCNT as an alternative strategy to ESCs, the ovine collagen gene was replaced by an expression cassette, targeting the expression of human factor IX to the mammary gland (McCreath *et al.*, 2000). More recently, pigs with an engineered deletion of the α-1,3-galactosyltransferase (*GGTA1*) gene, determining a cell-surface xenoepitope, have been generated (Denning *et al.*, 2001; Lai *et al.*, 2002). This epitope plays a key role in hyperacute rejection (HAR). Organs and tissues of pigs lacking the *GGTA1* gene, not expressing this epitope have a reduced HAR response after transplantation to humans, making these animals an interesting source for xenotransplantation. In 2008 Rogers and co-workers reported the production of porcine animal models for cystic fibrosis, by targeting the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene. Those animals represent expedient tools for a better understanding of this hereditary disease and to develop new prevention and treatment strategies (Rogers *et al.*, 2008).

2.3.1 Targeted knock-out of *CFTR*: a disease model for cystic fibrosis

2.3.1.1 Cystic fibrosis

Cystic fibrosis (CF) is one of the most common, genetically inherited disorders with recessive outcome among Caucasians (Ren, 2008). It was first described as an independent disease in 1938 by Andersen and colleagues, who investigated autopsy studies from malnourished infants (Andersen, 1938). In her publication ‘Cystic fibrosis of the pancreas and its relation to celiac diseases’ she was able to distinguish a disease, developing mucus plugging of the glandular ducts, termed ‘cystic fibrosis of the pancreas’, from other celiac syndromes (Davis, 2006). Additionally, the disease also was known as ‘generalized exocrinopathy’ because many exocrine glands were affected. This syndrome was characterized by fat and protein malabsorption due to pancreatic damage and a lack of pancreatic enzyme secretion, steatorrhea, growth failure and pulmonary infection, often representing the terminal event (Kreindler, 2010). Studies on the basic defect, at that time, usually focused on the abnormalities of mucus. One decade later, in 1951, Kessler and Anderson developed the connection between salt transport and cystic fibrosis of the pancreas and supported the hypothesis that ‘fibrocystic disease is associated with widespread abnormality of epithelial glands’ (Kessler *et al.*, 1951). In 1953, di Sant’Agnese postulated that Na^+ and Cl^- levels in the sweat of CF patients were markedly elevated, not representing the consequences of pancreatic dysfunction, pulmonary disease adrenal dysfunction or renal disease, but the elevated susceptibility to dehydration in CF was due to increased salt loss from the sweat glands (Di Sant'Agnese *et al.*, 1953). These findings resulted in the development of a diagnostic tool for CF, the sweat test (Gibson *et al.*, 1959). In the 1980s, by investigating sweat glands, pancreas and pulmonary tract, it became more and more obvious, that CF was a disease of altered anion transport. In 1988 Kopelman and co-workers reported that abnormal pancreatic secretion in CF could mainly be attributed to an altered Cl^- secretion, opening the door for researchers to find the affected gene (Kopelman *et al.*, 1988). In 1985, it was possible to localize the gene by linkage analysis to the long arm of chromosome 7 (Knowlton et al., 1985). Furthermore, more than 20 years ago, in 1989, Kerem and colleagues were able to identify the causative gene for CF called CF transmembrane conductance regulator (*CFTR*) (Kerem *et al.*, 1989).

2.3.1.2 *CFTR*: genetic structure and function

The *CFTR* gene encodes a 250 kb long, consisting of 1480 AA, protein with a molecular weight of 168 kDa, which acts as an apically localized chloride channel, mainly anchored to the outer membrane of epithelial cells of the sweat glands, pancreatic duct, airway, skin, intestine, biliary tree and vas deferens. Defects in the causative gene for CF lead to consequences like elevated sweat chloride concentration, lung disease, pancreatic insufficiency, intestinal obstruction, biliary cirrhosis, and congenital bilateral absence of the *vas deferens* (Davis, 2006). However, the most severe impacts caused by a defective CFTR protein are observed in the lung and the pancreas. The main function of the cAMP-mediated chloride channel, encoded by the *CFTR* gene, is the regulation of the ion and water homeostasis across epithelia. CFTR is a member of the ATP-binding cassette superfamily of proteins. Those transporters usually consist of two highly hydrophobic transmembrane domains, each of them containing six membrane-spanning segments, and two hydrophilic nucleotide binding domains. CFTR contains one additional, highly charged, so called regulatory domain (R-domain), responsible for the regulation of the channels function (Higgins, 1992).

2.3.1.3 *CFTR* mutations

Up to now 1900 mutations (according to the Cystic Fibrosis Mutation Database; CFMDB, 2011) are known, which proved to be responsible for the development of this monogenic disease. The most common among them is a deletion of a nucleotide triplet encoding a phenylalanine, called ΔF508. It accounts for almost 70% of all mutant *CFTR* alleles (Riordan, 2008). Based on their influence on the CFTR protein the different mutations are grouped in five distinct mutation classes including (i) the presence of large deletions and STOP-codons, both mostly leading to truncated and non-functional protein, (ii) mutations (e.g. ΔF508) affecting the post-translational folding of CFTR, (iii) mutations resulting in full-length protein, being incorporated into the cell membrane, but presenting a defective regulation impairing the channel gating function (iv) mutations leading to defective chloride conductance and (v) transcription dysregulations, ending in decreased amounts of functional CFTR (Proesmans *et al.*, 2008). Focusing on the pulmonary phenotype, a non-functional protein leads to an imbalance of the ion and water regulation in epithelia, resulting in increasing viscosity of the exocrine secretion with the consequences of ciliary dysfunction, mucus impaction and chronic endobronchial infections in the lung, representing the most common and severe phenotype of CF (Ren, 2008; Widdicombe, 2010).

2.3.1.4 CF animal models

For the development of curative therapies for CF, making one step towards the establishment of gene therapy approaches, it is inevitable to focus on the generation of expedient animal models. Carvalho-Oliveira and colleagues (2007) showed an overview of mouse models for CF, reflecting different mutation strategies. Surprisingly, all of those models lack the prominent lung phenotype (referring to Davidson *et al.*, 2001; Carvalho-Oliveira *et al.*, 2007). Guilbault and colleagues (2007) explained this fact by the decreased amount of mucous glands generally contained in mouse airways (Guilbault *et al.*, 2007). The differential expression of *CFTR* and CaCCs (Ca^{2+}-activated chloride channel) as well as the longevity, size of airways and immunology, may contribute to the differences in the lung disease characteristics between mice and humans. As a consequence, other animal models, which are able to reflect all aspects of this very complex disease were required, leading to the generation of *CFTR*-deficient ferrets (Sun *et al.*, 2008) and pigs (Rogers *et al.*, 2008), at least exhibiting a similar airway gland situation compared to human. The group around M. J. Welsh and R. S. Prather generated *CFTR*-null and *CFTR*-ΔF508 hetero- and homozygous pigs by using adeno-associated virus gene targeting in combination with SCNT (Rogers *et al.*, 2008; Welsh *et al.*, 2009). The *CFTR*-null and *CFTR*-ΔF508 heterozygous piglets were generated by integration of a neomycin-resistance cassette via homologous recombination to disrupt exon 10 of the porcine *CFTR* gene. The insertion of the cassette also introduced a premature STOP-codon at position 508, whereby the production of a functional protein is inhibited. Using a similar strategy, the ΔF508-mutation was mimicked by eliminating 3 bp, encoding Phe508 in the porcine exon 10. In humans, this mutation disrupts processing of the protein, resulting in the retention of nearly all human *CFTR*-ΔF508 in the ER (endoplasmic reticulum) followed by its degradation. Surprisingly, some porcine *CFTR*-ΔF508 is able to escape the ER retention, traffics to the Golgi apparatus, meaning there is still some *CFTR* activity left in contrast to human *CFTR* carrying the ΔF508 mutation (Rogers *et al.*, 2008). All *CFTR*$^{-/-}$ newborn piglets exhibited the earliest manifestation of CF in humans, a meconium ileus. In humans, around 15% of all CF infants exhibit this intestinal obstruction, whereas the penetrance of meconium ileus in *CFTR*$^{-/-}$ piglets was 100%. The occurrence of pancreatic insufficiency in *CFTR*$^{-/-}$ piglets was comparable to the human phenotype (Wine, 2010). Further studies showed other significant aberrations present at birth, including degenerative changes of the pancreas, focal biliary cirrhosis in the liver and gall bladders smaller than usual and often filled with mucus and congealed bile (Rogers *et al.*, 2008). Additional studies revealed an increase in mucus cells in the pancreas, liver, gall bladder, intestine and cystic duct (Meyerholz *et al.*, 2010). In another study, *CFTR*$^{\Delta F508/\Delta F508}$ pigs developed lung disease exceedingly similar to the human phenotype, indicating that the limited *CFTR* activity is not sufficient to prevent lung as well as gastrointestinal disease in CF (Ostedgaard *et al.*, 2011). Stoltz and colleagues investigated the progress of CF lung disease in *CFTR*$^{-/-}$ pigs, which were generated by breeding. They reported that homozygous knock-out pigs spontaneously develop the pulmonary CF phenotype, largely replicating that observed in human, reflecting the main features of inflammation, remodeling, mucus accumulation and infection (Stoltz *et al.*, 2010). Although it is not obvious whether homozygous *CFTR* knock-out pigs or humans develop more severe lung disease, the characteristic hallmarks of lung disease in both species are comparable and differ from the mouse. Those results also indicate that the pulmonary consequences of *CFTR* loss are not unique to primates (Welsh *et al.*, 2009).

The fact that the targeted disruption of the porcine *CFTR* gene reflected several of the major phenotypes known from CF-patients in pigs, opens the door for investigators to better understand this hereditary disease and to develop new prevention and treatment strategies (Mogayzel *et al.*, 2010).

2.3.2 Targeting of *GGTA1*: organ resources for xenotransplantation

2.3.2.1 Xenotransplantation: background

Currently, 11594 patients (evaluated by the Eurotransplant-International-Foundation, 2010) are registered on the active waiting list for donor transplants (including kidney, heart, liver, lung, pancreas), facing the lack of improvement in the number of deceased human organs that become available each year and restrict allotransplantation. Therefore, cross-species transplantation would offer the prospect of an unlimited supply of organs, tissue and cells for clinical transplantation. The pig as a main candidate for xenotransplantation of solid organs will only ever become reality in clinical application with genetic modifications of the porcine genome, to overcome hyperacute, acute antibody-mediated or cellular rejection (reviewed in Pierson *et al.*, 2009). Additionally, other issues also have become very prominent, such as development of thrombotic microangiopathy in the graft or systemic consumptive coagulopathy in the recipient (Bach *et al.*, 1994; Gollackner *et al.*, 2004). Hence, to address these problems, pigs that express one or more human thromboregulatory or anti-inflammatory genes, such as human thrombomodulin or human CD39 are being established (Petersen *et al.*, 2009; Le Bas-Bernardet *et al.*, 2011). Nevertheless, the results of preclinical transplantation of porcine cells, such as pancreatic islets (van der Windt *et al.*, 2009), neuronal cells (Lim *et al.*, 2010), hepatocytes (He *et al.*, 2011), or corneas (Choi *et al.*, 2011) are as well very encouraging, with survival times greater than one year in all cases.

Xenotransplantation (XT) is described as the transplantation of living cells, tissue or organs (so called xenografts or xenotransplants) from one species to another. One major obstacle in this field of research is the lack of a readily available source of such xenografts for transplantation, which would decrease the availability imbalance of donated human organs and the demand for transplantation (reviewed in Cooper, 2012). In general, although non-human primates are phylogenetically closely related to humans, they exhibit several disadvantages when used as organ source for xenotransplantation: (i) there is still little experience in breeding these animals in captivity, (ii) breeding would be cost-intensive, (iii) they have a relatively long generation period accompanied by small litter sizes, (iv) many considerable species are known as endangered, (v) there is little knowledge about the possibility to genetically modify these species and (vi) the very close relation between non-human primates and human, brings up moral and ethical concerns and the transmission of inter-species infections may arise more likely (reviewed in Dooldeniya *et al.*, 2003).

2.3.2.2 The pig as a donor animal for xenotransplantation

The pig represents a suitable source as xenograft donor, due to its similarities in size, physiology, anatomy, metabolism, pathology, organ development and disease progression to human (Rieben *et al.*, 2005; Sachs *et al.*, 2009), not to forget that SCNT works quite well in pigs (Lagutina *et al.*, 2007). The main focus of porcine organs used for XT lies on pancreatic islets (Hering *et al.*, 2006), kidneys (Yamada *et al.*, 2005), heart (Kuwaki *et al.*, 2005) and liver (Nagata *et al.*, 2007). It just has to be taken into account that there indeed is a risk of transmission of infectious agents from pig to humans. The use of designated pathogen-free (DPF) animals counteracts the impact of exogenous agents. In contrast, porcine endogenous retroviruses (PERVs), known as vertically transmitted proviruses, are present in all pig breeds, and they have the potential to infect human cells (Dieckhoff *et al.*, 2009). Hence, several approaches to compass this infectious risk were developed, including introduction of siRNAs/shRNAs targeting highly conserved PERV sequence regions (Dieckhoff *et al.*, 2008).

2.3.2.3 Xenograft rejection and *GGTA1*

The major problem of immunological xenograft rejection still remains. There are different types of rejection mechanisms known, following the generally accepted nomenclature including HAR (hyperacute rejection), AVR (acute vascular rejection) with its major component AHXR (acute humoral xenograft rejection) and chronic rejection (Schuurman *et al.*, 2002). An important step forward to circumvent this problem was the finding that the rejection mechanism is started by HAR and AHXR, which attack vascularized organs from pigs, after transplantation to primates, within minutes or days, respectively (Miyagawa *et al.*, 2010). HAR is mediated by ‘natural’ antibodies directed against a carbohydrate epitope, Galα1-3Galβ1-4GlcNAc (α-1,3-Gal), synthesized by the enzyme α-1,3-galactosyltransferase (α-1,3GalT), encoded by *GGTA1* (Yang *et al.*, 2007). *GGTA1* encodes a 371 amino acid protein, belonging to the family of glycosyltransferases 6, which catalyzes the terminal step in biosynthesis of the α-Gal-epitope. This epitope is expressed on the cell surface of all mammalian species, including pigs, but not in catarrhine primates, as Old World monkeys and Apes (including human). Those species produce high concentrations of anti-αGal antibodies, subsequently mediating HAR by the activation of the complement system and the coagulation cascade, when organs from α-Gal-positive species are transferred to α-Gal-negative species (Koike *et al.,* 2007; Puga Yung *et al.,* 2009). Therefore, the major focus in XT-research lies on the development of strategies to prevent HAR in a first step. Thus, absorbing α-1,3-Gal-specific antibodies from the recipients' blood with α-1,3-Gal antigen, the depletion or total inactivation of the complement, as well as the overexpression of α-2,3-siasyltransferase or α-1,2-fucosyltransferase to compete with *GGTA1* (Dai *et al.*, 2002), are taken into account to overcome this hurdle. As a self-evident solution, genetically modified pigs lacking a functional *GGTA1* expression have been generated (Lai *et al.*, 2002; Phelps *et al.*, 2003). The elimination of *GGTA1* prevented HAR after transplantation of pig hearts into baboons and extended the survival of the graft up to 2-6 months (Kuwaki *et al.*, 2005). Additionally, pigs deficient for *GGTA1* in combination with the overexpression of complement regulatory proteins, e.g. human decay-accelerating factor (hDAF) (Zaidi *et al.*, 1998) or CD59 (protectin) (Niemann *et al.*, 2001) have been developed. A promising strategy for the generation of *GGTA1* knock-out pigs, resulting in a very low level of inbreeding is achieved by the combination of SCNT with heterozygous somatic cells for αGal and crossbreeding of the resulting animals (Nottle *et al.*, 2007). This genetic background subsequently can be used to be combined with other candidate transgenes to develop multi-transgenic pigs required for clinical xenotransplantation (Klymiuk *et al.*, 2010).

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Apparatuses

Accu-jet® pro pipette controller	Brand, Wertheim
Agarose gel electrophoresis chamber	MWG Biotech, Ebersberg
Analytical balance	Sartorius, Göttingen
Gel documentation system	BioRad, Munich
Heating plate with magnetic stirrer	KA-processequipment, Staufen
Hybridization oven	H. Saur, Reutlingen
Microwave	Severin, Sundern
MS1 minishaker	IKA-process equipment, Staufen
Pipetman (1000, 200, 20)	Gilsen, Middleton
Pipettes (1000, 200, 100, 20, 10)	Eppendorf, Hamburg
pH-meter	WTW, Weilheim
Rolling device	Heidolph, Schwabach
Shaking-incubator 3031	GFL, Burgwedel
Thermomixer 5436	Eppendorf, Hamburg
UV-crosslinker	Biometra, Göttingen
Waterbath WB6 Medingen	Störk-Tronic, Stuttgart

Centrifuges

Sorvall RC5C plus	Thermo Scientific, Dreieich
Table centrifuge 5415D	Eppendorf, Hamburg
Table centrifuge 5810R (cooling)	Eppendorf, Hamburg

Thermocycler

ABIPrism 7000	Applied Biosystems, Weiterstadt
Biometra Uno Thermoblock	Biometra, Göttingen
GeneAmp PCR System 9700	Applied Biosystems, Weiterstadt

3.1.2 Consumables

Centrifugation tubes	Herolab, Wiesloch
Micro Amp^{TM} optical adhesive film	Applied Biosystems, USA
Micro Amp^{TM} optical 96-well reaction plate	Applied Biosystems, USA
Parafilm®	American Can Company, Greenwich
PCR reaction tubes (0.2 ml)	Life Science Brand, Wertheim
Pipettetips	Eppendorf, Hamburg
Pipette filter tips	Axygen, California, USA
Reaction tubes	Eppendorf, Hamburg
Tubes (50 ml, 15 ml)	Greiner, Frickenhausen

3.1.3 Chemicals

Acetic acid (glacial acetic acid)	Roth, Karlsruhe
Adenosine triphosphate	Sigma-Aldrich, Deisenhofen
Agar, granulated	Difco, Detroit, USA
Agarose	Invitrogen, Karlsruhe
Agarose ultra pure	Invitrogen, Karlsruhe
Ampicillin	ApliChem, Darmstadt
Bacto^{TM} Trypton	BD, Heidelberg
Bacto^{TM} Yeast Extract	Difco, Detroit, USA
Calcium chloride	Sigma-Aldrich, Deisenhofen
Chloramphenicol	Sigma-Aldrich, Deisenhofen
Chloroform	Merck, Darmstadt
Dithiothreitol (1 M)	Invitrogen, Karlsruhe
EDTA	Merck, Darmstadt
Ethanol	Roth, Karlsruhe
Ethidiumbromide (solution: 1%)	Merck, Darmstadt
D-(+)-Glucose	Sigma-Aldrich, Deisenhofen
Glycerol	Roth, Karlsruhe
Hydrochloric acid	Roth, Karlsruhe
IPTG (Isopropyl-beta-thio galactopyranoside)	Invitrogen, Karlsruhe
Kanamycin	Roth, Karlsruhe
Magnesium chloride (15 mM)	Qiagen, Hilden

Potassium acetate	Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Potassium hydrogen phosphate	Merck, Darmstadt
2-Propanol	Merck, Darmstadt
Saccharose	Roth, Karlsruhe
Sodium chloride	Merck, Darmstadt
Sodium dodecyl sulphate	Roth, Karlsruhe
Sodium hydroxide (platelets)	Roth, Karlsruhe
Tris	Roth, Karlsruhe
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Invitrogen, Karlsruhe

3.1.4 Enzymes, kits and other reagents

dNTPs (dATP, dCTP, dGTP, dTTP)	Fermentas, St. Leon-Rot
E.Z.N.A. Tissue DNA Mini Kit	Omega bio-tek, Norcross, USA
Gene Ruler™ (1 kb DNA ladder)	Fermentas, St- Leon-Rot
Geneticin (G418)	Invitrogen, Karlsruhe
Gentra Puregene Cell Kit	Qiagen, Hilden
MAXWELL®16 Cell LEV DNA Purification Kit	Promega, Mannheim
nexttec™ DNA Isolation clean columns	nexttec, Leverkusen
Nucleospin® Tissue Kit	Macherey-Nagel, Dueren
peqGOLD MicroSpin Tissue DNA Kit	Peqlab, Erlangen
Proteinase K (20 mg/ml)	Roche, Mannheim
pUC mix marker 8	Fermentas, St. Leon-Rot
QIAamp DNA Micro Kit	Qiagen, Hilden
QIAgen DNA Maxi Kit	Qiagen, Hilden
QIAexII Gel Extraction Kit	Qiagen, Hilden
QIAgen DNeasy® Blood & Tissue Kit	Qiagen, Hilden
Restrictionenzymes and -buffers	Fermentas, St. Leon-Rot
Restrictionenzymes and -buffers	New England Biolabs, Boston, USA
Ribonuclease A (RNase A; 0.2 U/µl)	Applied Biosystems, Weiterstadt

Spermidine	Sigma-Aldrich, Taufkirchen
SYBR® Green PCR Master Mix	Applied Biosystems, Weiterstadt
Taq Polymerase	Agrobiogen, Hilgertshausen
T4 DNA Ligase (2000 U/µl)	Fermentas, St. Leon-Rot
UNG (uracil-N-glycosylase)	Applied Biosystems, Weiterstadt
Wizard® genomic DNA-Purification Kit	Promega, Mannheim
10x ligation buffer	Fermentas, St. Leon-Rot
10x PCR buffer	Agrobiogen, Hilgertshauesen

3.1.5 Buffers, media and solutions

Unless otherwise noted, in a Millipore machine deionized water termed aqua bidest was used as solvent.

Chloroform-isoamylalcohol (CiA)

96 ml chloroform
4 ml isoamylalcohol
Stored at 4°C protected from light

DNA loading buffer (10x)

10% glycerol in aqua bidest
1 spatula tip of bromophenolblue (BPB)
Add 0.5 M NaOH until color turns blue
Stored at 4°C

DNA molecular weight standards

100 µl of 1 kb DNA ladder standard
100 µl 6x loading dye
400 µl aqua bidest
Stored at -20°C

dNTP-mix

2 mM dATP, dCTP, dGTP, dTTP
In aqua bidest
Stored at -20°C in suitable aliquots

Kawasaki buffer

20 mM Tris-HCl (pH 8.3)

1.5 mM $MgCl_2$

25 mM KCl

0.05% Tween 20

Aqua bidest

LB medium

5 g yeast extract

10 g peptone/tryptone

5 g NaCl

Ad 1000 ml aqua bidest

pH 7.0 (adjust with 5 M NaOH)

Autoclave

Medium was stored at room temperature

LB-Agar

2.5 g yeast extract

5 g peptone/tryptone

2.5 g NaCl

Ad 500 ml aqua bidest

pH 7.0 (adjust with 5 M NaOH)

7.5 g agar-agar

Autoclave

Keep liquid at 60°C

Add 500 µl respective antibiotic (ampicillin 50 mg/ml, chloramphenicol 12.5 mg/ml, kanamycin 25 mg/ml)

Pour into sterile 10 cm culture dishes (afterwards stored at 4°C)

Lysisbuffer for DNA isolation (high-salt precipitation)

100 µl PK buffer (10x)

10 µl 10% (w/v) SDS

4.4 µl DTT (1M)

Lysisbuffer for DNA isolation (PCiA extraction)

160 mM saccharose

80 mM EDTA pH 8.0

100 mM Tris/HCl pH 8.0

0.5% (w/v) SDS

Lysisbuffer for DNA isolation (spermidine-method; cutting buffer)

2.5 ml 1 M Tris/HCl pH 8.0

5.0 ml 0.5 M EDTA

1.0 ml 5 M NaCl

250 µl 1 M DTT

127 µl spermidine (500 mg/ml)

Ad 50 ml aqua bidest

Stored at 4°C

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 (10x)

200 mM Tris

1 M NaCl

40 mM EDTA

Stored at room temperature

Plasmid A

50 mM glucose

25 mM Tris/HCl pH 8.0

10 mM EDTA/NaOH pH 8.0

Stored at room temperature

Plasmid B

0.1 M NaOH
0.5% (w/v) SDS
Prepared freshly before use

Plasmid C

3 M KOAc
pH 4.8 (adjust with 9 M HOAc)
Autoclave
Stored at room temperature

Proteinase K (20mg/ml)

Stored at 4°C

RNaseA (20mg/ml)

Stored at -20°C

SOC medium (500 ml)

2.5 g yeast extract
10 g peptone/tryptone
0.25 g NaCl
9.32 g KCl
pH 7.0 (adjust with 5 M NaOH)
Autoclave
add 2 M $MgCl_2$ (final conc. 10 mM)
add 1 M glucose, (final conc. 20 mM)
Stored at room temperature

STE

10 mM Tris/HCl pH 8.0
100 mM NaCl
1 mM EDTA/NaOH pH 8.0
Stored at room temperature

TAE (50x)

242g Tris

100 ml 0.5 M EDTA (pH 8.0)

57 ml AcOH conc.

Ad 1000 ml aqua bidest

T-buffer

10 mM Tris/HCl

Adjust to pH 8.0 with HCl

Stored at room temperature

Tbf I (250ml)

30 mM KOAc pH 6.0

100 mM $CaCl_2$

15% (w/v) glycerol

Autoclave

Add 1 M $MnCl_2$ (final conc. 50 mM)

Stored at 4°C

Tbf II (20ml)

10 mM MOPS pH 7.0

75 mM $CaCl_2$

10 mM KCl

15% (w/v) glycerol

Autoclave

Stored at 4°C

TYM medium

5 g yeast extract

20 g peptone/tryptone

0.1 M NaCl

Autoclave

Add 2 M $MgCl_2$ (final conc. 10 mM)

Ad 1000 ml aqua bidest

Stored at room temperature

3.1.6 Plasmids and BACs

3.1.6.1 Plasmids

pGEM® T-Easy Vector System	Promega, Mannheim
CloneJET™ PCR Cloning Kit	Fermentas, St. Leon-Rot
pSV-β-galactosidase Control Vector	Promega, Mannheim
pBluescript KSII	Stratagene, La Jolla, USA
pBS302	kindly provided by Brian Saur
PL452	kindly provided by Neil Copeland
pPNTlox^2	kindly provided by Ingeborg Klymiuk
pPNT4	Conrad M. et al. (2003)
bGHpA	kindly provided by Marlon Schneider

3.1.6.2 BACs

CH242-248P18	BACPAC Resource, Chori, USA
CH242-21F3	BACPAC Resource, Chori, USA
CH242-372F22	BACPAC Resource, Chori, USA
PigI-170I3	BACPAC Resource, Chori, USA

3.1.7 Bacterial strains

DH10B	New England Biolabs, USA
SW106	NCI Frederick, USA
TOP10	Invitrogen, Karlsruhe

3.1.8 Software

Abi Prism 7000	Applied Biosystems, Weiterstadt
BioEdit Sequence Alignment Editor	Ibis Bioscience, USA
Double Digest ™	Thermo Fisher Scientific, USA
FinchTV Version 1.3.1	Geospiza Inc., USA
Macromedia Freehand MX	Adobe, USA
NEBcutter V2.0	New England Biolabs, USA
Primer Express® Software v2.0	Applied Biosystems, Weiterstadt

3.1.9 Oligonucleotides

Oligonucleotides were either designed by hand or with the Primer Express® Software v2.0.

3.1.9.1 Oligonucleotides for qPCR

ACTB1059fw	5’-CCACAGCGGAAGCTCAGTC-3’
ACTB1219rev	5’-CTGGGTACATGGTGGTGCC-3’
ACTB237fw	5’-TCTCCTTTGGAACTCTGCC-3’
ACTB390rev	5’-TTTACGGCAGCCTCGTCG-3’
pACTB129f	5’-CCCAGGTCAGTGGCCCACTG-3’
pACTB1686r	5’-CGCCCTAGATGCATGCTCGA-3’
pACTB100f	5’-GAACCCCAAAGCCAACCGTG-3’
pACTB1760r	5’-CGCACACCGGCCTTATTCCA-3’
CFTR1772f	5’-GACAGTACTGCTTAGTGGTCAG-3’
CFTR2060r	5’-GGTACAGGGAGTTGTAAAGACTG-3’
CFTR986f	5’-CCACCGAATCAGCATACTTAGG-3’
CFTR1132r	5’-TTAGCACCTGAGCTCTATCC-3’
CFTR6752fw	5’-AAGGGAGGCTCGGGACTG-3’
CFTR7118rev	5’-GAGAAGATGCTGGCCTTTTCC-3’
CFTR6822fw	5’-GGAGAAAGCCGCTAGAGCAA-3’
CFTR7199rev	5’-TTTCCACCCCAAACGCAG-3’
CFTR46f	5’-TTCAGGTGAGAGGGTGTCTA-3’
CFTR172r	5’-ACCCTCATTCTCGTCCAT-3’
CFTR402fw	5’-GGCGCCGAGAAGAGTAGGG-3’
CFTR621rev	5’-TTTCCACCCCAAACGCAG-3’
CFTR359fw	5’-CAAATGACATCACCGCAGGTC-3’
CFTR564rev	5’-TCCAAAGCTCAGCTAGACACCCT-3’
CFTR696f	5’-TGTGAAGCCATGGGAATAG-3’
CFTR853r	5’-CACTTTGCCTAAGACTCTGAAC-3’
GGTA10f	5’-GGAAGAGTGGTTCTGTCAATGC-3’
GGTA149r	5’-GGTGACTTGGCTGATAACTAGGAG-3’
GGTA126f	5’-CTCCTAGTTATCAGCCAAGTCACC-3’
GGTA492r	5’-CGGTATTTAAGGGCTCAGGGATAC-3’
GGTA2377fw	5’-CACTCCTTAGCGCTCGTTGAC-3’

GGTA2758rev	5’-ATTGGGTTTGCTGCCCCT-3’
GGTA3423fw	5’-TCATCAGTGGATTCACCCCAA-3’
GGTA3640rev	5’-CACCACGGGAATGCCTTC-3’
GGTA3323fw	5’-GCTGGTGATTCATTTGTGCCT-3’
GGTA 3516rev	5’-CTGTCAGAAGCGTCTCCAGCT-3’
GGTA131f	5’-CCTCGTTATCAGCCAAGTCACC-3’
GGTA297r	5’-CGGATCCTTAAGCCAAAGAG-3’
GGTA232f	5’-CCAATTAGGATCCAAGAGGAGG-3’
GGTA424r	5’-GCAAGTGTGGGATATGGAAG-3’
HPRT781i2fw	5’-GAGCTACAGTTGCCGGCCT-3’
HPRT943i2rev	5’-AGCGGAAACAAATCCAACTAGG-3’
HPRT834i2fw	5’-TGTCTGCGACCCACACCA-3’
HPRT987i2rev	5’-GCATGCATCAGTAAGGAACTGG-3’
HPRT3133i4fw	5’-CGAATCAGAGCTGTAGCCGC-3’
HPRT3297i4rev	5’-TGACGAATCCAACTAGGAACCA-3’
HPRT374i5fw	5’-GAGGGCTTAGGCAGTGGCA-3’
HPRT528i5rev	5’-AAACAGCGTAGGTCAGACCAGG-3’
HPRT3088f	5’-GTTCTACATGCTGATCCTGACC-3’
HPRT3425r	5’-CTCTGCCTAGCTACTCTGATGATG-3’
HPRT4152f	5’-CCTTATCCCTTCTCACTACTCAGG-3’
HPRT4478r	5’-CCCACTTCCACGAATCAATGCTAC-3’
HPRT4578f	5’-GTGCTTATTGCCTCTCACTC-3’
HPRT4744r	5’-GGCTCCAACAATAAGACTCC-3’
HPRT657f	5’-CATCTAGCCTCTCTGGAGTTAG-3’
HPRT788r	5’-CGACATGCTAATGCTCTTGC-3’
lepR1452fw	5’-GCATCCCATATCTGAACCCAAA-3’
lepR1616rev	5’-ACGGAATCAGGAATGACACATG-3’
pLEPR3119rev	5’-GCCTGGGTTTCTATCTCCCATG-3’
pLEPR3059fw	5’-GCAGCAATTCCCTACCGAAAG-3’
pLEPR3360fw	5’-CCTCCAGGAGAGCTGTTCACAC-3’
pLEPR2944rev	5’-CATCCTCACGAGTTATCTCCATGC-3’
pLEPR2834fw	5’-CTCTACTCTTGACAACTCCGGACC-3’
pLEPR3517rev	5’-GAACTTAGACGGTTAGGTCATACATCTTG-3

pLEPR3068fw	5’-CCCTACCGAAAGAGTCTTTCTCG-3’
MYC41f	5’-TACCGCTTTCAATCCGCGATGAGT-3’
MYC307r	5’-CCGAGGTCAGCGTTCATCTACATT-3’
MYC633f	5’-CTTGGTCCTCGGAGATGTTAAG-3’
MYC761r	5’-GCGGAGATTTGTCCTCGTTT-3’
MYC286f	5’-TGTAGATGAACGCTGACCTC-3’
MYC425r	5’-CTTACTCATAGGAGCTCAGGAC-3’
MYC949f	5’-TACTGGGTGTTGCAAGGA-3’
MYC1051r	5’-GGGTAGAGAGCTCAGTCTT-3’
pTM1966f	5’-GGACCATTTCATAGGGACAGACT-3’
pTM2106r	5’-CTTGGACATTATCCACCAGTGAA-3’

3.1.9.2 Oligonucleotides for vector constructs

CFTR-3armf	5’-ATGACATGCATGTCATGGGATCCATACCAG-3’
CFTR-3armr	5’-TACTTGCGGCCGCAAGTCCAAGTGATCAGTCC-3’
CFTR-5armf	5’-CTGGTTGGTACCTTCTGTCCTCGAGTGTC-3’
CFTR-5armr	5’-CACAAAACCCTCATTCTCGTC-3’
CFTR-lacZf	5’-CGGTTCCCCCAGAGACCATGGTCGTTTTACAACGTCGTGAC-3’
CFTR-lacZr	5’-GTCACGACGTTGTAAAACGACCATGGTCTCTGGGGGAACCG-3’
CFTR-STOPf	5’-CGGTTCCCCCAGAGACCATGCCTCGGGGACACCAAATATGG-3’
CFTR-STOPr	5’-CCATATTTGGTGTCCCCGAGGCATGGTCTCTGGGGGAACCG-3’
GGTA-3armf	5’-TGCCTTGGAGATTCCAGCTG-3’
GGTA-3armr	5’-ATGATTGCGGCCGCCATCATCCTGAACTTGAG-3’
GGTA-5armf	5’-AGATTGGGTACCGAATCTCTATATGCTGTG-3’
GGTA-5armr	5’-GATAACTAGGAGATTAGAG-3’
GGTA-STOPf	5’-CCTTTTCTTTTCCCAGGAGAAAATAATGCCTCGGGGACACCAAATATGG-3’
GGTA-STOPr	5’-CCATATTTGGTGTCCCCGAGGCATTATTTTCTCCTGGGAAAAGAAAAGG-3’
GGTA-sf	5’- CAG TGG GTT AAG GAT CTG-3’
lacZr	5’-GTTCGGATAATGCGAACAG-3’
STOPr	5’-CCAATTATGTCACACAG-3’

3.2 Methods

3.2.1 DNA amplification

3.2.1.1 End-point PCR

The PCR amplification of desired DNA-fragments was performed according to the given cycling protocol using the reaction mix with a total volume of 25 µl as noted below (table 3.1). The amplifications were carried out using the GeneAmp PCR System 9700 (Applied Biosystems, Weiterstadt) thermocycler.

Table 3.1: Mastermix and cycling protocol for end-point PCR.

PCR-mastermix (total volume 25µl)	
2.5 µl	10x PCR buffer
2.5 µl	MgCl$_2$ (15 mM stock)
2.5 µl	dNTPs (2 mM stock)
0.5 µl	forward primer (10 mM stock)
0.5 µl	reverse primer (10 mM stock)
0.2 µl	Taq-polymerase (5 U/µl)
1.0 µl	DNA-template*
16.3 µl	A.dest.

PCR cycling protocol			
denaturation	95°C	3 min	
denaturation	95°C	30 sec	32 x
annealing**	xx°C	30 sec	
elongation***	72°C	xx min	
final elongation	72°C	10 min	
cooling step	4°C	15 min	

* genomic DNA 50-200 ng/µl, plasmid 1-100 ng/µl, BAC 10-200 ng/µl; ** annealing temperature depends on the used primer pair; is calculated by primer designing software or can be estimated by the '4+2'-rule -5°C (2°C for A/T, 4°C for C/G); *** elongation time depends on the length of the desired PCR-amplicon; if Agrobiogen-Taq-Polymerase is used, 1 min correlates with 2 kb DNA;

A specific 2-step fusion PCR shown in table 3.2 was developed for the construction of the modification vectors pCFTR-STOP/lacZ and pGGTA-STOP/lacZ described in 4.1.2.

Table 3.2: Cycling protocol for the 2-step fusion PCR used for STOP-box and *lacZ* introduction

2-step-fusion-PCR				
denaturation	95°C	2 min		
denaturation	95°C	45 sec	3x	pre-amplification
annealing	68°C	1 min		
elongation	72°C	2 min		
denaturation	95°C	45 sec	32x	amplification
annealing	58°C	30 sec		
elongation	72°C	2 min		
cooling step	4°C	15 min		

3.2.1.2 qPCR (quantitative real-time PCR)

The qPCR-amplifications were carried out with the ABIPrism 7000 Detection System (Applied Biosystems, Weiterstadt) using the SYBR® Green PCR Master Mix (Applied Biosystems, Weiterstadt). Table 3.3 shows the standard qPCR-mastermix components for target and reference genes (*CFTR*, *GGTA1*, *HPRT*) calculated for a total volume of 12.5 µl per sample. A standard curve using DNA of Niere m cells isolated via PCiA purification (read 3.2.9.1), with the assigned values of 10000, 7500, 5000, 2500, 500, 250, 50 and 25 copies, was prepared for each targeting approach, where it was assumed that 15000 copies is equivalent to 100 ng of DNA. Calibrators, reflecting copy numbers of approximately 5000, were prepared for each standard curve, using the same initial Niere m DNA. Sample DNA for the screening procedure was isolated according to the high-salt precipitation protocol further described in 3.2.9.1. Samples, standard curve, including the calibrator, and reaction components, except UNG, usually stored at -20°C and held on ice during the whole procedure, were pre-warmed in the water bath to room temperature. All components were gently stirred before the reaction was, pipetted on ice, applied to Microamp™ optical 96-well reaction plates. After charging, the plates were covered using a Microamp™ optical adhesive film and used either immediately or stored at -20°C.

Table 3.3: Standard mastermix for qPCR approaches.

qPCR standard mastermix	
6.25 µl	SYBR® Green I
0.075 µl	UNG
0.2-0.6 µM	primer fw
0.2-0.6 µM	primer rev
ad 12.5 µl	A.dest.

The standard thermal profile of the qPCR performance consists of (i) an initial activation step (for UNG) at 50°C for 2 min, followed by (ii) an initial denaturation step at 95°C for 10 min completed by (iii) a 40 cycle repeat of denaturation at 95°C for 15sec and primer annealing and extension at 60°C/63°C for 1.5 min. Finally, the plates were again heated from 60°C to 95°C to obtain a dissociation curve of the PCR products.

3.2.2 Agarose gel electrophoresis

The agarose gel electrophoresis allows separation of DNA fragments according to their size. For separation of DNA, TAE-agarose gels (0.7-3.0%) were used. After boiling the gel-solution in the microwave until the agarose was completely dissolved and cooling down of the suspension to approximately 60°C, ethidium bromide (1 mg/ml) was added (9 µl/100 ml). Samples were mixed with 10x BPB loading dye to observe the progress of the electrophoresis and were applied to the slots of the electrophoresis gel as well as a DNA molecular standard (1 kb DNA ladder or pUC8 marker) for estimating the gel band sizes. Ethidium bromide fluoresces under UV light, when intercalated into double stranded DNA, enabling the detection of the DNA bands by a gel electrophoresis documentation system (BioRad). Depending on the experiment, for fragments which were further processed agarose UltraPure^{TM} was used and fragments were excised under UV-light. For standard detection approaches Universal agarose was used.

3.2.3 Gel elution

The elution of DNA out of agarose gels was carried out according to the modified QIAex$^{®}$II Gel Extraction Kit (Qiagen, Hilden) protocol.

The gel bands of interest were excised and put into a 1.5 ml reaction tube. The gel slices were weighed. Three times the volume of buffer QX I was added to the tube, which then was placed onto a heating block at 50°C until the agarose had dissolved completely. Subsequently, 8 µl of QIAEX II were added to the tube, getting mixed by inverting the tube several times. To ensure an adequate DNA binding to the gel matrix, the tube was hold for 10 more minutes on the heating block. The sample then was centrifuged for 30 sec at 5400 g, the supernatant was removed and the pellet washed with 500 µl QX I solution. After an additional centrifugation for 30 sec at 5400 g the supernatant was removed again and the pellet was washed twice with 500 µl of PE buffer. The pellet then was air-dried for approximately 10 min and was afterwards resuspended in 20 µl T-buffer. Gel electrophoresis is used to estimate the concentration of the eluted DNA.

3.2.4 Restriction digest

The utilized amount of DNA was incubated for 90 minutes (for large amounts of DNA the restriction digest was performed overnight) with the appropriate restriction endonuclease, its recommended buffer at the recommended temperature in a total volume of 20 to 50 µl. In general 0.25 units of the restriction enzyme per µg DNA were used. Respective restriction enzyme recognition sites were determined using NEBcutter V2.0, appropriate restriction conditions for enzyme combinations are suggested by DoubleDigest^{TM}. Depending of the experiment different amounts of DNA have been digested. In term of analysis approximately 1 µg, for subsequent ligations 2 µg of DNA were digested. Restriction digests, unless otherwise noted by the supplier, were incubated o/N at 37°C.

Testing the integrity of BAC-DNA, a restriction digest of 10/20 µg BAC-DNA with the appropriate enzyme (e.g. EcoRI, XbaI, NsiI, PvuII) was performed overnight. The digest total volume was loaded onto a 0.7/1.0% agarose gel and run overnight at 0.5/2V/cm.

3.2.5 PCiA purification of restriction digests

PCiA-extraction was used to achieve the required purity of the digested DNA-fragments (e.g. if they were further used for ligations).

After the restriction digest volume was adjusted to 150 µl with A. dest., 100 µl of PCiA were added. The mixture was shaken for at least 2 minutes and then centrifuged for 3 minutes at RT and 16100 g. The supernatant was transferred to a new reaction tube, 15 µl NaOAc (3 M) and 400 µl EtOH (100% and ice cold) were added, mixed and the tubes then were maintained at -80°C for 30-60 minutes. Afterwards the tubes were centrifuged again for 30-45 minutes at 4°C and 16100 g. The precipitated pellet was washed once with 70% EtOH, air-dried for 5 minutes and dissolved in an appropriate volume of T-buffer.

3.2.6 Ligation

In general, an appropriate amount of vector was ligated with a 3 times stoichiometric excess of insert in the presence of 1 µl ligase and 2 µl of 10x ligase buffer. The ligation mix was adjusted with A. dest. to a total volume of 20 µl. The mix then was incubated at RT for at least 1.5 hours, but ideally overnight. For further applications the ligase was inactivated by a heating step at 65°C for 15 min.

3.2.7 Heat shock and electro-transformation

Two different methods, heat-shock-based transformation and electroporation, to introduce foreign DNA into different strands of *E. coli* bacterial cells have been used. The *E. coli* cells were prepared to make them competent for DNA uptake, according to the following protocols.

Heat shock-competent cells

The protocol for heat shock competent *E. coli* TOP10 cells was adapted from the protocol used at the Gene Center, LMU-Munich.

A 5 ml overnight culture TOP 10 cells was cultivated in LB medium at 37°C. On the next day 250 ml TYM medium were inoculated and incubated while shaking until this daily culture reaches an OD_{600} between 0.7 and 0.8 (approx. 2-4 hours). This bacterial suspension then was divided in 6 x 40 ml. Those reaction tubes were cooled down in an ice water bath for 5 to 10 min and then were centrifuged for 10 min at 3700 g and 4°C. After discarding the supernatant, each pellet was resuspended in 12 ml Tbf I solution, incubated for 10 min at 4°C and then centrifuged again for 10 min at 2500 g and 4°C. The supernatant was discarded, each pellet was resuspended in 1.6 ml Tbf II solution and the bacterial suspension subsequently was split into aliquots of 30-60 µl per Eppendorf cup. The ready to use aliquots were stored at -80°C.

Competent cells for electroporation (including recombineering)

The protocol to produce competent cells for electroporation was adapted from Liu and colleagues (or http://recombineering.ncifcrf.gov). For the general uptake of DNA by electroporation, the *E. coli* strain DH10B (Invitrogen, Karlsruhe) was used. Two other *E. coli* strains, SW106 for recombineering and SW 106 for Cre-mediated recombination, have been identically prepared (table 3.4).

Table 3.4: Protocols for competent cells.

competent cells for electroporation		
DH10B (general procedure)	**SW106 (recombineering)**	**SW106 (Cre-recombination)**
5 ml o/N culture in LB	5 ml o/N culture in LB	5 ml o/N culture in LB
1 ml o/N culture*	1 ml o/N culture*	1 ml o/N culture*
OD_{600} = 0.6/0.8	OD_{600} = 0.4/0.5	OD_{600} = 0.3/0.4
	15 min at 42°C water bath	add 1 ml arabinose**
		shake 1 h at 32°C
*** per 100 ml LB ** conc. 100 mg/ml; 1 ml per 100 ml o/N**		

General washing procedure

For this general step, the cells should be kept at 4°C at any time. The cell suspension was cooled down with occasional shaking for 10 min. Then the cells were harvested by centrifuging the suspension for 10 min at 5000 g and 4°C. After carefully removing the supernatant with a pipette, the pellet was resuspended in 50 ml ice-cold A. dest. Following a centrifugation for 10 min at 5000 g and 4°C, the supernatant was removed and the pellet was resuspended in 20 ml ice-cold A. dest. The centrifugation was repeated and the pellet resuspended in 1 ml ice-cold 10% glycerol/A.dest. Subsequently 50-80 µl aliquots of this suspension have been prepared. Preferentially, those cells should be used freshly for electroporation (1.75 kV), but can be stored at -80°C for several months.

3.2.7.1 Heat shock transformation

An aliquot (30-60 µl) of chemically-competent *E. coli* TOP10 cells, usually stored at -80°C, was slowly thawed. Up to 10 µl of the inactivated ligation batch were added to the bacteria solution, gently mixed and incubated on ice for 20 minutes. Applying a heat shock for 45 sec at 42°C in the water bath was followed by an incubation step of 2-3 minutes on ice. Subsequently 1 ml of SOC (or LB) medium was added to the suspension, gently mixed and incubated for 45 minutes at 37°C. The bacterial suspension then was centrifuged for 5 minutes at RT and 2300 g. The pellet was resuspended in 100-200 µl of the supernatant and plated on LB-agar plates containing the appropriate antibiotic (ampicillin 50 µg/ml, kanamycin 25 µg/ml and chloramphenicol 12.5 µg/ml). Optionally, if pGEM®Teasy vector was used the LB agar plates were pre-treated with 40 µl IPTG (100 mM) in combination with X-Gal (20 mg/ml) enabling the differentiation between positive and negative plasmid colonies (also termed 'blue-white-selection'). The plates were incubated at 37°C overnight. After counting the grown colonies, plates were conserved by wrapping them with Parafilm® and stored at 4°C.

3.2.7.2 Electroporation

Electro-competent cells were used to transfer DNA by applying an external electrical field, increasing the electrical conductivity and permeability of the cell membrane. This process is known to be approximately 10 times as efficient as chemical transformation. The procedure was performed using the BioRad Pulser.

Cuvettes were pre-cooled on ice and 1 ml LB aliquots in 1.5 ml reaction tubes on RT were prepared. The DNA was mixed with one aliquot of electro-competent cells (Plasmid: 1 ng, BAC: 20-100 ng). After transferring this mixture to a cuvette and pulsing it with 1.75 kV (the resulting time constant should range between 4.0 and 5.0), 1 ml LB medium was added to the cuvette, was resuspended and transferred to a reaction tube.

The cells then were stored in the incubator:

- DH10B cells at 37°C for 45 min
- SW106 cells at 32°C for 45 min
- SW106 for recombineering at 32°C for 2 h

The cells then were centrifuged for 5 min at 2300 g and RT, most of the supernatant should be removed if possible (100-200 µl should be left in the tube, otherwise plate whole suspension). Subsequently the cells were resuspended, which appear hairy and fluffy, and the suspension was plated on agar plates containing the appropriate antibiotic:

- BAC: chloramphenicol
- Plasmid: ampicillin, kanamycin
- BAC modification: kanamycin (introduction of neokan^R) or blasticidin (cassette exchange from neokan^R to blasticidin in homozygous targeting)

Finally, the plates were incubated overnight at 32°C/37°C.

3.2.8 Recombineering

To introduce the STOP- or the lacZ-box into the wild-type BACs CH242-248P18 and PigI-170I3 for *CFTR* and CH242-21F3 for *GGTA1*, the bacterial recombination based method called recombineering was used. The procedure was performed according to the given recombineering protocols (Liu *et al.*, 2003).

For this project the *E. coli* strain SW106 was used, which facilitates a heat induced bacterial recombination by the λ-phage encoded proteins *exo*, *bet* and *gam,* which are transcribed by the λPL promoter. The promoter is repressed by cI857, so at 32°C the expression is blocked. Increasing the temperature to 42°C enables the transcription of *exo*, *bet* and *gam*. Because of the possibility to induce the recombineering machinery by a heating step, the strain has to be grown at 32°C in regular LB medium (doubling time: approx. 50 min). The exo gene encodes a 5’-3’ exonuclease, producing single strand overhangs at linear DNA fragments. Those overhangs are stabilized by the bet protein. The gam protein protects the introduced single-stranded DNA from degradation mediated by the *E. coli* RecBCD protein. The introduction of linear DNA fragments, which exhibit homology to DNA molecules contained in the SW106 cells, results, mediated by the recombination proteins, in the desired recombination between the homologous regions of the DNA molecules. Additionally, in SW106 bacterial cells an *arabinose* induced Cre recombinase is encoded, needed for targeted exchange or excision of antibiotic resistance cassettes, using the Cre-loxP system, in which the Cre recombinase exclusively mediates recombination between palindromic loxP sites. The bacterial strain itself contains no antibiotic resistance. For a more convenient handling of the BAC-constructs after the recombineering procedure, they were retransformed into the *E. coli* strain DH10B enabling cultivation in regular LB medium at 37°C.

3.2.9 DNA isolation methods

Several DNA isolation protocols, described below, have been used to isolate genomic, plasmid and BAC-DNA. DNA from small amounts of cells was isolated using the high-salt precipitation method. Genomic DNA isolation was performed according to a standard PCiA-based protocol or using the spermidine-method. BAC-DNA was prepared endotoxin-free following a modified protocol using a combination of the QIAgen DNA Maxi Kit (Qiagen, Hilden) and the E.Z.N.A® Tissue DNA Mini Kit (Peqlab, Erlangen).

3.2.9.1 Genomic DNA isolation

Genomic DNA from porcine ear cartilage tissue was isolated and purified according to the protocols either described by Sambrook (2001) (PCiA-method) or using the spermidine-method adapted from the lab protocol of Dr. Josef Platzer.

Targeting experiments were performed with primary porcine kidney cells (pKC) as target cells. Several DNA isolation methods for genomic DNA were tested. This turned out to be challenging due to the small amount of available cells per targeted clone and in parallel the very high purity of the DNA required for subsequent qPCR application. In this experiment six conventional isolation methods (Phenol-Chloroform-Isoamylalcohol extraction, Kawasaki buffer isolation, high-salt precipitation, spermidine-purification, Gentra Puregene Cell Kit, Wizard® genomic DNA-Purification Kit), five column-based isolation methods (Nucleospin® Tissue Kit, peqGOLD MicroSpin Tissue DNA Kit, Qiagen DNeasy Blood & Tissue Kit, QIAamp® DNA Micro Kit, E.Z.N.A® Tissue DNA Kit), one filtration-based (nexttec™ DNA Isolation clean columns) and one isolation method based on magnetic particles were tested according to the suppliers protocol.

PCiA-method

The samples were minced with a scalpel and transferred to a reaction cup. 400 µl lysis buffer plus 20-30 µl Proteinase K (20 mg/ml) were added per sample and mixed by agitation. The samples were held o/N at 60°C. If the tissue was not completely digested after one night another 10-20 µl Proteinase K have been added and incubated for additional 3-4 hours. After the sample was centrifuged for 5 min at 16100 g and RT, the supernatant was transferred to a new tube, whereas undigested parts (hair etc.) should be retarded. 400 µl 4.5 M NaOAc were added to the tube which was then mixed by inverting it several times. Thereafter 600 µl PCiA were added to each tube, which then were put onto the rolling device for gently and homogenous mixing of the solution. The tubes then were centrifuged for 5 min at 16100 g and RT. After the transfer of the resulting supernatant to a new tube the PCiA step was repeated. Then, 0.7 volumes of Isopropanol (approx. 650 µl) were added and mixed by inverting the tube several times. Subsequently, DNA was precipitating. This DNA fiber was washed in 70% EtOH two times and then transferred to a tube containing 70% EtOH, where it stayed ideally o/N. The 70% EtOH is removed again and the DNA pellet should be air-dried for 6-10 min. Finally the pellet was dissolved in an appropriate volume of T-buffer and the DNA concentration was measured using the spectrophotometer.

Spermidine-method

Tissue samples were placed in reaction tubes, 400 µl of the mastermix to lyse the tissue were added and then the tubes were incubated for a minimum of 3-4 hours at 60°C. After the sample was centrifuged for 5 min at 16100 g and RT, the supernatant was transferred to a new tube, whereas undigested parts (hair etc.) should be retarded. Then, 0.7 volumes of Isopropanol were added and mixed by inverting the tube several times. Subsequently, DNA was precipitating. This DNA fiber was washed in 70% EtOH two times and then transferred to a tube containing 70% EtOH, where it stayed ideally o/N. The 70% EtOH was removed again and the DNA pellet should be air-dried for 6-10 min. Finally the pellet was dissolved in an appropriate volume of T-buffer and the DNA concentration was measured using the spectrophotometer.

High-salt precipitation

The sample was incubated for at least one hour at 60°C with 110 µl of the mastermix. 2 µl Proteinase K (20 mg/ml) were added and the mix was incubated for another hour at 60°C. After adding 30 µl NaCl (4.5 M) the mixture should be placed immediately on ice for 10 min. Then the sample was centrifuged at 16100 g for 20 min and RT. The supernatant was transferred to a new reaction tube and mixed by inverting the tube gently with 0.7 volumes Isopropanol. After the centrifugation at 16100 g for 20 min and RT the supernatant was removed, the pellet was washed one or two times with 500 µl 70% EtOH. Subsequently, the DNA was left in 70% EtOH overnight. Finally, the 70% EtOH was removed, the pellet was air-dried for 6-10 min and then dissolved in 35 µl T-buffer.

Kawasaki buffer isolation

To lyse the cells 100 µl Kawasaki buffer (see 3.1.5) were added per each sample. After adding 5 µl Proteinase K (20 mg/ml) the sample was incubated for 1 h at 55°C. The Proteinase K was inactivated by a heating step for 15 min at 95°C. Thereafter the tubes were put immediately on ice for several minutes. The samples then were centrifuged for 1 min at 16100 g and the supernatant was directly used for PCR.

3.2.9.2 Plasmid DNA isolation

Single colonies were picked from LB-agar plates with a sterile inoculating loop and transferred to 15 ml culture tubes containing 2.5-5 ml LB medium with the appropriate antibiotic. After an overnight incubation at 37°C in the shaking incubator the bacteria suspension was centrifuged for 10 minutes at 1300 g. Optionally, from the overnight cultures glycerol stocks to conserve the bacterial culture for subsequent inoculations have been made by mixing 900 µl 60% glycerol with 300 µl o/N culture. The DNA was isolated according to the protocol adapted from (Sambrook, 2001) (Volumes are capable for 2.5 ml bacteria culture; using 5 ml culture, a 1.5 fold amount of solutions is necessary, as is needed for BAC isolation).

The supernatant was discarded; the pellet then was resuspended in 750 µl STE and transferred to 1.5 ml (2.0 ml) reaction tubes. After centrifugation of the samples for 5 minutes at 4500 g and RT the supernatant was discarded, the pellet was resuspended in 200 µl plasmid A, 400 µl plasmid B were added, the suspensions was mixed 5-7 times and the samples were incubated for 5 min on ice. Then 300 µl plasmid C were added, the solution was mixed again 5-7 times, held on ice for 3 min followed by a centrifugation for 10 min at 16100 g and RT. The resulting supernatant was incubated with 4 µl RNaseA (20 mg/ml) for 45 min at 37°C. Thereafter, 300 µl PCiA were added per sample, which then was shaken for at least 1 min and then centrifuged for 2.5 min at 16100 g and RT. The aqueous phase was transferred to a new tube. 0.7 volumes (approx. 650 µl) iPrOH were added to precipitate the DNA, followed by a centrifugation for 10 min at 16100 g and RT and a washing step of the pellet in 700 µl 70% EtOH, at least for a few hours, better o/N. The samples then were centrifuged for 2.5 min at RT and 16100 g, the EtOH was removed, the pellet was air-dried for 6 min and finally dissolved in 55 µl T-buffer. The DNA concentration of each sample was determined by the spectrophotometer.

BAC-DNA isolation by heating step for PCR screening

A number of grown BAC colonies were picked from agar plates, were inoculated in 5 ml LB medium + antibiotic (kanamycin) and grown at 32°C overnight. 10 µl of the overnight culture were mixed with 20 µl T-buffer in PCR tubes. This mixture was heated up and denatured for 10 min at 95°C. After cooling the suspension down for 15 min at 4°C, the tubes were centrifuged for 5 min at RT and 1500 g. The supernatant (1 µl) containing the BAC-DNA was used for the screening PCR using primer pairs, selective for the wild-type sequence (in case of a random integration of the modified construct), and for the introduced sequence (STOP-box, *lacZ* reporter gene).

BAC-DNA isolation (modified protocol; endotoxin-free)

The BAC-DNA was prepared endotoxin-free to assure an optimal transfection rate in pKCs and pFFs. The modified protocol used represents a combination of the Endofree plasmid Maxi Kit using the QIAGEN-tips 500 and the buffer set (Qiagen, Hilden) and the E.Z.N.A.® Endo-free Plasmid Midi Kit, using the ETR-solution. BACs in SW106 cells are cultured at 32°C, in DH10B cells at 37°C.

A pre-culture of 3 ml LB medium supplied with the respective antibiotic was inoculated with the BAC of interest and left on the shaking incubator at 32°C (SW106)/37°C (DH10B) for at least 4 hours. After that a 100-200 ml o/N culture was inoculated. The o/N culture was centrifuged for 15 min at 4600 g and 4°C, the supernatant was discarded and the pellets resuspended in 10 ml P1 (+ RNase). After adding 10 ml P2 and a 5 min incubation at RT, 10 ml P3 (ice-cold) were added and incubated on ice for 15 min. Afterwards the samples were centrifuged for 30 min at 16100 g and 4°C, the supernatants were transferred to new tubes and 0.1 volumes (approx. 3 ml) ETR solution were added. The tubes were inverted for mixing 7 times and then held on ice for 10 min. To enable the next centrifugation step, the solution had to be split into 15 ml tubes, which then were incubated for 15 min at 42°C (water bath) and centrifuged for 10 min at 5000 g and RT. The supernatant was transferred to a new tube, 1 ml of prewarmed A. dest. was added and the previous centrifugation step was repeated, followed by the transfer of the supernatant to a 50 ml tube. 0.5 volumes of 100% EtOH were added and incubated for 2 min at RT. In the meantime the QIAtip 500 column was equilibrated by applying 10 ml QBT solution. The DNA solution was applied to the equilibrated column and entered the resin by gravity followed by a 2 times washing step with 30 ml QC solution. Afterwards the DNA was eluted with 15 ml QF solution, 10.5 ml iPrOH were added, the solution was then split into 15 ml tubes and centrifuged for 30-40 min at 16100 g and 4°C. Subsequently, the supernatant was discarded, the pellet was washed with 5 ml 70% EtOH overnight, the supernatant was removed on the next day and finally the pellet was resuspended in an appropriate volume of T-buffer. The DNA concentration was determined in a spectrophotometer.

3.2.10 DNA sequencing

DNA sequencing was achieved by capillary sequencing at the Helmholtz Center Munich. The DNA for sequencing was purified by PEG-precipitation and prepared for the sequencing service according to the following protocols.

PEG-precipitation

To achieve higher purities of plasmid DNA for sequencing, a precipitation step using PEG-$MgCl_2$ was performed according to (Sambrook, 2001). A reaction mix containing equal amounts of plasmid DNA, PEG-$MgCl_2$ and H_2O (20 µl each) was equilibrated for 10 minutes at RT. The mixture then was centrifuged for 20 minutes at RT and 16100 g, the pellet was washed in 70% EtOH, air-dried and dissolved again in 20 µl T-buffer.

Sequencing

The sequencing was carried out according to the protocol of the Helmholtz Center sequencing service. The amount of DNA-template in ng was calculated as follows: DNA amount (ng) = (length of fragment in bp / 100) x 1.5.

The DNA samples were diluted with A. dest. to the desired concentration. Constructs were sequenced using oligonucleotides termed T7 and M13, which are prone to sequence the fragments inserted into the multiple cloning site of the pGEM vector. Additionally DNA fragments were sequenced with self-designed primers or primers designed with the Primer Express software. The regular mastermix and the standard cycler protocol are given in table 3.5.

Table 3.5: Mastermix and cycling protocol for sequencing.

Sequencing mix	
4 µl	5x sequencing buffer
1 µl	BigDye
1 µl	primer (10 µM stock)
2 µl	template
2 µl	A.dest.

Sequencing cycler protocol		
95°C	1 min	
95°C	5 sec	40x
50°C	10 sec	
60°C	4 min	

EtOH precipitation

After adding 2.5 µl 125 mM EDTA and 30 µl 100% EtOH to the sequenced samples the solution was transferred to 1.5 ml tubes, which then were incubated on ice for 15 min. The samples then were centrifuged for 30 min at 16100 g and 4°C. Thereafter the pellet was washed in 50 µl 70% EtOH (overnight). Subsequently the samples were centrifuged for 2.5 min at 16100 g and RT, the pellets were air-dried for 6 min and dissolved in 30 µl A. dest. Finally, the samples were transferred to a sequencing plate (ABgene® 96-well plate) and stored at -20°C until they were sent to the Helmholtz Center sequencing service.

Data-analysis

The electropherograms of the sequences were analyzed with the DNA sequencing chromatogram trace viewer FinchTV 1.3.1 and the biological sequence alignment editor BioEdit.

4 RESULTS

4.1 Modification and preparation of BACs

A total number of four different modification plasmid vectors (pCFTR-STOP/lacZ and pGGTA-STOP/lacZ) were designed to introduce the desired alterations by recombineering into the respective BACs, carrying the target gene of interest (*CFTR* or *GGTA1*). The constructs pCFTR/GGTA-lacZ and their further application are described in 4.6.

4.1.1 Searching for wild-type BACs

For the *CFTR* gene, two different BACs, carrying the respective genomic sequence of interest, which is subsequently altered by recombineering (Copeland *et al.*, 2001) with the respective modification vectors, were chosen. CH242-248P18, including a pTARBAC1.3 backbone and PigI-170I3, supplied with a pBeloBACII backbone, were tested by a restriction digest (PvuII) for their integrity. The fragments on the agarose gel, when compared with the *in silico* pattern, give information if the supplied BACs actually contain the correct region of interest. CH242-248P18 was used for further experiments.

The verification procedure concerning the BACs containing the *GGTA1* gene (CH242-21F3 and CH242-372F22; both with pTARBAC1.3 backbones) was similar. The wild-type BACs were digested with PvuII and the resulting fragments were compared with the *in silico* pattern. Finally, BAC CH242-21F3 was used for further experiments.

4.1.2 Design and construction of the modification vectors

In order to obtain the complete knock-out of a desired gene, either RNA or protein synthesis have to be eliminated. In case of *CFTR* both, the RNA transcription and the protein translation start, are located in exon I. For the knock-out of the *GGTA1* gene, exon IV was targeted, eliminating the transcriptional and translational start as well. For this reason, a STOP-box containing a HIS3 (encodes imidazoleglycerol-phosphat dehydratase) yeast protein termination sequence as well as a SV40-pA (simian virus-polyadenylation) signal (Sauer, 1993) coupled to a neomycin/kanamycin resistance cassette (neokan^R), was introduced behind the initial ATG codon of the respective exon by fusion PCR. The modification vectors pCFTR-STOP and pGGTA-STOP were similarly designed and constructed. Restriction digests and ligations were carried out as described in methods 3.2.4 and 3.2.6, respectively. PCRs were performed according to the standard protocol in 3.2.1.1 with varying annealing temperature and elongation time, given in brackets.

pCFTR-STOP and pGGTA-STOP

The 2 kb floxed neomycin resistance cassette, lox^{2}neo (NEO), derived from the vector pPNTlox^2 was exchanged by a PGK (phosphoglycerate kinase) promoter driven, floxed 2.2 kb neomycin/kanamycin resistance (neokan^R) cassette from pL452 via arabinose induced Cre-recombination. Neokan^R was cloned by NotI/BamHI into the pBSK II vector (pBSK-NEO). The 0.5 kb 3’arm-fragment was amplified from genomic pig DNA by conventional PCR using the primer pairs CFTR-3armf/CFTR-3armr (58°C annealing, 1 min elongation) and GGTA-3armf/GGTA-3armr (58°C annealing, 1 min elongation). The amplified fragments were digested with NotI/NsiI and then ligated into the lox^{2}neo-pBSK II vector (pBSK-3arm-NEO). The STOP-box, derived from pBS302, was cloned via BamHI into the pBSK II vector (pBSK-STOP). The 0.8 kb 5’arm-fragment was amplified from genomic BAC-DNA (CH242-248P18, *CFTR*; CH242-21F3, *GGTA1*) using primers CFTR-5armf/CFTR-STOPr and GGTA-5armf/GGTA-STOPr, respectively (annealing 58°C, elongation 30 sec). In parallel, the STOP-fragment was amplified from the pBS302 vector using the primer pair CFTR-STOPf/STOPr and GGTA-STOPf/STOPr (annealing 58°C, elongation 1 min). The 5’arm- and STOP-PCR-fragments were eluted from an agarose gel and subsequently used as templates for a two-step-fusion-PCR using the flanking primers CFTR-5armf/STOPr and GGTA-5armf/STOPr according to the cycling protocol described in table 3.2. The PCR fusion-amplificates were directly digested with KpnI-BglII and then ligated into the KpnI-BglII digested pBSK-STOP vector (pBSK-5arm-ATG-STOP). The resulting 5arm-ATG-STOP fragment was cloned by a HindIII-KpnI digest into the pBSK-3arm-NEO vector resulting in the modification vectors pCFTR-STOP and pGGTA-STOP (figure 4.1). The generated modification vectors subsequently were used to be introduced into the respective BAC mediated by recombination proteins in SW106 *E. coli* cells.

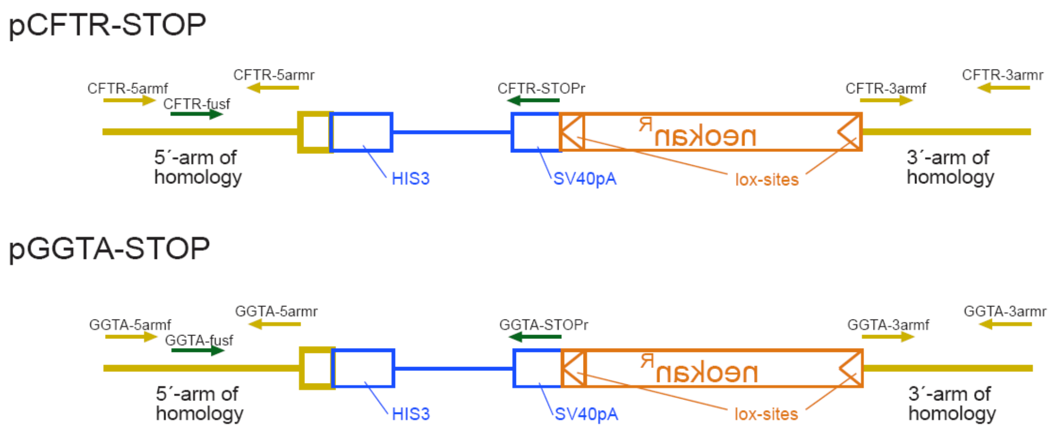


Figure 4.1: **Illustration of the modification vector construction of pCFTR-STOP and pGGTA-STOP.**

Each modification construct consists of (i) a 5’-arm of homology and (ii) a 3’-arm of homology, amplified from genomic DNA by primer pairs indicated with yellow arrows, (iii) a floxed neomycin/kanamycin resistance (neokan^R) cassette driven by a PGK (phosphoglycerate kinase) promoter and (iv) a STOP-box, containing a HIS3 (encodes imidazoleglycerol-phosphat dehydratase) yeast protein termination sequence as well as a SV40-pA (simian virus-polyadenylation) signal. Primer pairs used to append the STOP-box directly to the ATG of the porcine gene of interest (*CFTR* or *GGTA1*) by a 2-step fusion PCR are indicated by green arrows.

4.1.3 Modification of wild-type BACs

The modification vector pCFTR-STOP and the respective wild-type BAC (CH242-248p18) were prepared according to the plasmid preparation protocol (adapted from Sambrook, 2001) described in 3.2.9.2. Thereafter, the isolated BAC-DNA was transferred to recombineering competent SW106 *E. coli* cells (500 ng BAC-DNA per aliquot) by electroporation. The bacterial suspension was plated after the electro-transformation step on agar plates containing the suitable antibiotic (kanamycin). Cells were grown overnight at 32°C. A resulting colony was picked, tested for the correct uptake of the BAC by conventional PCR and prepared as given in table 3.4 (SW106-recombineering). In parallel, the purified plasmid DNA was digested overnight (see 3.2.4) with KpnI/Cfr42I and the respective fragments were eluted from the agarose gel.

Aliquots of the SW106 cells, containing the BAC of interest, were used for the electrotransformation step as described in 3.2.7.2. Two different amounts of linearized modification-fragment were used: 20 ng and 100 ng. After 2 hours recovery at 32°C (allowing activity of recombineering enzymes), the cells were plated on agar plates (containing kanamycin) and were incubated overnight at 32°C. Clones appearing after recombineering on LB + Kan plates, were picked and prepared for screening PCR as described in 3.2.9.2 (heating step isolation).

The isolated BAC-DNA modified with the pCFTR-STOP constructs was screened with the primer pair CFTR-6456f/CFTR-7160r (annealing 60°C, elongation 90 sec) for the wild-type sequence and with the primers CFTR-6456f/STOPr (annealing 54°C, elongation 90 sec) detecting the correct integration of the modification construct. BAC-DNA used for the *GGTA1* approach, was screened after modification with the constructs pGGTA-STOP using the primers GGTAsf/GGTA5armr (annealing 56°C, elongation 90 sec) for the wild-type sequence and GGTAsf/STOPr (annealing 60°C, elongation 90 sec) for correct integration of the modification construct.

The *CFTR* BAC modification approach resulted in 627 colonies from 4 agar plates (2 x 20 ng and 2 x 100 ng of linearized STOP fragment). 24 of them have been picked and analyzed by PCR as described above. 14 out of 24 clones could be dedicated as correctly modified. Colony 7 was used for further experiments. The *GGTA1* BAC modification resulted in a total number of 182 colonies. Again 24 colonies have been screened, of which 3 carried the correct modification. The results of the BAC modifications are shown in figure 4.2.

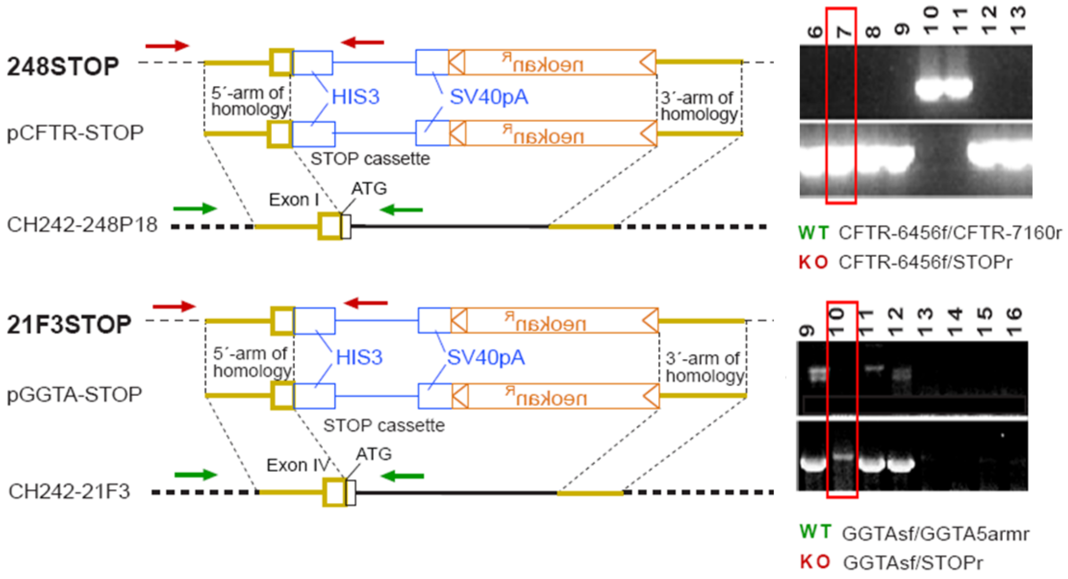


Figure 4.2: Modification scheme for wild-type BACs by recombineering.

Recombineering-competent SW106 cells containing the BAC of interest have been used for electrotransformation with the respective modification fragment, primarily excised from pCFTR-STOP or pGGTA-STOP, respectively. The picture indicates a selective overview of the PCR-screened modified BACs. In case of *CFTR* 14 out of 24 clones were screened positive (clone 7 was used for further experiments), in case of *GGTA1* 3 out of 24 have been correctly modified (clone 10 was used for further experiments). Screening primers for the wild-type sequence are indicated by green arrows, for the knock-out sequence by red arrows.

For a more easy-handling, correctly modified BAC clones (in SW106 cells), termed p248-STOP or p21F3-STOP, respectively, were re-transformed into recombineering deficient *E. coli* strains (DH10B). The procedure was carried out similar to the electrotransformation described above. Aliquots of electrocompetent DH10B cells (produced as described in 3.2.7) were transformed with 20 ng as well as 100 ng of the respective BAC-DNA. The cell suspension was plated on agar plates containing the suitable antibiotic (kanamycin or chloramphenicol) and incubated overnight at 37°C. A number of the resulting colonies were picked, and prepared for screening PCR as well. The remaining screening steps were carried out equally as already described above.

4.1.4 Preparation of the modified BACs for transfection approaches

Finally, the correctly modified BAC-constructs were prepared for the transfection step into porcine kidney cells following the endotoxin-free isolation protocol described in 3.2.9.2. After DNA isolation, the constructs have been linearized with AscI as restriction enzyme of choice. The restriction digest was carried out according to the standard protocol for BAC digestion given in 3.2.4 using adequate amounts of enzyme for the amount of DNA to be digested. The number of BAC-DNA isolations per construct and the resulting amount of DNA, after linearization, is listed in table 4.1.

Table 4.1: Overview of BAC-DNA isolations per construct.

p248STOP		**p21F3STOP**	
# isolation	**DNA amount [µg]**	**# isolation**	**DNA amount [µg]**
1	27.4	1	9.0
2	21.7	2	4.0
3	15.0	3	5.5
4	10.0	4	33.5
5	14.0		
6	38.0		
7	42.8		
8	19.2		
9	34.6		
10	14.0		
11	18.0		
12	20,7		
13	17,6		
14	31.8		
	324.8		**51.5**

4.2 Introduction of modified BAC-DNA into porcine primary cells

The necessary cell culture work for this project, including (i) preparation of target cells, (ii) cell transfection, (iii) antibiotic-based selection for construct integrations into the cells, (iv) the microscopical screening for single cell clones and (v) the propagation of cell clones for DNA isolation and cryopreservation for nuclear transfer experiments was carried out under the direction of Dr. Annegret Wünsch at the Chair for Molecular Animal Breeding and Biotechnology.

4.2.1 Preparation of target cells

Two different target cell populations were established from different material according to an isolation protocol using Collagenase II for cell dissociation: (i) primary porcine fetal fibroblasts (pFF) obtained from a day 27 male pig fetus and (ii) primary porcine kidney cells (pKC) from approximately three months old male pigs. After verification of the cells for the correct karyotype (*sus scrofa* = 38 chromosomes) and capability for nuclear transfer the primary cells were further cultured and transfected after two to three passages. As standard culture medium Dulbecco Modified Eagle Medium (DMEM) with 10-15% (v/v) fetal calf serum and additional supplements was used. Both cell populations were growing as monolayer on collagen-coated plates and detached using trypsin/EDTA.

4.2.2 Transfection of target cells

Transfection was performed via the electroporation based Nucleofector® (AMAXA® Basic Nucleofector Kit Primary Fibroblasts, Lonza, Köln) according to the manufacturers protocol. In brief, 0.5-1 x 10^6 target cells (pFF or pKC) were transfected after harvesting from the plates with circular or linearized BAC-DNA. DNAs p248STOP, p248lacZ, p21F3STOP, p21F3lacZ were isolated according to the protocol described in 3.2.9.2 and linearized, if needed, with AscI (see 3.2.4).

4.2.3 Selection for construct integration and screening for single cell clones

48 hours (except one time 24 hours) after transfection of the target cells, the selection for clones with integrated BAC-constructs carrying the neomycin resistance cassette was started. Different amounts of transfected cells (partly in combination with non-transfected wild-type cells) were seeded on 96-well plates

and cultured in regular growth medium supplemented with geneticin (G418) in the appropriate concentration (0.6 mg/ml for pFF, 1.2 mg/ml for pKC) for one week. Thereafter, the plates were screened for wells containing one colony of a single cell clone. Those single cell colonies were expanded to 30-90% confluence. Different strategies were faced: (i) after harvesting the cells with at least 90% confluence from the 96-well plates the cell pellets were frozen in a reaction tube for subsequent DNA-isolation (see 3.2.9.1) at -80°C, (ii) the cells were transferred to another 96-well plate when they were minimum 30% confluent to promote proliferation; after reaching confluence, they were harvested and frozen for DNA isolation (see above); (iii) to generate a backup sample for nuclear transfer, the cell colonies were split 1:2 on new 96-well plates; after reaching 90-100% confluence, the cells were removed by trypsinization from the plates. One aliquot was used for DNA isolation as needed for qPCR-screening, the other one was conserved as backup sample in cryopreservation medium (90% fetal calf serum + 10% DMSO) at -80°C, enabling the reactivation of candidate clones for nuclear transfer.

The *CFTR* knock-out approach included 23 AMAXA nucleofections using porcine kidney cells as target cells, resulting in 1151 clones for subsequent analysis (for a detailed overview see table 4.2).

Table 4.2: Nucleofection overview for *CFTR* targeting.

p248STOP	# epos	DNA [µg]	# cells (10^6)	# cells per well*	clones
100309	2	10/10	1	2000	51
270309	4	10/10/10/10	1	2000	164
310309	2	10/4.7	0.74	2000	19
200609	4	7/7/5.6/5.6	1	2000	83
060709	5	5.6/5.7/7.6/7.6/2.5	1	2000	329
300709	2	9/9	1	2000	59
110809	2	5.6/5.6	1	2000	165
120909 (A)	1	3.2	0.5	600 / 600	53
120909 (B)	1	6.7	1	1300 / 1100	228
total CFTR	**23**	**168**	**8.24**		**1151**
***transfected/wild-type**					

In case of the *GGTA1* gene knock-out using pKCs as target cells five transfections with the p21F3STOP construct resulted in 306 clones for further investigation (a detailed overview of the p21F3STOP transfections is listed in table 4.3).

Table 4.3: Nucleofection overview for *GGTA1* targeting.

p21F3STOP	**# epos**	**DNA [µg]**	**# cells (10^6)**	**# cells per well***	**clones**
310309	3	6.4/3/3.9	0.74	2000	36
120909(A)	1	4.5	1	2400/2400	150
120909(B)	1	6.6	1	2400/2400	120
total GGTA	**5**	**24.4**	**2.74**		**306**
*** transfected/wild-type**					

The genomic DNA of the generated cell clones was isolated followed by the screening for correct construct integration into the porcine genome of the somatic cell according to the LOWA-assay.

4.3 Isolation of genomic DNA from cell clones

After transfection of the target cells with the respective gene constructs for the targeting approach (p248-STOP or p21F3-STOP), single cell clones have been generated, preselected and screened for vector integration. Each of the single cell clones was propagated to confluence on a 96-well plate well, bearing the problem of very small amounts of primary cells (maximum of 8000-10000) available per clone. The isolated DNA is demanding appropriate purity for further qPCR-application. Hence, several DNA isolation methods needed to be tested.

In this experiment 13 different isolation methods, including six conventional isolation methods (Phenol-Chloroform-Isoamylalcohol extraction, Kawasaki buffer isolation, high-salt precipitation, spermidine-purification, Gentra Puregene Cell Kit, Wizard® genomic DNA-Purification Kit), five column-based isolation methods (Nucleospin® Tissue Kit, peqGOLD MicroSpin Tissue DNA Kit, Qiagen DNeasy Blood & Tissue Kit, QIAamp® DNA Micro Kit, E.Z.N.A® Tissue DNA Kit), one filtration-based (nexttec™ DNA Isolation clean columns) and one isolation method based on magnetic particles (MAXWELL® 16 cell LEV Purification Kit), were tested. In each case five samples containing 7500 pFFs (this amount complies with the average cell number grown in one well of a 96-well culture plate) and five samples containing 3750 cells (reflecting cell clones growing not that good) were isolated according to the provided protocols. Due to the small amount of available cells, it was not possible to measure the resulting DNA concentration by a spectrophotometer as usual. Standard qPCR runs provided an insight, which isolation methods met the demands for further approaches. In table 4.4 an overview of the 13 different methods regarding DNA-yield and reproducibility of the qPCR results is shown.

Table 4.4: Overview of 13 different isolation methods.

	DNA isolation method	**DNA-yield**	**reproducibility**
conventional	PCiA	sufficient	high SD
	Kawasaki	low	/
	High-salt precipitation	sufficient	yes
	Spermidine	sufficient*	/
	Puregene	sufficient	high SD
	Wizard®	low	/
column-based	Nucleospin®	low	/
	peqGOLD	low	/
	DNeasy	low	/
	QIAamp	low	/
	E.Z.N.A®	low	/
filtration-based	Nexttec™	sufficient	high SD
magnetic particles	MAXWELL®	sufficient	high SD

*** Agent interferes with SYBR green I detection in qPCR; SD: standard deviation**

In case of the conventional methods tested, the Kawasaki buffer isolation was not applicable for qPCR, due to insufficient DNA yield and purity. The isolation of DNA by spermidine for the SYBR green I assay was also not practicable, due to inhibiting effects correlating with the concentration of the DNA samples tested. The Wizard® genomic DNA-Purification Kit yielded too low amounts of DNA, the initially used PCiA-method and the Gentra Puregene Cell Kit showed too high standard deviations when tested in qPCR applications.

The five column-based methods and the filtration based method all resulted in a too high loss of DNA during the procedure, making them not feasible for this approach.

The magnetic-beads-based method used, showed a very high variety in the binding capacity of the DNA to the magnetic particles, resulting as well in very high standard deviations during qPCR, whereas the high-salt precipitation method showed convincing results in the qPCR assay. Additionally, the easy-handling due to the requirement of nontoxic reagents for the isolation and the expedient time-management pointed out the high-salt precipitation as method of choice.

4.4 Screening of cell clones for correct vector integration

Due to the long homology regions, preparing BACs as ideal tools for targeting experiments, it is necessary to think of alternative methods to detect correct vector integrations in the porcine genome beside Southern blot technology and conventional PCR.

4.4.1 LOWA-assay

The ‘loss of wild-type allele’-assay (LOWA), known as a qPCR-based method to detect copy numbers of wild-type alleles of one target gene compared to respective reference genes throughout the genome, provides an excellent method to screen hundreds of clones for heterozygous as well as homozygous alterations (figure 4.3). Due to possible variations concerning the qPCR efficiency throughout different loci the search for appropriate reference genes and primer pairs was necessary.

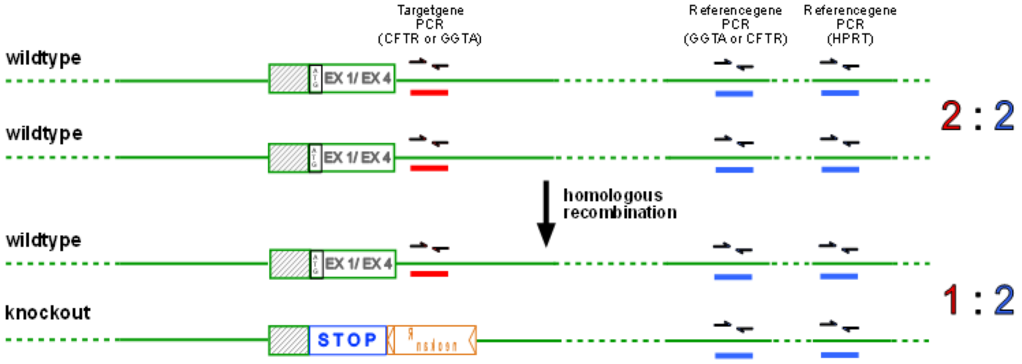


Figure 4.3: Schematic illustration of the LOWA-assay for *CFTR/GGTA1* targeting.

The copy numbers of the target gene and two selected reference genes (*CFTR*, *GGTA1*, *HPRT*) were detected by qPCR. Primer pairs were selected to detect wild-type alleles in each case, and the copy number ratio between the target gene and each reference gene was calculated. In wild-type cells or cells with random integration of the vector the ratio was 2:2. After targeted introduction of the vector construct, the ratio between the target gene and each reference gene was reduced to 1:2, indicating the loss of one wild-type allele for the desired DNA region.

4.4.2 Optimization of qPCR conditions

Several primer pairs for the target loci *CFTR* or *GGTA*1 as well as for four other reference genes (β-actin, *ACTB;* Hypoxanthine-phosphoribosyl-transferase, *HPRT;* porcine Leptinreceptor, *lepR*; Myelocytomatosis oncogene, *MYC*) have been designed (table 4.5) and purchased from Thermo Scientific (Ulm, Germany). All 31 primer pairs, either designed by hand or using the Primer Express® Software v2.0 (Applied Biosystems, Weiterstadt), have been initially tested by conventional PCR, each showing bands on agarose gels at the desired size. The conventional PCR was carried out according to the protocol given in 3.2.1.1 with an annealing temperature of 60°C/63°C and elongation for 45 sec.

Table 4.5: Overview of the pre-tested primer pairs for qPCR optimization.

GGTA1	CFTR	HPRT	ACTB	lepR	MYC
2377f/2758r	6752f/118r	781i2f/943i2r	237f/390r	3059f/3119r	41f/307r
3423f/3640r	6822f/7199r	**834i2f/987i2r**	1059f/1219r	1452f/1616r	633f/761r
3323f/3516r	359f/564r	3133i4f/3297i4r			286f/425r
10f/149r	**402f/621r**	374i5f/528i5r			949f/1051r
126f/492r	1772f/2060r	3088f/3425r			
131f/297r	986f/1132r	4152f/4478r			
232f/424r	46f/172r	4578f/4744r			
	696f/853r	657f/788r			
7	8	8	2	2	4

In order to evaluate the appropriate primer pairs for the subsequent qPCR application to test the targeted cells for events of homologous recombination by the LOWA-assay, several conditions have to be kept. Thus, (i) the amplificate should show sizes ranging between 150 to 500 bp, (ii) the run conditions relating to extension temperature and primer concentration should be at least similar throughout the compared genes (iii) the PCR efficiency should amount to almost 100% marked by a slope of -3.322 of the standard curve and (iv) the correlation factor (R^2), obtained by a standard curve, should not be less than 0.99.

In an initial qPCR approach, genomic DNA from porcine fetal fibroblasts (pFF) has been isolated by PCiA extraction, diluted with T-buffer to concentrations of 125 ng, 12.5 ng and 1.25 ng (a no template control, NTC, was also added) and tested at run conditions of 60°C extension temperature and a primer concentration of 0.25 µl per 12.5 µl total volume per sample. Promising primer pairs then were tested in additional qPCR-set-ups by increasing the annealing temperature to 63°C and the primer concentration to 0.5 µl or 0.75 µl per 12.5 µl mastermix total volume.

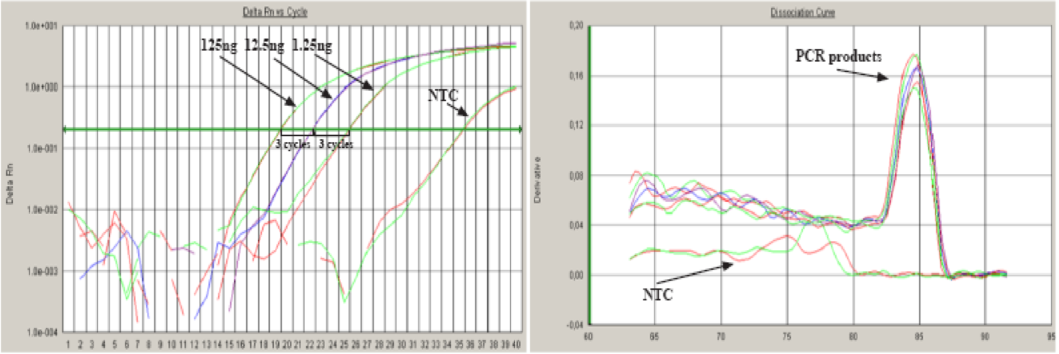
Optimization of the evaluated 31 primer pairs revealed for the LOWA-assay: the *CFTR* gene has been detected with primer pair CFTR402f-CFTR621r at run conditions of 60°C extension temperature and 0.25 µl primer per 12.5 µl (0.2 µM) total volume per PCR mix and the reference genes of choice (*HPRT, GGTA1*) have been detected with the primer pairs HPRT834i2f-987i2r with 63°C and 0.25 µl primer (0.2 µM) and GGTA3423f-GGTA3640r with 63°C and 0.75 µl primer (0.6 µM), respectively (table 4.6).

Table 4.6: Overview of the primer pairs for qPCR representing the best candidates for target and reference gene.

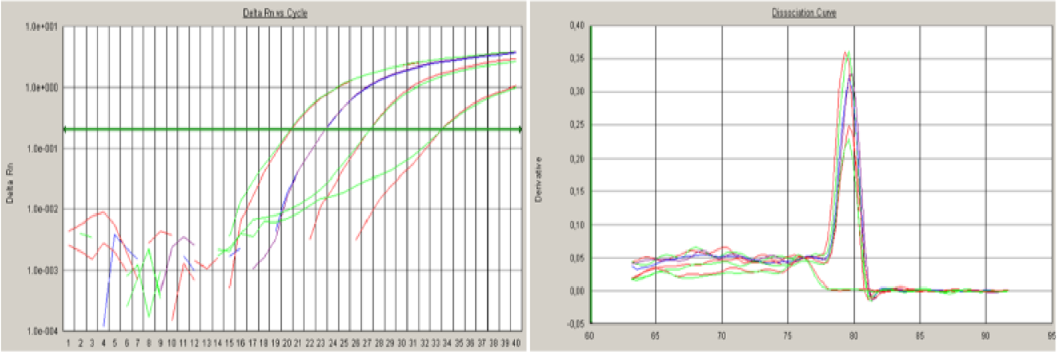
gene	primer	fragment length in bp	run conditions	slope	R^2
ACTB	1059f/1219r	160	0.2 µM/63°C	-3,266	0,999
HPRT	**834i2f/987i2r**	**153**	**0.2 µM/63°C**	**-3,206**	**0,995**
GGTA1	**3423f/3640r**	**217**	**0.6 µM/63°C**	**-3,404**	**0,997**
CFTR	**402f/621r**	**219**	**0.2 µM/60°C**	**-3,374**	**0,997**
lepR	3059f/3119r	60	0.2 µM/63°C	-3,231	0,997
MYC	41f/307r	266	0.2 µM/63°C	-2,904	0,991
	949f/1051r	102	0.2 µM/60°C	-2,794	0,963

The amplification plots and the associated dissociation curves for the three chosen primer pairs are shown in figure 4.4. For the *GGTA1* approach target gene and reference gene were simply switched (*CFTR* now reference gene and *GGTA1* evaluated as target gene).

CFTR402f-621r



GGTA3423f-3640r



HPRT834i2f-987i2r

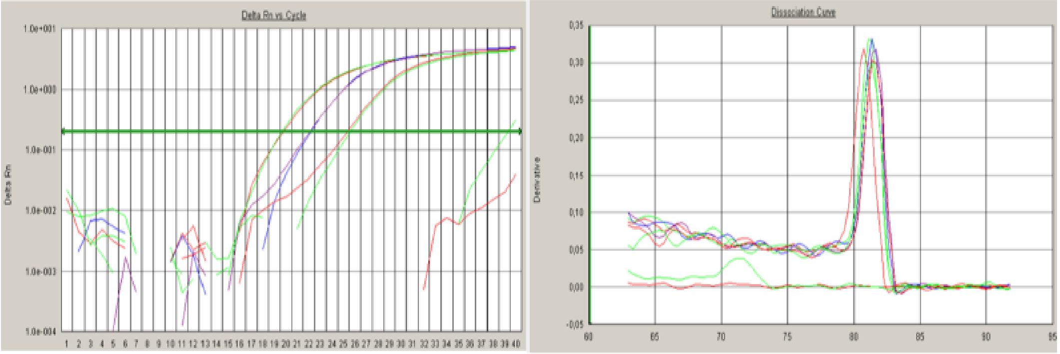
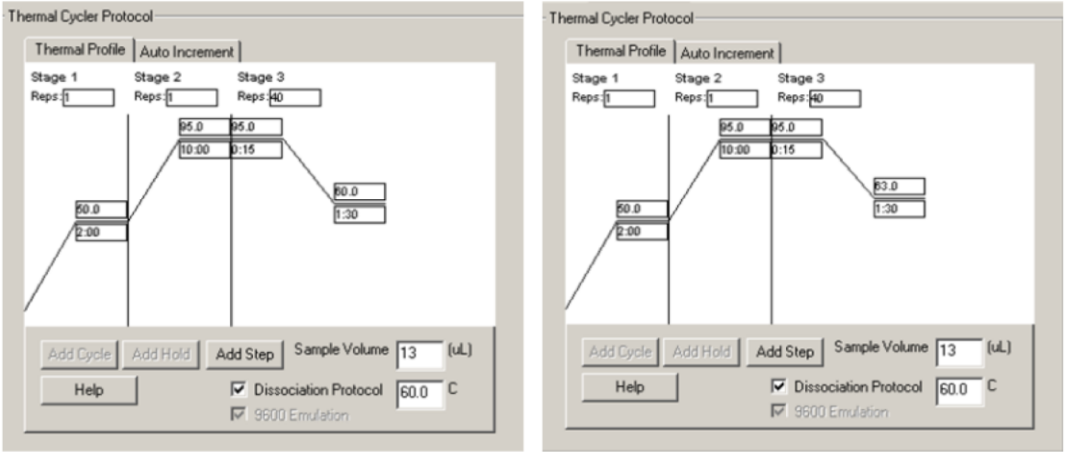


Figure 4.4: Amplification plots and dissociation curves.

The primer pairs CFTR402f-621r, GGTA3423f-3640r and HPRT834i2f-987i2r were tested in an initial qPCR approach using isolated pFF DNA diluted to concentrations of 125 ng, 12.5 ng and 1.25 ng. The screenshots represent the amplification plots on a logarithmic scale and the respective dissociation curves as an output of the ABIPrism 7000 detection system. The amplification plots indicate a serial 10-fold dilution as the single DNA curves cross the threshold (indicated by the light green horizontal line) every 3 cycles, representing a valid result for further proceeding with this PCR primer pair. The peaks of the dissociation or melting curve demonstrate a particular type of molecule (the amplicon) dissociating at a particular temperature. NTCs (no template controls) should display an almost flat line without any or too high peaks, otherwise primer dimer formation according to secondary structures might affect the amplification reaction.

The thermal profile of the qPCR performance consists of (i) an initial activation step at 50°C for 2 min, followed by (ii) an initial denaturation step at 95°C for 10 min completed by (iii) a 40 cycle repeat of denaturation at 95°C for 15 sec and primer annealing and extension at 60°C (for *CFTR*) and 63°C (*HPRT, GGTA1*) respectively, for 1.5 min (illustrated in figure 4.5).



CFTR

Thermal Cycler Protocol

Stage 1 Reps: 1
Stage 2 Reps: 1
Stage 3 Reps: 40
50.0 2:00
95.0 10:00
95.0 0:15
60.0 1:30
Sample Volume 13 (uL)
☑ Dissociation Protocol 60.0 C
☑ 9600 Emulation

HPRT/GGTA

Thermal Cycler Protocol

Stage 1 Reps: 1
Stage 2 Reps: 1
Stage 3 Reps: 40
50.0 2:00
95.0 10:00
95.0 0:15
63.0 1:30
Sample Volume 13 (uL)
☑ Dissociation Protocol 60.0 C
☑ 9600 Emulation

Figure 4.5: The qPCR thermal profile used for cell clone screening.

Three stages including an initial activation step (for UNG) for 2 min at 50°C, an initial denaturation step for 10 min at 95°C and a 40 cycle repeat of denaturation for 15 sec at 95°C and primer annealing and elongation for 90 sec at 60°C (in case of *CFTR*) and 63°C (in case of *HPRT*/*GGTA1*). After the last cycle the samples again were heated from 60°C to 95°C to obtain a dissociation curve of the PCR amplicons.

4.4.3 Routine setup for qPCR screening

Different PCR conditions for three different genes required the correlation of the copy numbers from three separated PCR runs. Thus, all screening experiments were conducted on three different plates each detecting *CFTR*, *GGTA1* or *HPRT*, respectively. One Microamp^{TM} optical 96-well plate contains the following samples: (i) genomic pig DNA at positions A1-H2 with concentrations of 10000, 7500, 5000, 2500, 500, 250, 50 and 25 copies pipetted in duplicates representing a standard curve, (ii) a triplicate of genomic pig DNA at positions H9, 10 and 11 in a concentration of around 5000 copies working as a calibrator between the three plates of one evaluation set, (iii) a no template control (NTC) at position H12 representing a negative control for the system and (iv) a maximum of 38 clones pipetted in duplicates (illustrated in figure 4.6).

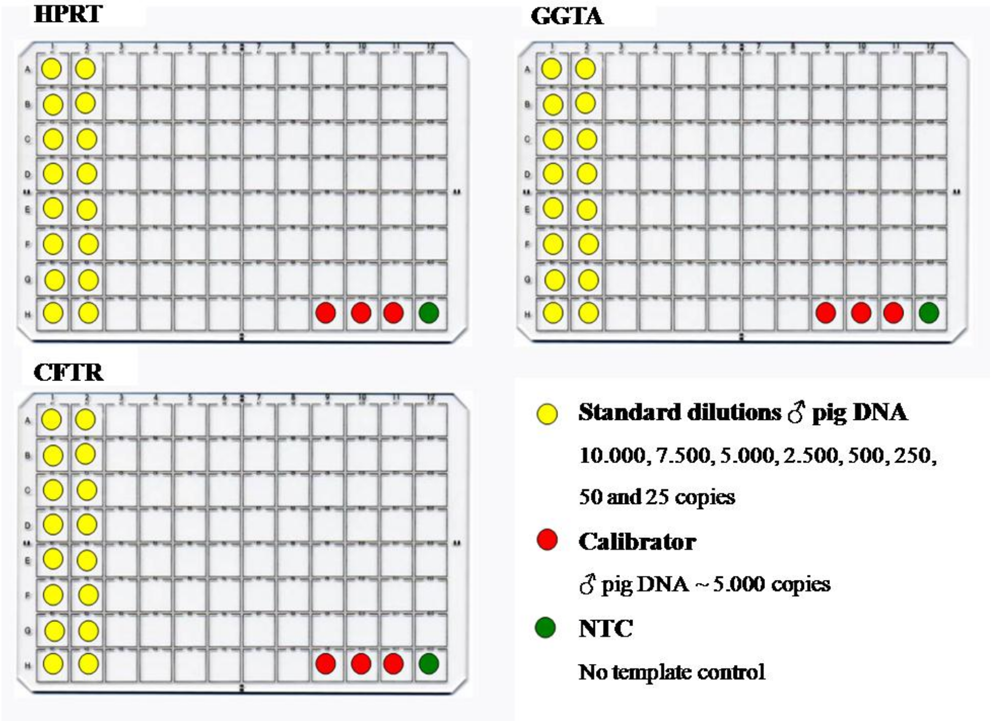


Figure 4.6: Routine setup for qPCR screening.

Standard dilutions for the standard curve in the given concentrations (assuming that 100 ng of porcine DNA is equivalent to 15000 copies), a calibrator and a no template control (NTC) are set on a Microamp^{TM} optical 96-well detection plate. It is possible to evaluate 38 different clones (duplicates) per setup. Plates are held on ice during pipetting, samples are added using filter tips.

4.4.4 Clone determination

The template number of the clones was calibrated for each amplicon using a male porcine genomic DNA with a defined copy number (~5000 copies). The calibrated copy numbers were compared by calculating the ratios *GGTA1*/*CFTR*, *HPRT*/*CFTR* and *GGTA1*/*HPRT*. For visual illustration later on, the *GGTA1*/*HPRT* ratio was omitted. In the case of random integration the number of the *CFTR* target locus was assumed to be the same as for the reference genes, thus, the calculated ratios should be around 1.0 whereas in the case of successful targeting, there would be only one remaining wild-type *CFTR* copy per diploid genome, changing the ratios with the *CFTR* copy number as divisor near 2.0.

Of course, this presumption of the copy number ratios is only valid for identical PCR efficiencies for all detected genes with a respective PCR efficiency of 100%. For this reason, it was necessary to adjust the mathematical calculation to determine positive cell clones as follows. The qPCR raw data was analyzed by the calculation of the copy number ratios of each reference gene divided by the target gene copy number. Clone DNA was pipetted in duplicates (as described in 4.4.3), the mean value (MV) as well as the two times standard deviation (2 SD) of each clone were determined. Clones exceeding the value of the 2 SD of the mean value of all ratio values per setup-plate were termed as ‘candidates’. Assuming a normal distribution of the ratio values, clones are termed as candidates with a statistical likelihood of 97.5%. An illustrated example of the candidate evaluation is given in figure 4.7-A. Those candidates are confirmed as correctly targeted in a second qPCR run using a different set of clones to ensure a different background situation (figure 4.7-B).

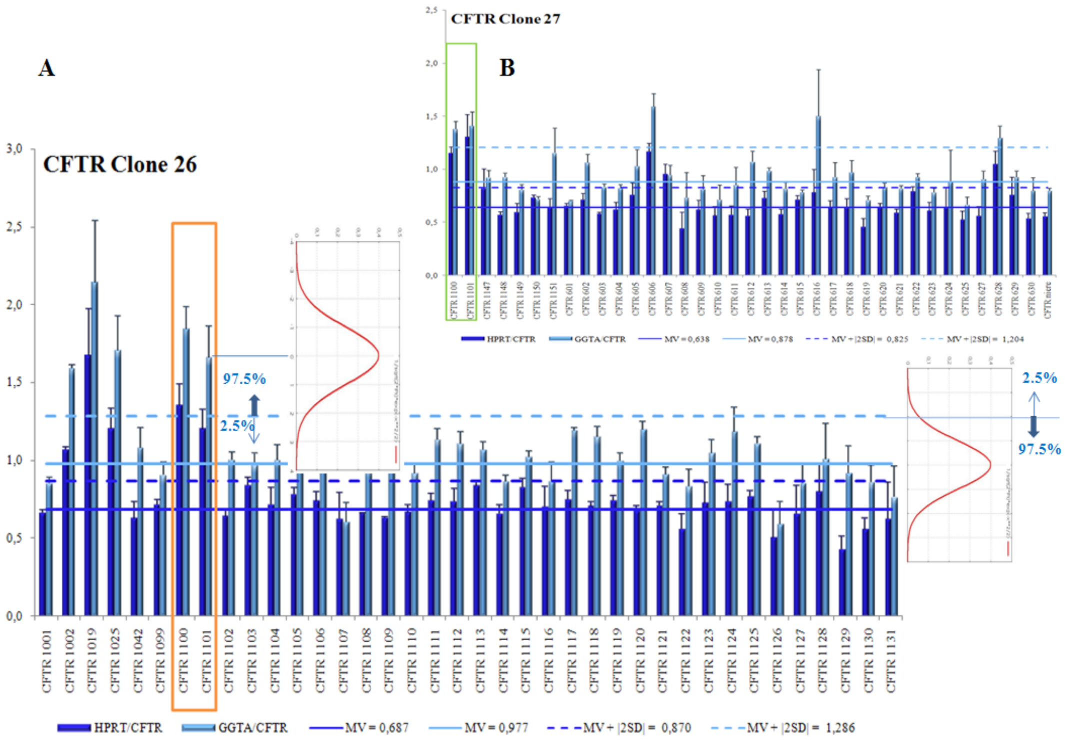


Figure 4.7: **Illustrated example of the candidate evaluation.**

The copy number ratios of each reference gene divided by the target gene copy number are used for calculating the mean value (mv; indicated by continuous lines) and the two times standard deviation (2 SD; indicated by dotted lines) among the investigated clone DNA. Clones exceeding the 2 SD-value are termed ‘candidates’. (A) Assuming a normal distribution of the ratio values, CFTR 1100 and CFTR 1101, represent correctly targeted candidates with a statistical likelihood of 97.5%. (B) The candidates are verified in a second qPCR setup, using different background clones.

Clones showing calibrated copy numbers below 30 (cn < 30) and standard deviations of their ratio values higher than 0.5 (SD > 0.5) in any of the 4 values were termed ‘not determinable’ and not considered for further processing. Additionally, not detectable wells due to pipetting errors or too low DNA amounts, leading to clones which were not evaluable also were summarized in this category. All other samples meeting the quality criteria (cn > 30, SD < 0.5) were used for the final targeted clone evaluation.

4.4.5 LOWA-screening results

The LOWA-results for the *CFTR* screening were summarized in table 4.7 and for the *GGTA1* screening in table 4.8.

Table 4.7: ***CFTR*** **knock-out screening.**

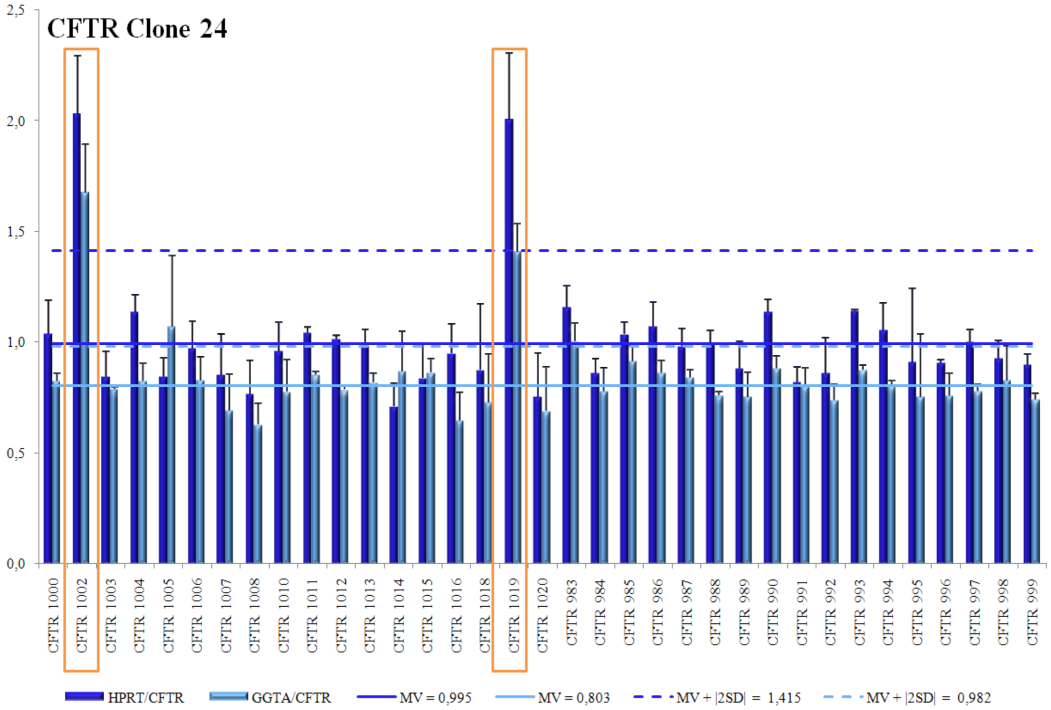
p248STOP	# epo	DNA [µg]	# clones	positive clones	confirmed
100309	2	20	51	0	0
270309	4	40	164	1	1
310309	2	14.7	19	0	0
200609	4	25.2	83	1	1
60709	5	29	329	7	6
300709	2	18	59	1	0
110809	2	11.2	165	2	0
120909	2	9.9	281	9	6
total pKC	**23**	**168**	**1151**	**21**	**14**

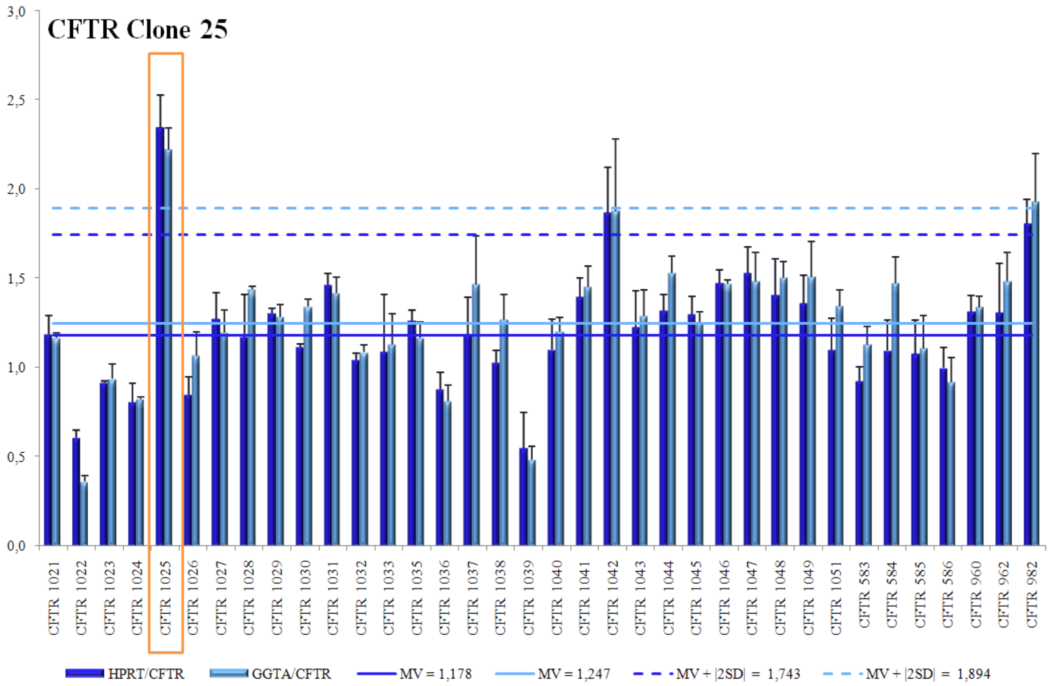
The 23 transfections of pKCs with different amounts of endotoxin-free prepared and linearized p248STOP-DNA resulted in the generation of 1151 single cell clones. After isolation of the clone DNA and evaluation of 31 clone sets (each consisting of one target gene plate and two reference gene plates) for qPCR-screening, 1034 of those clones were categorized as ‘determinable’. 14 out of 21 candidate clones were finally confirmed as correctly targeted after a second qPCR-validation. This led to a targeting efficiency of 1.35%.

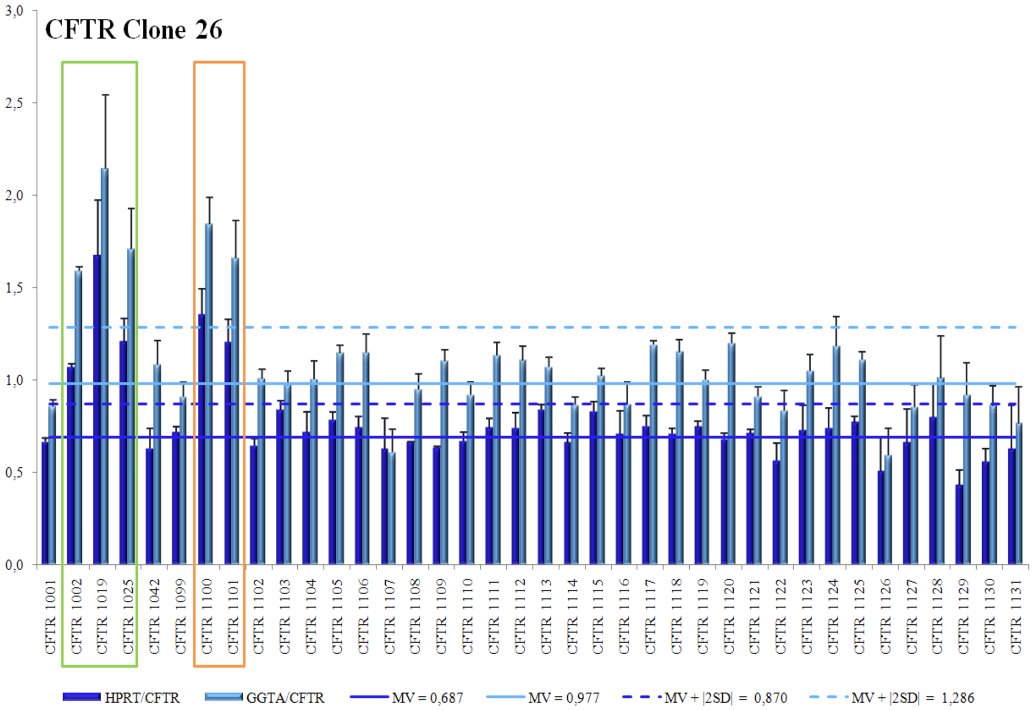
Table 4.8: ***GGTA1*** **knock-out screening.**

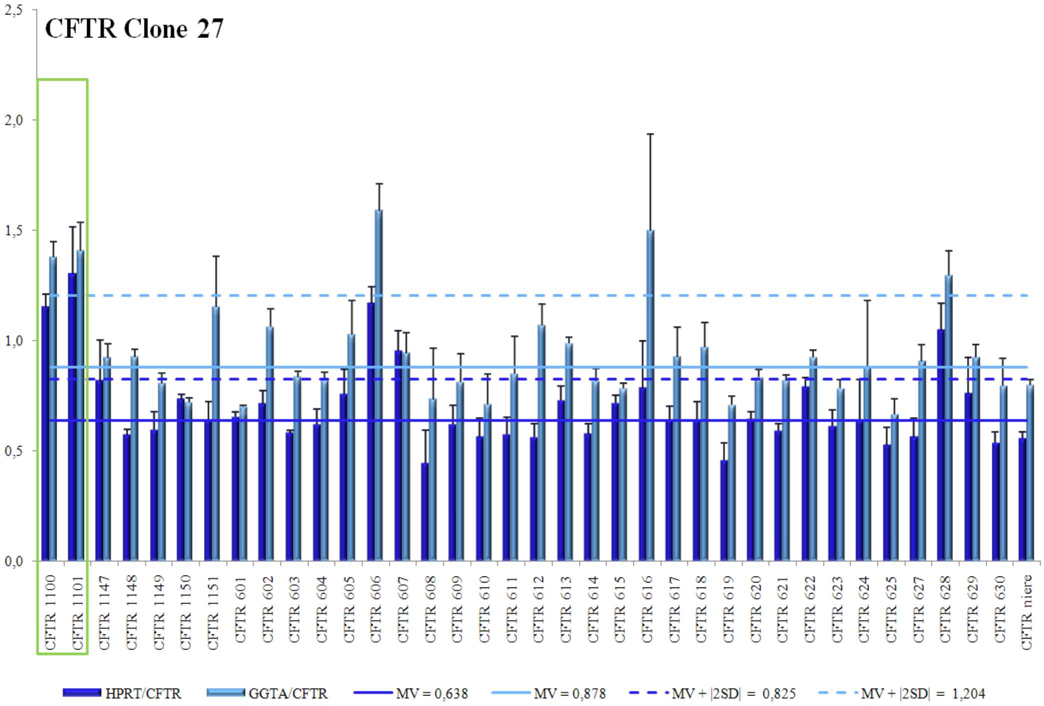
p21F3STOP	# epo	DNA [µg]	# generated clones	candidates	confirmed
310309	3	13.3	36	0	0
120909	2	11.1	269	11	8 + 1mc*
total pKC	**5**	**24.4**	**305**	**11**	**8 + 1mc***
*** mc: mixed clone**					

A total number of five electroporations using endotoxin-free prepared and linearized p21F3STOP DNA resulted in the generation of 305 neomycin resistant clones which were evaluated in eight clone sets. 230 clones were determinable from which 8 out of 11 candidates could be validated as correctly targeted. One candidate, marked as mixed clone, showed less prominent qPCR confirmation properties, presumably mediated by a mixed population, containing targeted and non-targeted cells. Nevertheless, this clone was used for nuclear transfer as well. The targeting efficiency in this case, assuming the mixed clone as positive candidate, was 3.91%. The illustrations of the correctly targeted clones for each targeting approach are shown in figure 4.8 for *CFTR* and 4.9 for *GGTA1*. Only clones subsequently used for SCNT have been considered. Finally, the correctly targeted clones were thawed, cultured and prepared for nuclear transfer.






CFTR Clone 26
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CFTR 1001
CFTR 1002
CFTR 1019
CFTR 1025
CFTR 1042
CFTR 1099
CFTR 1100
CFTR 1101
CFTR 1102
CFTR 1103
CFTR 1104
CFTR 1105
CFTR 1106
CFTR 1107
CFTR 1108
CFTR 1109
CFTR 1110
CFTR 1111
CFTR 1112
CFTR 1113
CFTR 1114
CFTR 1115
CFTR 1116
CFTR 1117
CFTR 1118
CFTR 1119
CFTR 1120
CFTR 1121
CFTR 1122
CFTR 1123
CFTR 1124
CFTR 1125
CFTR 1126
CFTR 1127
CFTR 1128
CFTR 1129
CFTR 1130
CFTR 1131
HPRT/CFTR
GGTA/CFTR
MV = 0,687
MV = 0,977
MV + |2SD| = 0,870
MV + |2SD| = 1,286


CFTR Clone 27
2,5
2,0
1,5
1,0
0,5
0,0
CFTR 1100
CFTR 1101
CFTR 1147
CFTR 1148
CFTR 1149
CFTR 1150
CFTR 1151
CFTR 601
CFTR 602
CFTR 603
CFTR 604
CFTR 605
CFTR 606
CFTR 607
CFTR 608
CFTR 609
CFTR 610
CFTR 611
CFTR 612
CFTR 613
CFTR 614
CFTR 615
CFTR 616
CFTR 617
CFTR 618
CFTR 619
CFTR 620
CFTR 621
CFTR 622
CFTR 623
CFTR 624
CFTR 625
CFTR 627
CFTR 628
CFTR 629
CFTR 630
CFTR niere
HPRT/CFTR
GGTA/CFTR
MV = 0,638
MV = 0,878
MV + |2SD| = 0,825
MV + |2SD| = 1,204

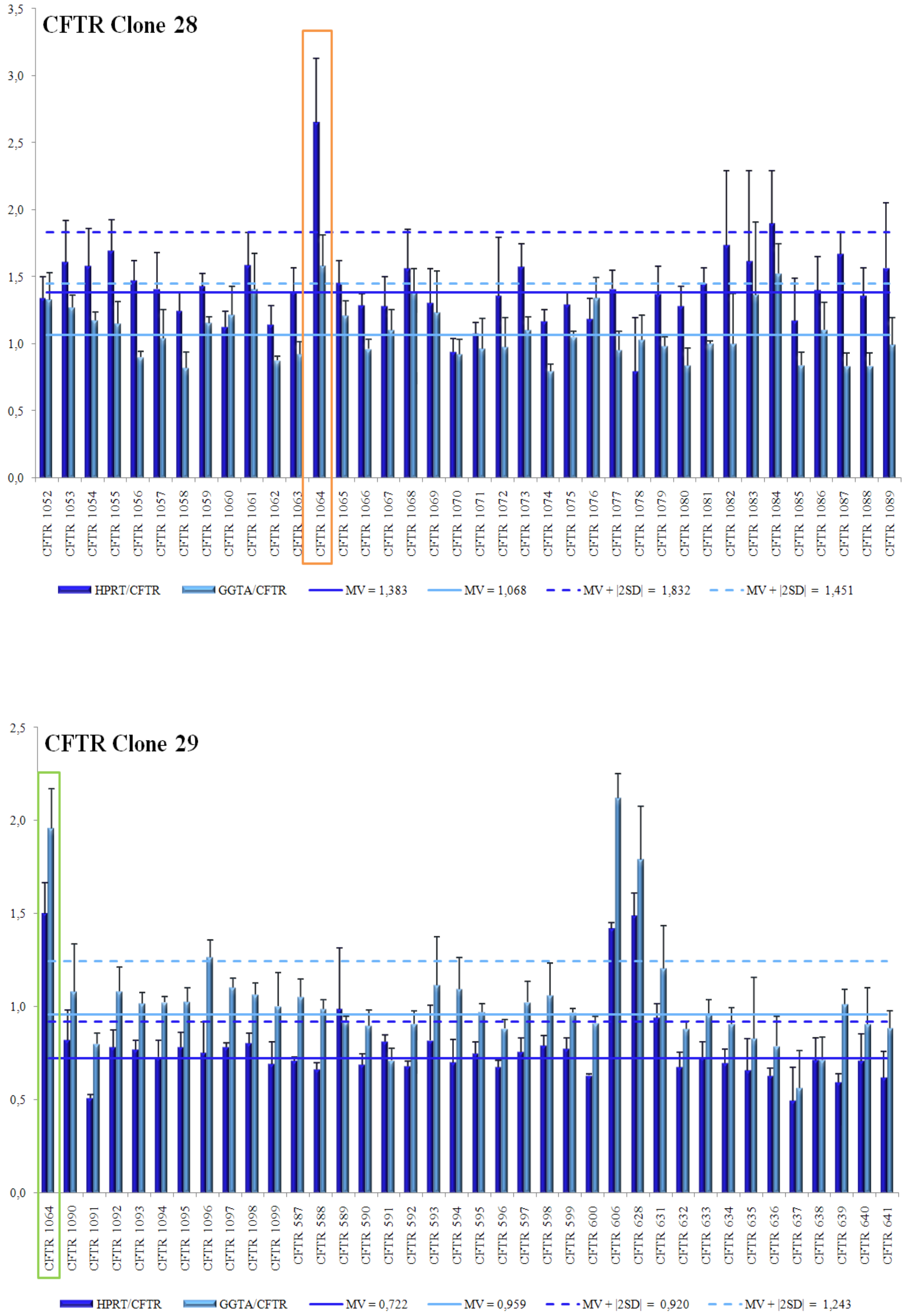
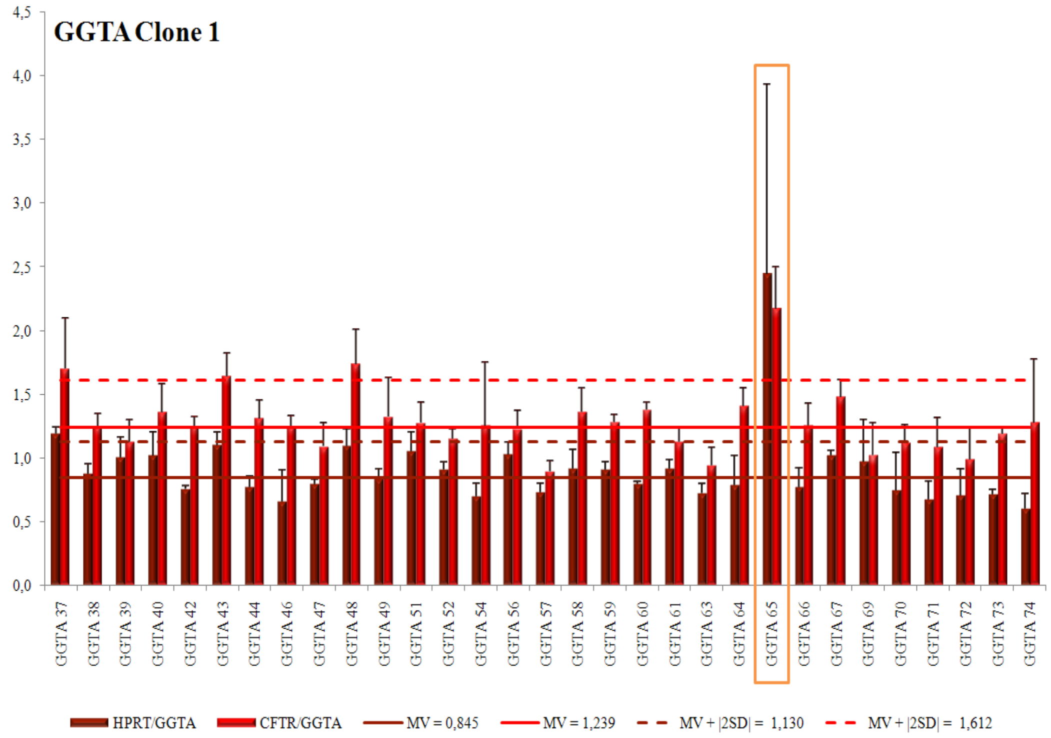
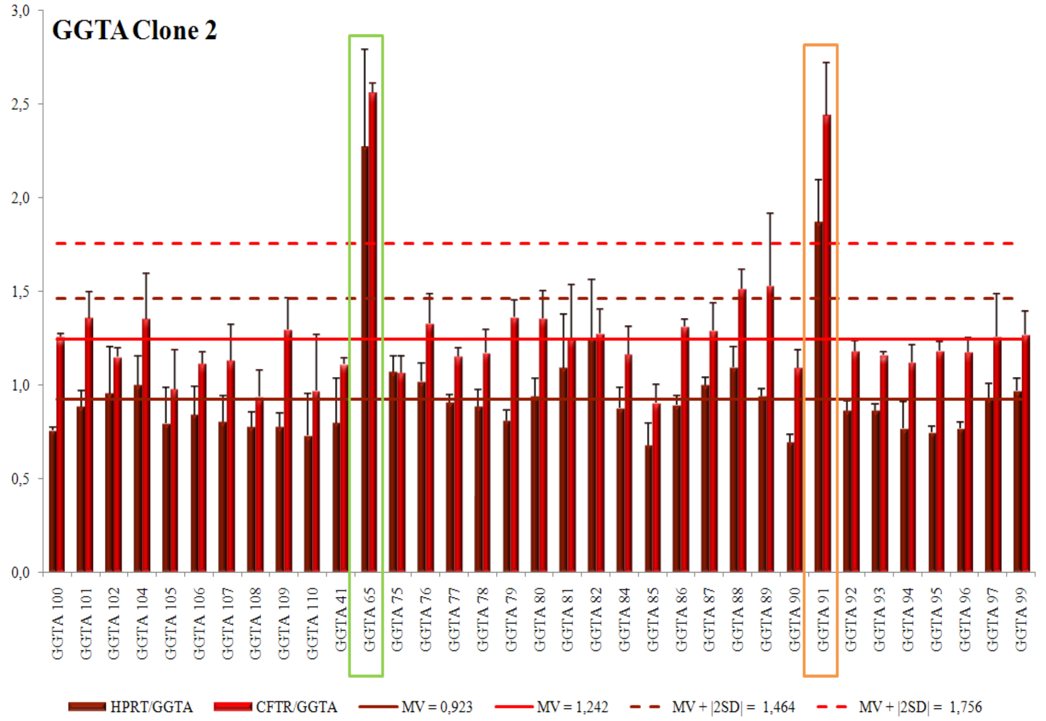


Figure 4.8: Illustration of clone sets for *CFTR*.

CFTR1002, 1019, 1025, 1100, 1101 and 1064 exceed the 2 SD-value, indicated by dotted lines, therefore considered as correctly targeted candidates. All clones are demonstrated in a first evaluation and a second confirmation run. MV: mean value, 2 SD: two times standard deviation, continuous line: mean value, dotted lines: MV plus 2 SD, orange boxes: candidates after a first qPCR evaluation, green boxes: verified candidates in a second evaluation on a different clone set, to ensure different background situations. Only clones, subsequently used for further proceeding have been considered.


GGTA Clone 1
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
GGTA 37
GGTA 38
GGTA 39
GGTA 40
GGTA 42
GGTA 43
GGTA 44
GGTA 46
GGTA 47
GGTA 48
GGTA 49
GGTA 51
GGTA 52
GGTA 54
GGTA 56
GGTA 57
GGTA 58
GGTA 59
GGTA 60
GGTA 61
GGTA 63
GGTA 64
GGTA 65
GGTA 66
GGTA 67
GGTA 69
GGTA 70
GGTA 71
GGTA 72
GGTA 73
GGTA 74
HPRT/GGTA
CFTR/GGTA
MV = 0,845
MV = 1,239
MV + |2SD| = 1,130
MV + |2SD| = 1,612


GGTA Clone 2
3,0
2,5
2,0
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1,0
0,5
0,0
GGTA 100
GGTA 101
GGTA 102
GGTA 104
GGTA 105
GGTA 106
GGTA 107
GGTA 108
GGTA 109
GGTA 110
GGTA41
GGTA 65
GGTA 75
GGTA 76
GGTA 77
GGTA 78
GGTA 79
GGTA 80
GGTA 81
GGTA 82
GGTA 84
GGTA 85
GGTA 86
GGTA 87
GGTA 88
GGTA 89
GGTA 90
GGTA 91
GGTA 92
GGTA 93
GGTA 94
GGTA 95
GGTA 96
GGTA 97
GGTA 99
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CFTR/GGTA
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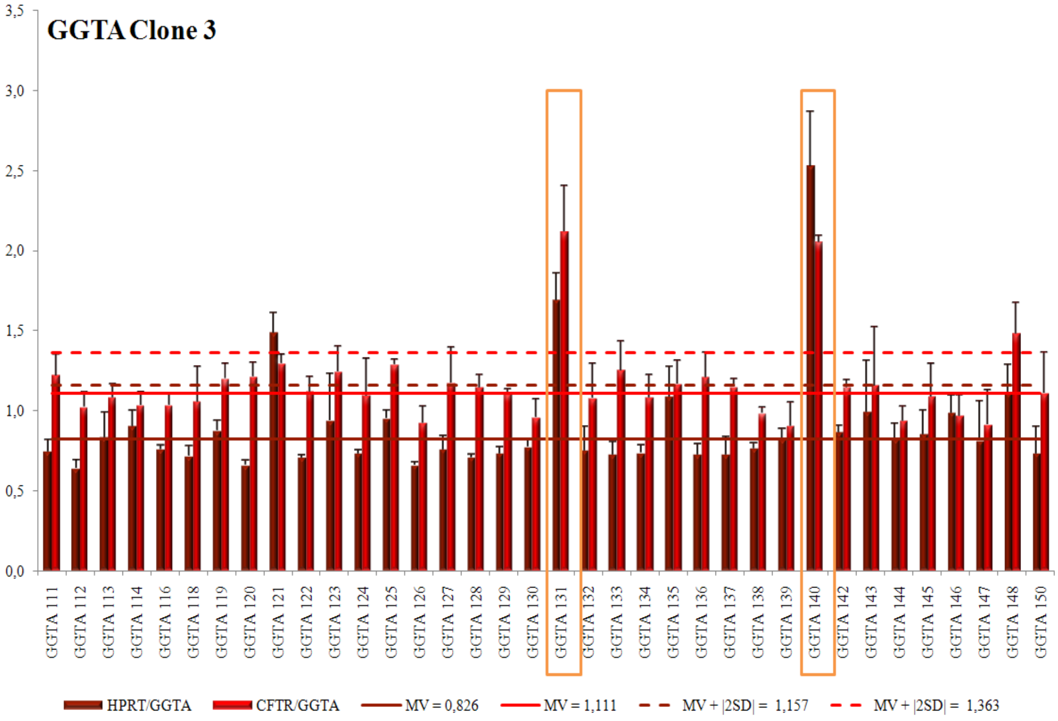
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GGTA 116
GGTA 118
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GGTA 120
GGTA 121
GGTA 122
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GGTA 125
GGTA 126
GGTA 127
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GGTA 147
GGTA 148
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CFTR/GGTA
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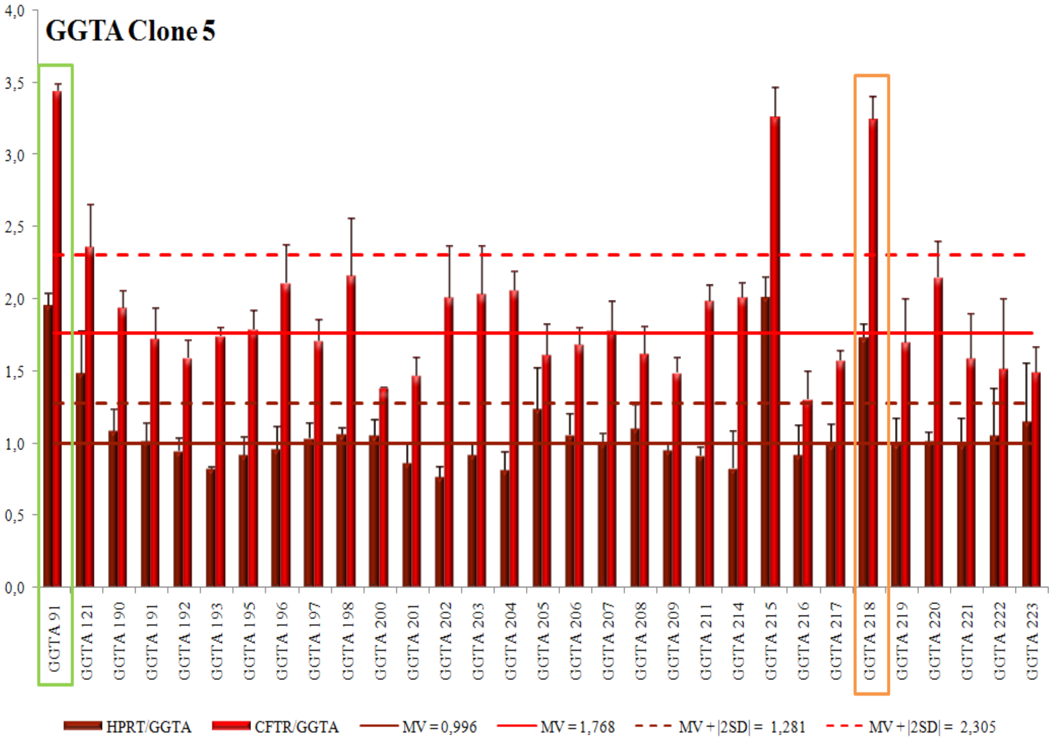
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GGTA 191
GGTA 192
GGTA 193
GGTA 195
GGTA 196
GGTA 197
GGTA 198
GGTA 200
GGTA 201
GGTA 202
GGTA 203
GGTA 204
GGTA 205
GGTA 206
GGTA 207
GGTA 208
GGTA 209
GGTA 211
GGTA 214
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GGTA 220
GGTA 221
GGTA 222
GGTA 223
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CFTR/GGTA
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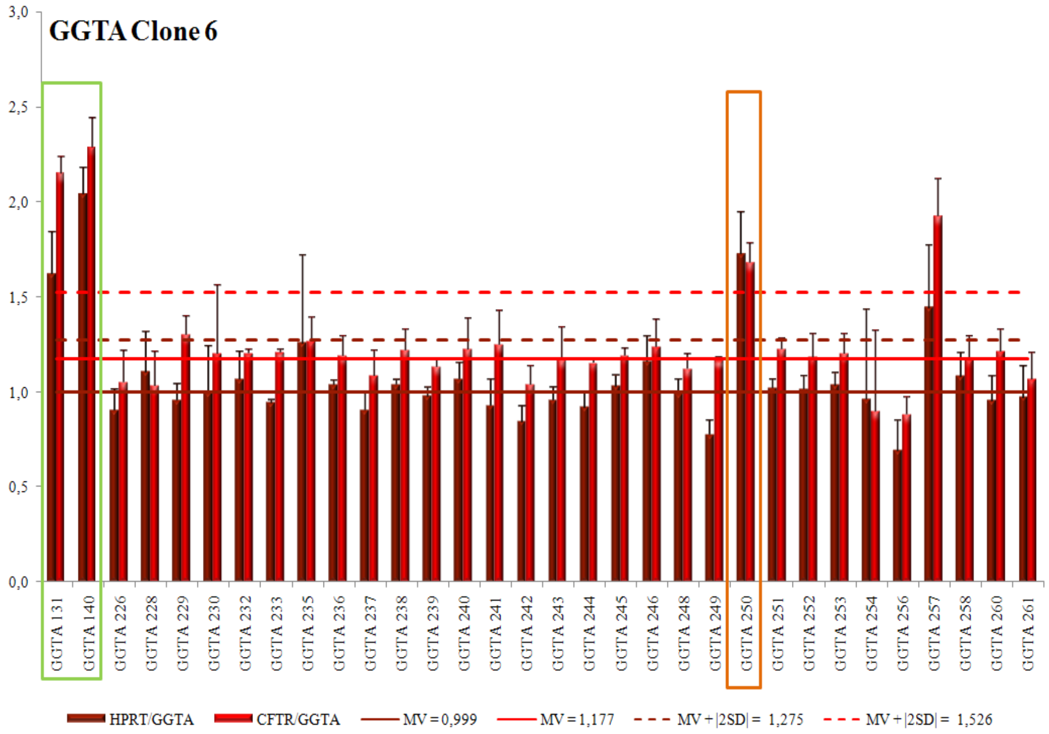
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GGTA 140
GGTA 226
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GGTA 236
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GGTA 238
GGTA 239
GGTA 240
GGTA 241
GGTA 242
GGTA 243
GGTA 244
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GGTA 248
GGTA 249
GGTA 250
GGTA 251
GGTA 252
GGTA 253
GGTA 254
GGTA 256
GGTA 257
GGTA 258
GGTA 260
GGTA 261
HPRT/GGTA
CFTR/GGTA
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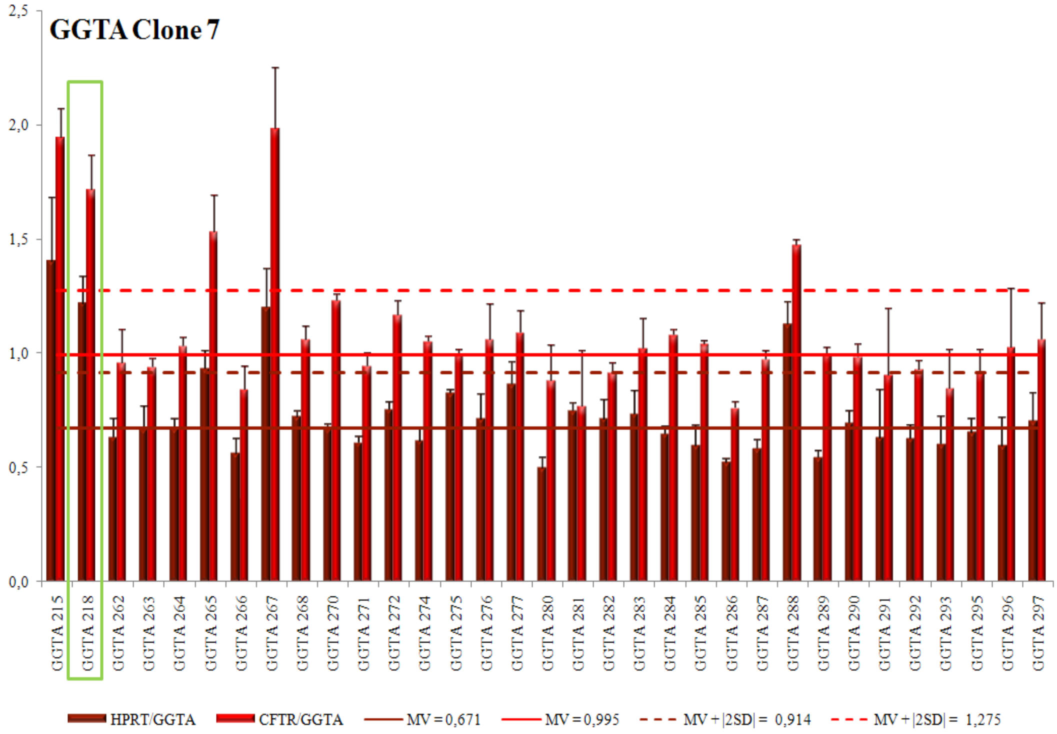
MV + |2SD| = 1,526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GGTA 287
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GGTA 290
GGTA 291
GGTA 292
GGTA 293
GGTA 295
GGTA 296
GGTA 297
HPRT/GGTA
CFTR/GGTA
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MV + |2SD| = 1,275

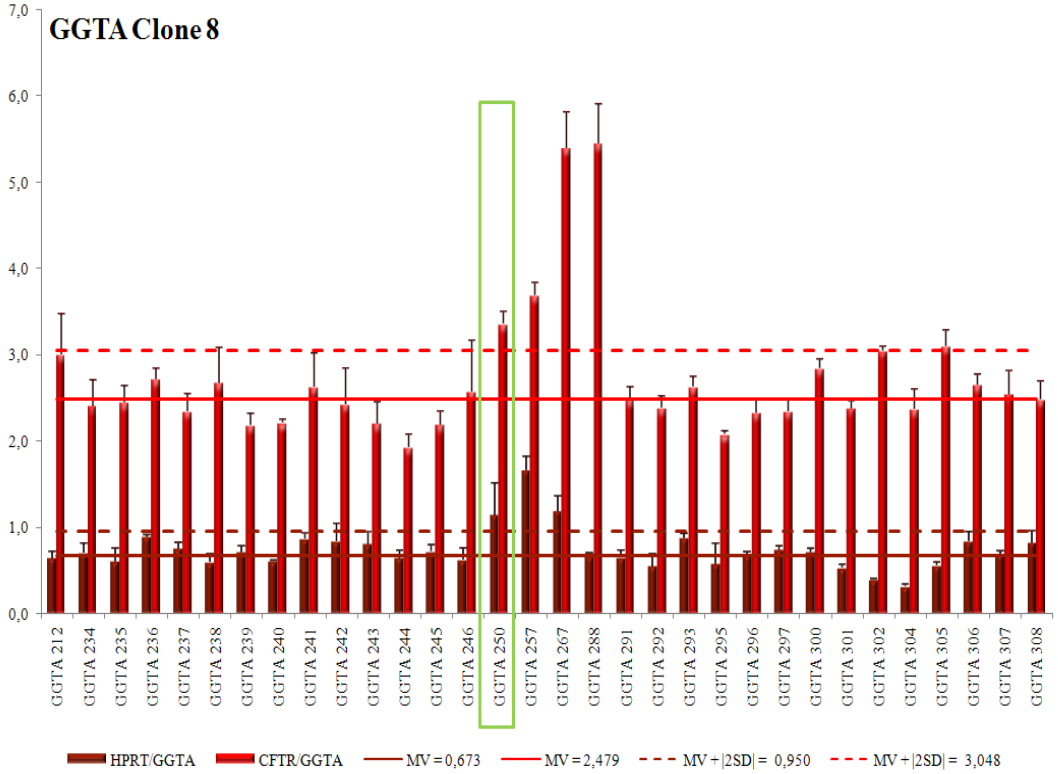


Figure 4.9: Illustration of clone sets for *GGTA1*.

GGTA 65, 91, 131, 140, 218 and 250 exceed the 2 SD-value, indicated by dotted lines, therefore considered as correctly targeted candidate. All clones are illustrated in a first evaluation and a second confirmation run. MV: mean value, 2 SD: two times standard deviation, continuous line: mean value, dotted lines: MV plus 2 SD, orange boxes: candidates after a first qPCR evaluation, green boxes: verified candidates in a second evaluation on a different clone set, to ensure different background situations. Only clones, subsequently used for further proceeding have been considered.

An overview of the candidate clones, regarding their copy number ratios (cnr), the mean value (MV) detected on the respective clone set and the ratio of both of them to visualize the change of the copy number ratios after correct targeting by dividing cnr/MV, is shown in table 4.9.

Table 4.9: Overview of candidate clones for SCNT.

candidate	MV H/C	MV G/C	cnr H/C	cnr G/C	cnr/MV (H/C)	cnr/MV (G/C)
CFTR1002	1.00	0.80	2.30	1.68	**2.30**	**2.09**
	0.69	0.98	1.06	1.59	**1.54**	**1.62**
CFTR1019	1.00	0.80	2.01	1.41	**2.02**	**1.76**
	0.69	0.98	1.70	2.14	**2.46**	**2.18**
CFTR1025	1.18	1.25	2.34	2.22	**1.98**	**1.78**
	0.69	0.98	1.20	2.14	**1.74**	**1.73**
CFTR1064	1.38	1.07	2.56	1.60	**1.92**	**1.49**
	0.72	0.96	1.50	1.96	**2.08**	**2.04**
CFTR1100	0.69	0.98	1.35	1.84	**1.96**	**1.88**
	0.64	0.88	1.15	1.37	**1.80**	**1.56**
CFTR1101	0.69	0.98	1.20	1.66	**1.74**	**1.70**
	0.64	0.88	1.30	1.40	**2.03**	**1.59**

candidate	MV H/G	MV C/G	cnr H/G	cnr C/G	cnr/MV (H/G)	cnr/MV (C/G)
GGTA65	0.85	1.24	2.45	2.17	**2.88**	**1.75**
	0.92	1.12	2.27	2.56	**2.47**	**2.06**
GGTA91	0.92	1.12	1.87	2.44	**2.03**	**1.97**
	1.00	1.77	1.95	3.44	**1.95**	**1.94**
GGTA131	0.83	1.11	1.69	2.12	**2.04**	**1.91**
	1.00	1.18	1.62	2.15	**1.62**	**1.82**
GGTA140	0.83	1.11	2.53	2.05	**3.04**	**1.85**
	1.00	1.18	2.04	2.28	**2.04**	**1.93**
GGTA218	1.00	1.77	1.73	3.24	**1.73**	**1.83**
	0.67	1.00	1.22	1.71	**1.82**	**1.71**
GGTA250*	1.00	1.18	1.72	1.68	**1.72**	**1.42**
	0.67	2.45	1.14	3.35	**1.70**	**1.37**

MV: mean value; C: *CFTR*; G: *GGTA1*; H: *HPRT*; cnr: copy number ratio; cnr/MV: ratio between cnr and MV; * indicates mixed clone;

4.5 Generation of the respective porcine animal model

4.5.1 Nuclear transfer and embryo transfer experiments

Nuclear transfer (NT) and embryo transfer (ET) technologies were carried out by Dr. Mayuko Kurome and Dr. Barbara Keßler at the Chair for Molecular Animal Breeding and Biotechnology. Practical techniques therefore are just briefly summarized below.

In vitro matured (IVM) oocytes, treated according to the protocol for oocyte maturation (reviewed in Kurome et al, 2006) were used for the somatic cell nuclear transfer (SCNT) experiment. After in vitro maturation, oocytes showing extrusion of the first polar body were actually used for enucleation. In the case of the targeting approach using the p248STOP- and p21F3STOP-constructs, pairs or triplets of selected pKCs were pooled and used as nuclear donor cells after cell cycle synchronization by serum starvation for 48 hours. Single donor cells were inserted into the perivitteline space of the enucleated oocytes. The membranes of oocyte and donor cell were fused by an electric pulse, followed by the activation of the oocyte mediated by a direct pulse. Reconstructed oocytes were cultured for one or two days until they have been used for ET. Six to seven months old estrus synchronized gilts were used as recipients for embryo transfer. The reconstructed embryos, cultured for two days after NT, were transferred laparoscopically to the right oviduct of the synchronized gilt. In case of *CFTR* 3 NT/ET experiments resulted in the establishment of two pregnancies, with an overall outcome of seven fetuses and five piglets. A total number of three NT/ET experiments using correctly targeted cell clones modified with the p21F3STOP construct, led to the establishment of two pregnancies with an overall outcome of ten fetuses, three alive piglets and one still born. The results of the NT/ET experiments for the STOP constructs (p248STOP, p21F3STOP) are illustrated in table 4.10. Finally, the gained fetuses and piglets were rescreened for the assumed targeted deletion of the *CFTR* and *GGTA1* gene, respectively.

Table 4.10: Results of NT/ET experiments for targeting approaches.

p248STOP	cell pool	maturation rate	#NT embryos	fused oocytes	embryos transferred	pregnancy	outcome*
041209	1019, 1100	142/173	121	103	93	1	7f
111209	1002, 1101	143/161	116	107	105	1	5p
220110	1025, 1064	93/138	85	75	66	0	0
p21F3STOP	**cell pool**	**maturation rate**	**#NT embryos**	**fused oocytes**	**embryos transferred**	**pregnancy**	**outcome***
290110	65, 91, 131	111/186	98	83	79	0	0
110210	140, 218	105/158	100	82	82	1	3p/1sb
170210	250**, 218	158/178	139	110	110	1	10f

***f: fetus; p: piglet; sb: still born; **indicates a mixed population**

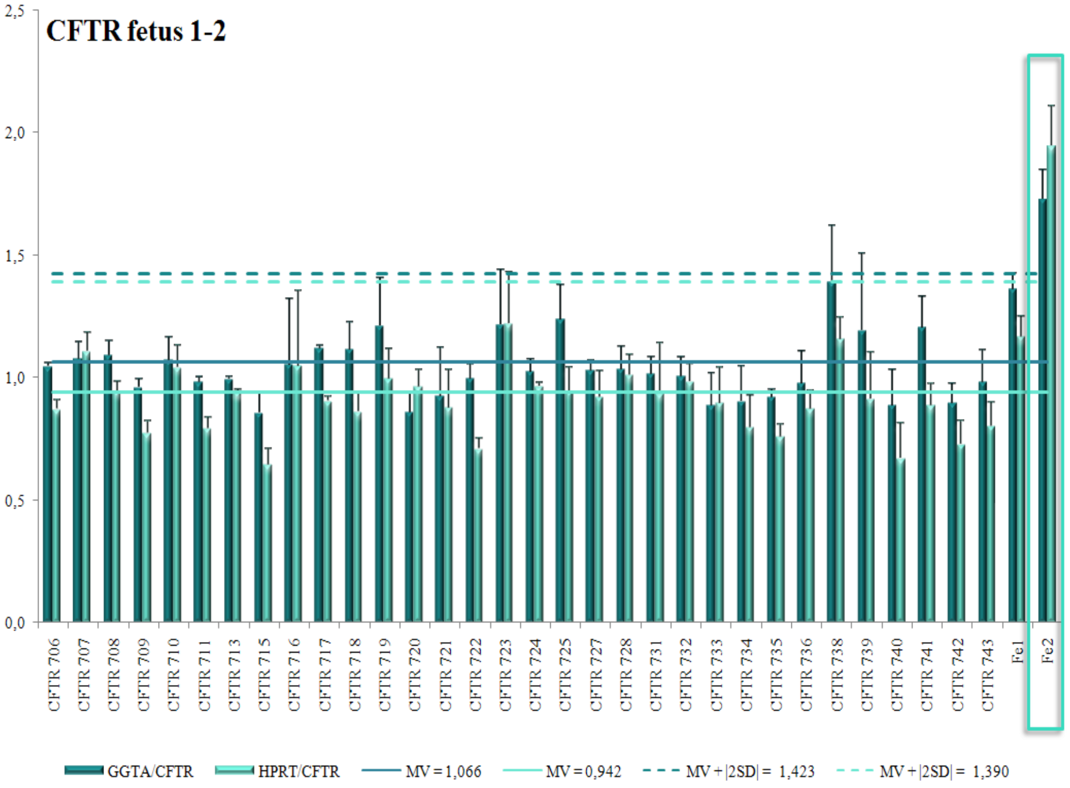
4.5.2 Evaluation of fetuses and piglets

4.5.2.1 *CFTR* results

Three NT/ET experiments (shown in table 4.10) have been performed, resulting in the establishment of two pregnancies. The NT1 pregnancy was terminated at day 59, allowing the evaluation of the kidney-tissue samples of the seven obtained fetuses for correct targeting. The NT2 pregnancy delivered five piglets (#9978-#9982) at term, from which ear tips have been taken. Kidney DNA from fetuses and DNA from ear cartilage tissue of the piglets was isolated according to the protocols given in 3.2.9.1. Subsequently, the isolated, purified DNA was tested by qPCR regarding its *CFTR*-heterozygosity.

4.5.2.1.1 Fetus verification

The isolated fetus DNA was added to a set of pKC cell clones, isolated by high-salt precipitation and obtained from the *CFTR* targeting before, providing a comparable background situation. The plates were simultaneously prepared as known from the LOWA-evaluation of the targeted *CFTR* clones described in 4.4.3 and 4.4.4. Fetus one was not confirmed as targeted, whereas fetus two to seven were confirmed as correctly heterozygously targeted. The results of the LOWA-evaluation of the fetal kidney cell DNA are shown in figure 4.10. An overview of the obtained fetuses, regarding their copy number ratios (cnr), the mean value (MV) detected on the respective clone set and the ratio of both of them to visualize the change of the copy number ratios after correct targeting by dividing cnr/MV, is shown in figure 4.11.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1,5
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CFTR 708
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CFTR 735
CFTR 736
CFTR 738
CFTR 739
CFTR 740
CFTR 741
CFTR 742
CFTR 743
Fe1
Fe2
GGTA/CFTR
HPRT/CFTR
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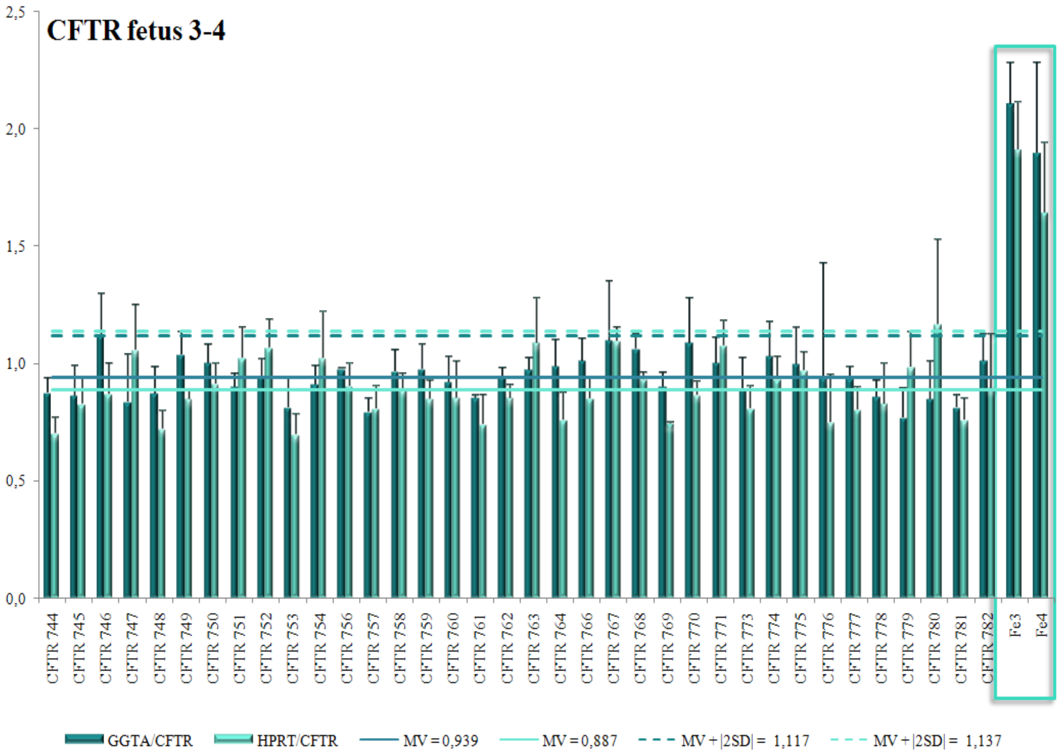
MV = 0,942
MV + |2SD| = 1,423
MV + |2SD| = 1,390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CFTR 745
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CFTR 774
CFTR 775
CFTR 776
CFTR 777
CFTR 778
CFTR 779
CFTR 780
CFTR 781
CFTR 782
Fe3
Fe4
GGTA/CFTR
HPRT/CFTR
MV = 0,939
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MV + |2SD| = 1,117
MV + |2SD| = 1,137

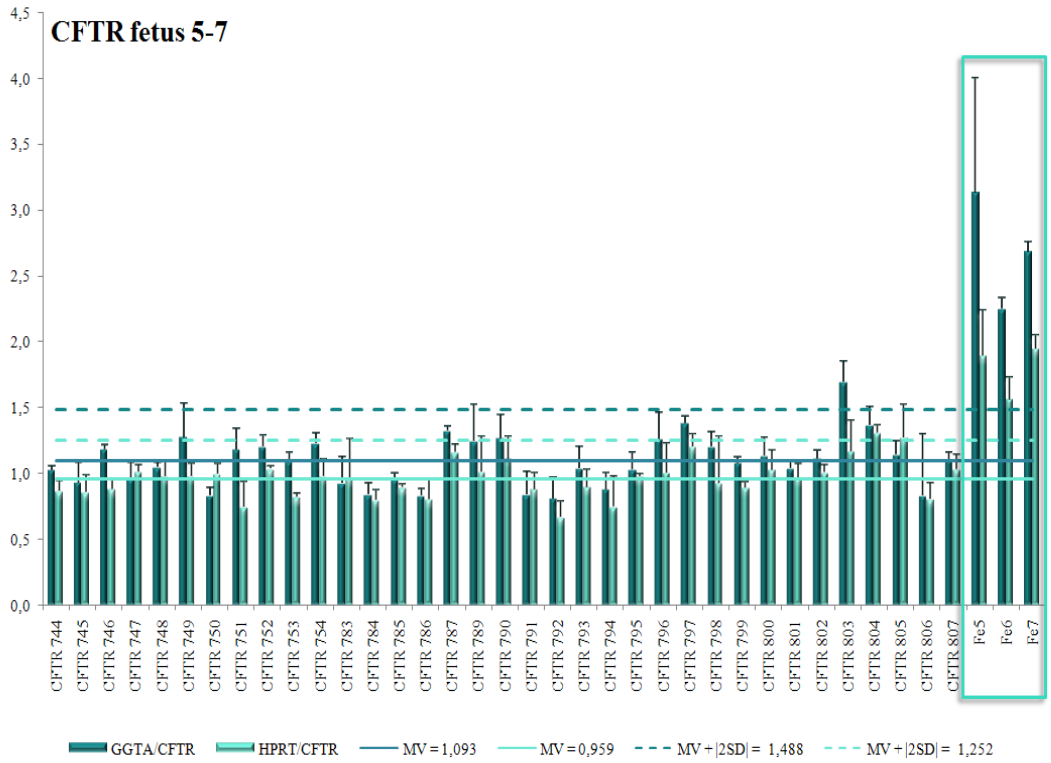
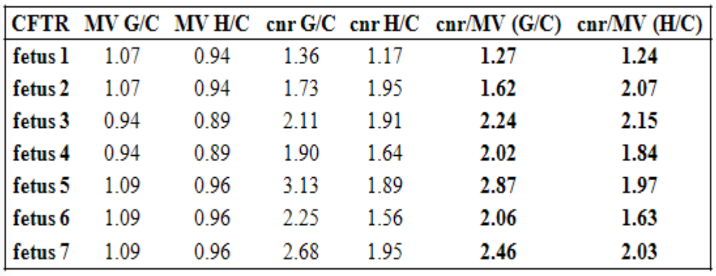


Figure 4.10: qPCR confirmation of the 7 heterozygous knock-out fetuses.

From kidney samples primary pKCs were isolated, cultured, the DNA was isolated by high-salt precipitation and used for LOWA-screening by qPCR. Fetus 1 was slightly below the predetermined 2 SD-values. Fetuses 2-7 were verified, as their copy number ratios exceed the 2 SD-value, to be heterozygous knock-outs. Background was given by high-salt isolated pKC-clones obtained from the *CFTR* targeting.



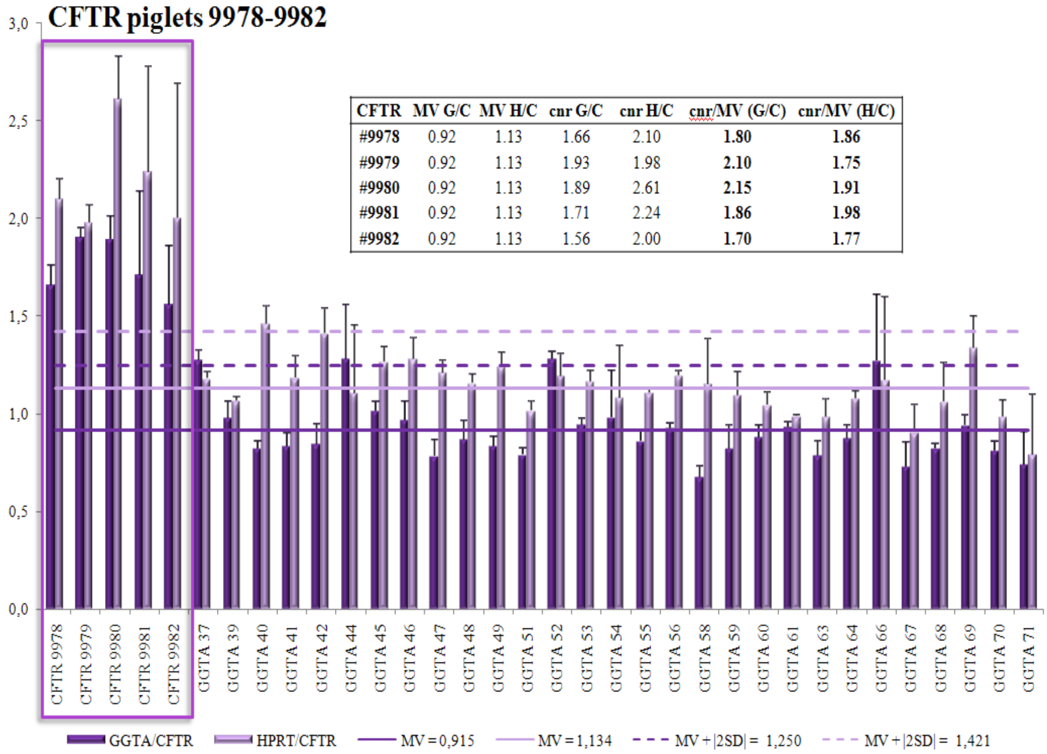
CFTR	MV G/C	MV H/C	cnr G/C	cnr H/C	cnr/MV (G/C)	cnr/MV (H/C)
fetus 1	1.07	0.94	1.36	1.17	**1.27**	**1.24**
fetus 2	1.07	0.94	1.73	1.95	**1.62**	**2.07**
fetus 3	0.94	0.89	2.11	1.91	**2.24**	**2.15**
fetus 4	0.94	0.89	1.90	1.64	**2.02**	**1.84**
fetus 5	1.09	0.96	3.13	1.89	**2.87**	**1.97**
fetus 6	1.09	0.96	2.25	1.56	**2.06**	**1.63**
fetus 7	1.09	0.96	2.68	1.95	**2.46**	**2.03**

Figure 4.11: Evaluation of the *CFTR*-fetuses.

The copy number ratios of each fetus were related to the whole plate mean value of copy number ratios obtained from the respective clone set by dividing those values. For a correct targeting, the result should be around 2.00. Due to clone set variability a range of 1.60 to 2.90 is considered. MV: mean value; C: *CFTR*; G: *GGTA1*; H: *HPRT*; cnr: copy number ratio; cnr/MV: ratio between cnr and MV.

4.5.2.1.2 Piglet verification

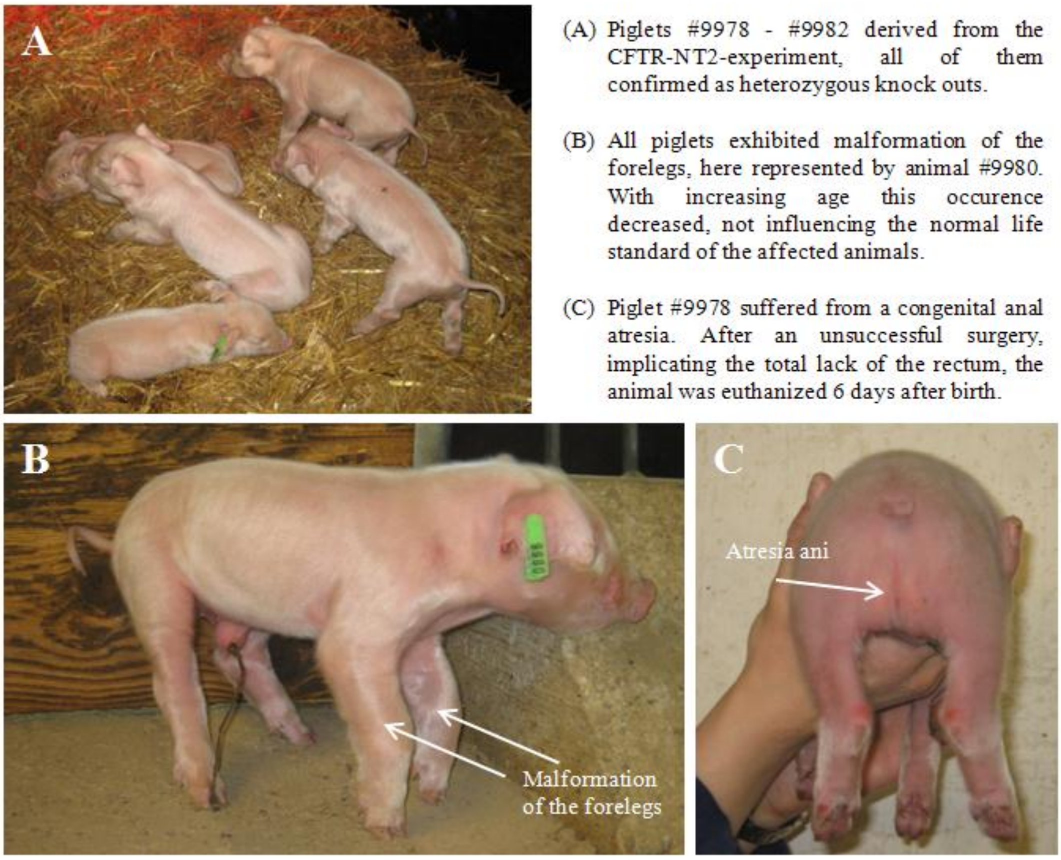
The isolated ear fibroblast DNA of the five obtained piglets was analyzed with the LOWA-assay for a heterozygous allele status. The results of the qPCR-evaluation are shown in figure 4.12. All animals reflected copy number ratios indicating a successful heterozygous targeting of the *CFTR* gene. As background, DNA isolated by high-salt precipitation from clones used for the *GGTA1* approach had been used. The *CFTR* heterozygous knock-out litter including the animals #9978-#9982 is shown in figure 4.13-A. Surprisingly, all animals established a malformation of the forelegs, shown in figure 4.13-B. With increasing age those malformations decreased, not impairing the animals' welfare. Animal #9978 suffered from a congenital atresia ani (figure 4.13-C). After an unsuccessful surgery, implicating the total lack of the rectum, the animal was euthanized on day six. Animal #9981 died with the age of four months of cystitis and rupture of the bladder, but, as assumed, not due to consequences of its *CFTR*-heterozygosity.



CFTR	MV G/C	MV H/C	cnr G/C	cnr H/C	cnr/MV (G/C)	cnr/MV (H/C)
#9978	0.92	1.13	1.66	2.10	**1.80**	**1.86**
#9979	0.92	1.13	1.93	1.98	**2.10**	**1.75**
#9980	0.92	1.13	1.89	2.61	**2.15**	**1.91**
#9981	0.92	1.13	1.71	2.24	**1.86**	**1.98**
#9982	0.92	1.13	1.56	2.00	**1.70**	**1.77**

Figure 4.12: qPCR confirmation of the 5 *CFTR*$^{-/+}$ piglets.

From ear tissue fibroblasts were isolated, cultured, the DNA was purified by high-salt precipitation and used for LOWA-screening by qPCR. All 5 piglets were verified as heterozygous knock-outs. The copy number ratios of each piglet were related to the whole plate mean value of copy number ratios obtained from the respective clone set by dividing those values. For a correct targeting, the result should be around 2.00. Due to clone set variability a range of 1.70 to 2.20 is considered. MV: mean value; C: *CFTR*; G: *GGTA1*; H: *HPRT*; cnr: copy number ratio; cnr/MV: ratio between cnr and MV.



(A) Piglets #9978 - #9982 derived from the CFTR-NT2-experiment, all of them confirmed as heterozygous knock outs.

(B) All piglets exhibited malformation of the forelegs, here represented by animal #9980. With increasing age this occurence decreased, not influencing the normal life standard of the affected animals.

(C) Piglet #9978 suffered from a congenital anal atresia. After an unsuccessful surgery, implicating the total lack of the rectum, the animal was euthanized 6 days after birth.

Figure 4.13: Piglets derived from the NT-2 experiment.

The *CFTR* heterozygous knock-out litter (#9978-#9982) is shown in (A). Malformations of the forelegs are demonstrated in (B). The congenital atresia ani of piglet #9978 is indicated in (C).

4.5.2.1.3 Production of homozygous animals and outlook

The residual three boars (#9979, #9980, #9982) were used to establish additional heterozygous pigs which subsequently are going to be used for interbreeding to obtain homozygous knock-outs. Mating results of the heterozygous animals with wild-type German landrace sows are shown in table 4.11.

Table 4.11: Mating results of *CFTR*$^{+/-}$ boars.

CFTR$^{-/+}$ **boar**	**litter**	**male/female**	**hemizygous knock-out**
# 9979	170411	7/7	1/3
# 9980	140311	8/0	2/0
# 9982	141211	6/3	4/2
		21/10	**7/5**

The results are in line with the expected Mendelian distribution. Animal #9981, which was euthanized, due to cystitis and bladder rupture with the age of four months, was used to establish a heterozygous kidney cell line to target the second

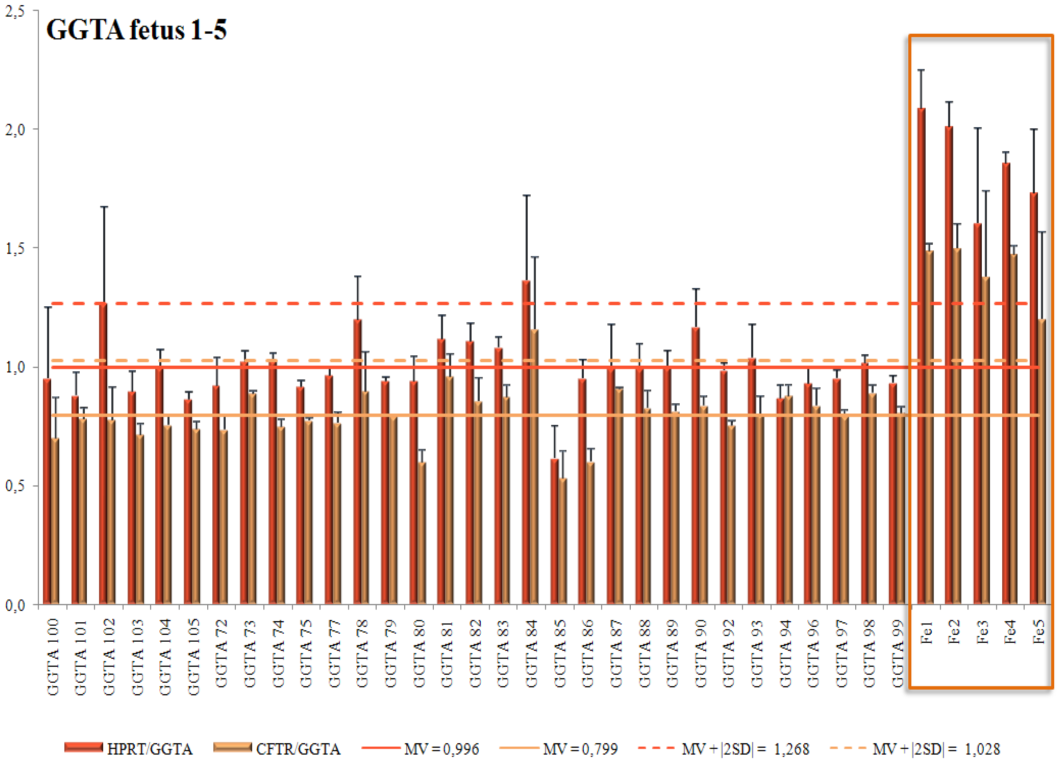
CFTR allele. For this reason the floxed neokan® cassette was exchanged through arabinose induced Cre-mediated excision by a blasticidin resistance (bsr®) cassette. *CFTR*$^{-/+}$ cells have been nucleofected with the targeting vector, carrying the bsr® resistance gene, subsequently were preselected by blasticidin S. A total number of 213 resistant colonies have been generated, 173 have been screened using the LOWA-assay and two of them could be determined as *CFTR*$^{-/-}$ (carried out by Dr. Nikolai Klymiuk and Anne Richter). One of those homozygous knock-out clones was used for SCNT. An overall number of 348 embryos were transferred to four synchronized gilts, two pregnancies were established and the delivery at term resulted in litters of nine and two piglets, respectively. Two piglets were still born, all the others were alive, but had to be euthanized after a maximum of 37 hours (for further information read Klymiuk *et al.*, 2011).

4.5.2.2 *GGTA1* results

Three NT/ET experiments (table 4.10) have been performed. NT2 (GGTA140, GGTA218) delivered three alive and one still born piglets at term. The pregnancy resulting from NT3 (GGTA250, GGTA218) was terminated at day 58, allowing the evaluation of the kidney-tissue samples of the ten obtained fetuses for correct targeting. From the piglets (#9987-#9990), ear tips have been taken. DNA has been isolated according to the protocol described in 3.2.9.1, except for #9990, as it was not possible to isolate DNA from the last (still born) piglet. Kidney DNA from fetuses also was isolated as described in 3.2.9.1. Subsequently, the isolated, purified DNA was tested by qPCR regarding its *GGTA1*-heterozygosity.

4.5.2.2.1 Fetus verification

The isolated fetus DNA was evaluated by adding it to sets of pKC cell clones, isolated by high-salt precipitation and obtained from the *GGTA1* targeting before, providing a comparable background situation. The plates were equally prepared as described in the LOWA-evaluation of the targeted *GGTA1* clones (4.4.3 and 4.4.4) Fetus 6, 8 and 9 were not confirmed as targeted, whereas fetus 1, 2, 3, 4, 5, 7 and 10 were confirmed as *GGTA1*$^{-/-}$. The results of the LOWA-evaluation of the fetal kidney cell DNA are shown in figure 4.14.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GGTA 97
GGTA 98
GGTA 99
Fe1
Fe2
Fe3
Fe4
Fe5
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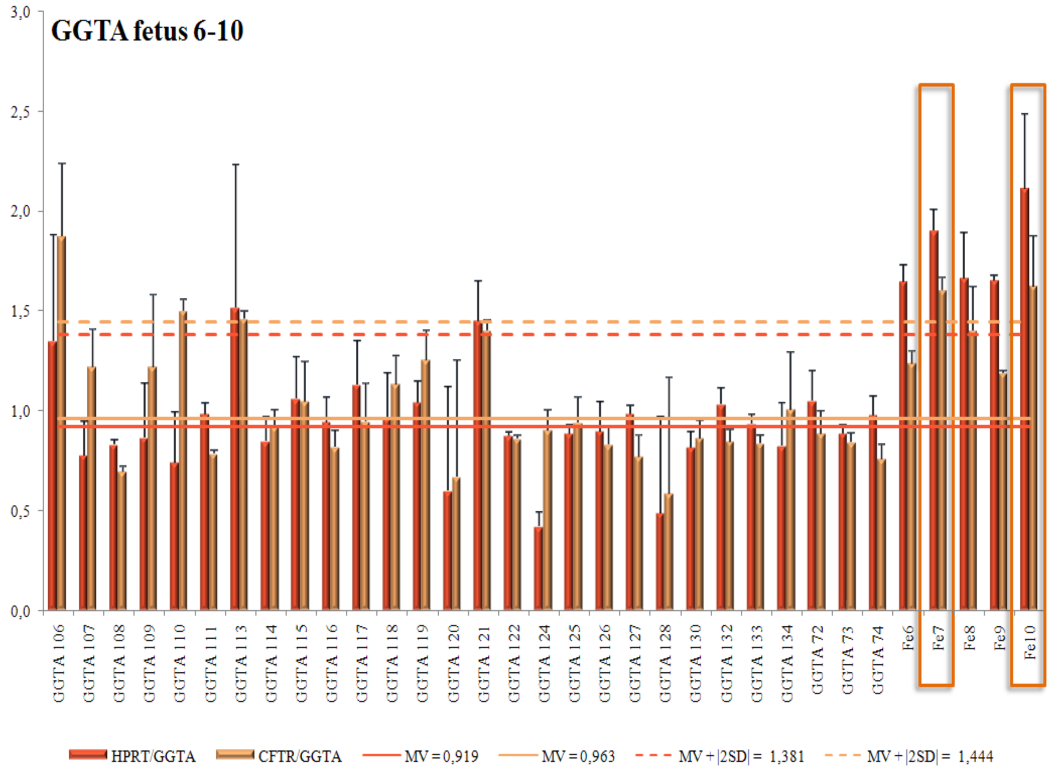
CFTR/GGTA
MV = 0,996
MV = 0,799
MV + |2SD| = 1,268
MV + |2SD| = 1,028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GGTA 122
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GGTA 125
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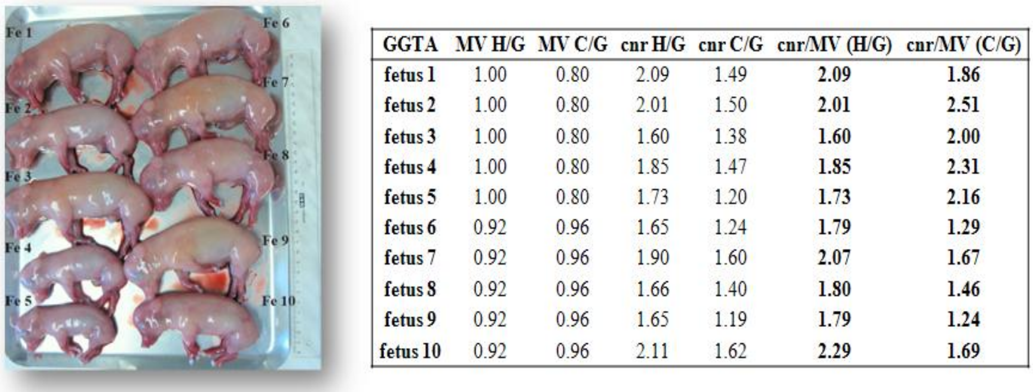
GGTA 127
GGTA 128
GGTA 130
GGTA 132
GGTA 133
GGTA 134
GGTA 72
GGTA 73
GGTA 74
Fe6
Fe7
Fe8
Fe9
Fe10
HPRT/GGTA
CFTR/GGTA
MV = 0,919
MV = 0,963
MV + |2SD| = 1,381
MV + |2SD| = 1,444

Figure 4.14: qPCR confirmation of the 10 *GGTA1*-fetuses.

DNA, isolated by high-salt precipitation, gained from pKCs of the fetal kidney samples, was used for LOWA-evaluation. Copy number ratios of fetus 6, 8 and 9 were below the predetermined 2 SD-values. Fetuses 1-5, 7 and 10 were verified, as their copy number ratios exceed the 2 SD-value, to be heterozygous knock-outs. Background was given by high-salt isolated pKC-clones obtained from the *GGTA1* targeting.

An overview of the obtained fetuses, regarding their copy number ratios (cnr), the mean value (MV) detected on the respective clone set and the ratio of both of them to demonstrate the change of the copy number ratios after correct targeting by dividing cnr/MV, is shown in figure 4.15.



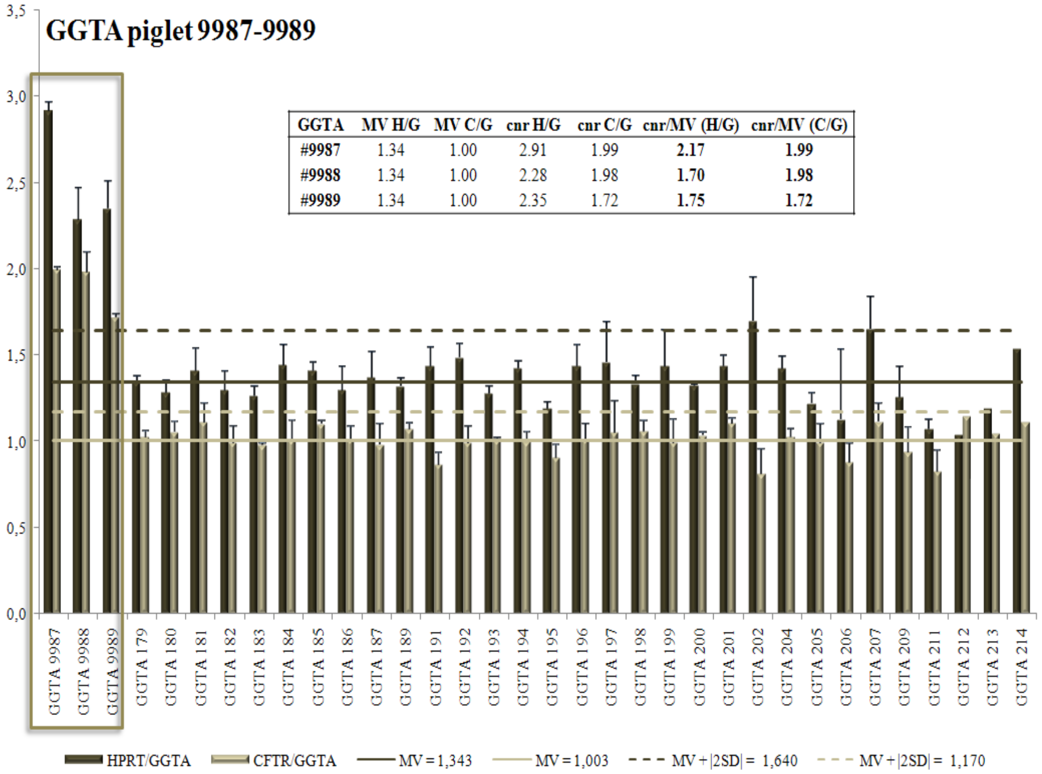
GGTA	MV H/G	MV C/G	cnr H/G	cnr C/G	cnr/MV (H/G)	cnr/MV (C/G)
fetus 1	1.00	0.80	2.09	1.49	**2.09**	**1.86**
fetus 2	1.00	0.80	2.01	1.50	**2.01**	**2.51**
fetus 3	1.00	0.80	1.60	1.38	**1.60**	**2.00**
fetus 4	1.00	0.80	1.85	1.47	**1.85**	**2.31**
fetus 5	1.00	0.80	1.73	1.20	**1.73**	**2.16**
fetus 6	0.92	0.96	1.65	1.24	**1.79**	**1.29**
fetus 7	0.92	0.96	1.90	1.60	**2.07**	**1.67**
fetus 8	0.92	0.96	1.66	1.40	**1.80**	**1.46**
fetus 9	0.92	0.96	1.65	1.19	**1.79**	**1.24**
fetus 10	0.92	0.96	2.11	1.62	**2.29**	**1.69**

Figure 4.15: Evaluation of the *GGTA1*-fetuses.

The copy number ratios of each fetus were related to the whole plate mean value of copy number ratios obtained from the respective clone set by dividing those values. For a correct targeting, the result should be around 2.00. Due to clone set variability a range of 1.60 to 2.60 is considered. MV: mean value; C: *CFTR*; G: *GGTA1*; H: *HPRT*; cnr: copy number ratio; cnr/MV: ratio between cnr and MV

4.5.2.2.2 Piglet verification

DNA of the three live born piglets was isolated from the taken ear tips and subsequently analyzed by the LOWA-assay for a heterozygous allele status. The results of the qPCR evaluation are shown in figure 4.16. All produced animals could be determined, regarding their copy number ratios, as heterozygously targeted for the *GGTA1* gene. As background, DNA isolated by high-salt precipitation from clones used for the *GGTA1* approach before had been used. An overview of the copy number ratio change as an indication of correct targeting is given in figure 4.16 as well.



GGTA	MV H/G	MV C/G	cnr H/G	cnr C/G	cnr/MV (H/G)	cnr/MV (C/G)
#9987	1.34	1.00	2.91	1.99	**2.17**	**1.99**
#9988	1.34	1.00	2.28	1.98	**1.70**	**1.98**
#9989	1.34	1.00	2.35	1.72	**1.75**	**1.72**

Figure 4.16: qPCR confirmation of the 3 heterozygous *GGTA1*$^{-/+}$ piglets.

From ear cartilage tissue fibroblasts were isolated and cultured, the DNA was purified by high-salt precipitation and used for LOWA-screening by qPCR. All 3 piglets were verified as heterozygous knock-outs. The copy number ratios of each piglet were related to the whole plate mean value of copy number ratios obtained from the respective clone set by dividing those values. For a correct targeting, the result should be around 2.00. Due to clone set variability a range of 1.70 to 2.20 is considered. MV: mean value; C: *CFTR*; G: *GGTA1*; H: *HPRT*; cnr: copy number ratio; cnr/MV: ratio between cnr and MV.

4.5.2.2.3 Outlook

Boar #9988 was euthanized with the age of three months to obtain a *GGTA1*$^{-/+}$ kidney cell line, which subsequently can be used for the targeting of the second *GGTA1* allele. In order to perform a homozygous targeting, the neokan^{R} cassette from one allele is exchanged by a blasticidin (bsr$^{®}$) resistance cassette, enabling the preselection of nucleofected clones with the bsr$^{®}$ targeting vector, by blasticidin S. After generation of homozygous knock-out clones finally the neokan^{R} and the bsr$^{®}$ cassette are going to be removed by arabinose-induced Cre-mediated cassette excision utilizing the lox sites of the respective cassette. Resulting clones are going to be used for NT/ET experiments to obtain *GGTA*$^{-/-}$ animals without any antibiotic resistance background. Additionally, the generation of *GGTA*$^{-/-}$ animals can be achieved by breeding.

4.6 Reporter gene strategy: a side project

In order to evaluate if BAC vectors are suitable tools for additive gene transfer, a side project was pursued in the context of this doctoral thesis. One possibility to determine the distribution of one gene of interest is to construct an expression vector which substitutes the respective gene by a reporter gene, visualizing the activity of the endogenous promoter. On that account, the *lacZ* gene (encoding β-galactosidase) was introduced, similar to the STOP-box, behind the ATG of exon I and IV of the *CFTR* and the *GGTA1* gene, respectively. Thus, the *lacZ* reporter, terminating the transcription by a bGH-pA (bovine growth hormone-polyadenylation) signal, is transcribed instead of the respective gene. The modification vectors pCFTR-lacZ and pGGTA-lacZ were similarly designed and constructed. Restriction digests and ligations were carried out as described in 3.2.4 and 3.2.6, respectively. PCRs were performed according to the standard protocol shown in 3.2.1.1 with varying annealing temperature and elongation time, given in brackets.

4.6.1 Design and construction: pCFTR-lacZ and pGGTA-lacZ

The 2 kb floxed neomycin resistance cassette, lox^{2}neo (NEO), derived from the vector pPNTlox^2 was exchanged by a PGK (phosphoglycerate kinase) promoter driven, floxed 2.2 kb neomycin/kanamycin resistance (neokan^R) cassette from pL452 via arabinose induced Cre-recombination. Neokan^R was cloned by NotI/BamHI into the pBSK II vector (pBSK-NEO). The 0.5 kb 3'arm-fragment was amplified from genomic pig DNA by conventional PCR using the primer pairs CFTR-3armf/CFTR-3armr (58°C annealing, 1 min elongation) and GGTA-3armf/GGTA-3armr (58°C annealing, 1 min elongation). The amplified fragments were digested with NotI/NsiI and then ligated into the lox^{2}neo-pBSK II vector (pBSK-3arm-NEO). The *lacZ*-fragment, derived from the pSV-β-Galactosidase Control Vector (Promega, Mannheim) was cloned via NcoI/XbaI into the bGHpA/pGEM vector (kindly provided by Marlon Schneider) (pGEM-lacZ). The 0.5 kb 5'arm-fragment was amplified from genomic BAC-DNA (CH242-248P18, *CFTR*; CH242-21F3, *GGTA1*) using primers CFTR-5armf/CFTR-lacZr and GGTA-5armf/GGTA-lacZr, respectively (annealing 58°C, elongation 30 sec). In parallel, the *lacZ*-fragment was amplified from the pSV-β-Galactosidase Control Vector using the primer pair CFTR-lacZ/lacZr and GGTA-lacZf/lacZr (annealing 58°C, elongation 1 min). The 5'arm- and the *lacZ*-PCR-fragment were eluted from an agarose gel and subsequently used as templates for a 2-step-fusion-PCR using the flanking primers CFTR-5armf/lacZr and GGTA-5armf/lacZr according to the cycling protocol described in table 3.2. The PCR fusion-amplificates were directly digested with KpnI/EcoRV and then ligated into the KpnI/EcoRV digested pGEM-lacZ vector (pGEM-5arm-ATG-lacZ). The resulting 5arm-ATG-lacZ fragment was cloned by a SalI/KpnI digest into the pBSK-3arm-NEO vector resulting in the modification vectors pCFTR-lacZ and pGGTA-lacZ (figure 4.17). The resulting plasmids have been used for the modification of the respective BACs by recombineering.

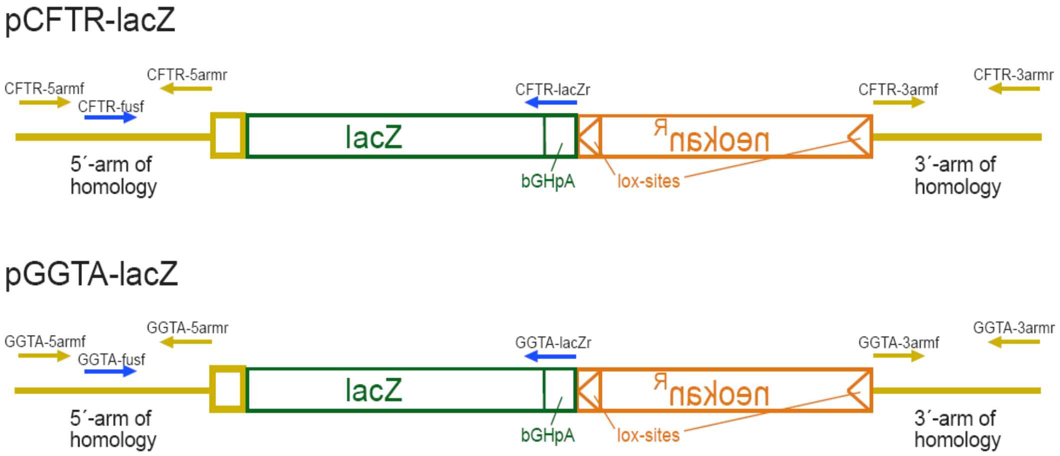


Figure 4.17: Construction of the pCFTR-lacZ and pGGTA-lacZ modification vectors.

The modification vectors for the introduction of the *lacZ* gene into the porcine genome contain (i) a 5'-arm of homology and a (ii) 3'-arm of homology, amplified from genomic DNA by primer pairs indicated with yellow arrows, (iii) a floxed neomycin/kanamycin resistance (neokan^R) cassette driven by a PGK (phosphoglycerate kinase) promoter and (iv) the *lacZ* reporter, terminating the transcription by a bGH-pA (bovine growth hormone-polyadenylation) signal, which subsequently is transcribed instead of the respective gene. Primer pairs to fuse the reporter gene to the respective ATG are indicated with blue arrows.

4.6.2 Modification of wild-type BACs

The modification vectors pCFTR-lacZ and pGGTA-lacZ and the respective wild-type BACs (CH242-248p18 and CH242-21F3) were prepared according to the plasmid preparation protocol (adapted from Sambrook, 2001) described in 3.2.9.2. Thereafter, the isolated BAC-DNA was transferred to recombineering competent SW106 *E. coli* cells (500 ng BAC-DNA per aliquot) by electroporation. The bacterial suspension was plated after the electro-transformation step on agar plates containing the suitable antibiotic (kanamycin). Cells were grown over night at 32°C. A resulting colony was picked, tested for the correct uptake of the BAC by conventional PCR and prepared as given in table 3.4 (SW106-recombineering). In parallel, the purified plasmid DNA was digested overnight (read 3.2.4) with KpnI/Cfr42I and the respective fragments were eluted from the agarose gel. Aliquots of the SW106 cells, containing the BAC of interest, were used for the electrotransformation step as described in 3.2.7.2. Two different amounts of linearized modification-fragment were used: 30 ng and 100 ng. After two hours recovery at 32°C (allowing activity of recombineering enzymes), the cells were plated on agar plates (containing kanamycin) and were incubated overnight at 32°C. Clones appearing after recombineering on LB + Kan plates, were picked and prepared for screening PCR as described in 3.2.9.2 (heating step isolation). The isolated BAC-DNA modified with the pCFTR-lacZ construct was screened with the primer pair CFTR-6456f/CFTR-7160r (annealing 60°C, elongation 90 sec) for the wild-type sequence and with the primers CFTR-6456f/lacZr (annealing 54°C, elongation 90 sec) detecting the correct integration of the modification construct. BAC-DNA used for the *GGTA1* approach, was screened after modification with the construct pGGTA-lacZ using the primers GGTAsf/GGTA5armr (annealing 56°C, elongation 90 sec) for the wild-type sequence and GGTAsf/lacZr (annealing 60°C, elongation 90 sec) for correct integration of the modification constructs. The *CFTR* BAC modification approach resulted in 214 colonies from 4 agar plates (2 x 30 ng and 2 x 100 ng of linearized *lacZ*-fragment). 24 of them have been picked and analyzed by PCR as described above. 4 out of 24 clones could be dedicated as correctly modified. Colony 2 was used for further experiments. The *GGTA1* BAC modification resulted in a total number of 83 colonies. Again 24 colonies have been screened, of which 3 carried the correct modification. The results of the BAC modifications are shown in figure 4.18.

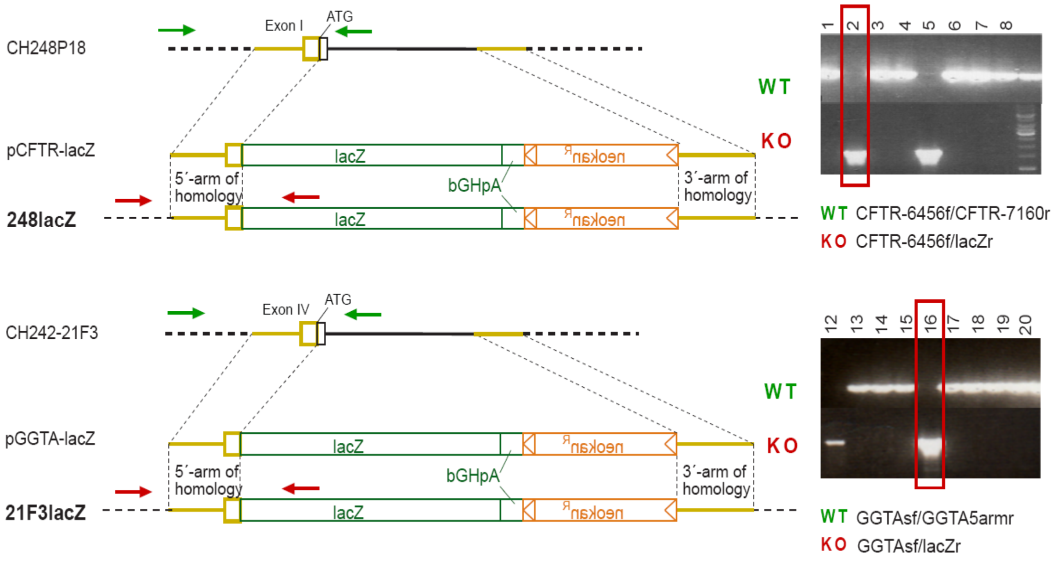


Figure 4.18: Modification scheme for wild-type BACs by recombineering.

SW106 cells containing the BAC of interest have been used for electrotransformation with the respective modification fragment, primarily excised from pCFTR-lacZ or pGGTA-lacZ, respectively. The picture indicates a selective overview of the PCR-screened modified BACs. In case of *CFTR* 4 out of 24 clones were screened positive (clone 2 was used for further experiments), in case of *GGTA1* 3 out of 24 have been correctly modified (clone 16 was used for further experiments). Screening primers for the wild-type sequence are indicated by green arrows, for the knock-out sequence by red arrows.

Correctly modified BAC clones (in SW106 cells), termed p248-lacZ or p21F3-lacZ, respectively, subsequently were re-transformed into recombineering deficient *E. coli* strains (DH10B) to allow incubation steps at regular 37°C. The procedure was carried out similar to the electrotransformation described above. Aliquots of electrocompetent DH10B cells (produced as described in 3.2.7) were transformed with 20 ng as well as 100 ng of the respective BAC-DNA. The cell suspension was plated on agar plates containing the suitable antibiotic (kanamycin or chloramphenicol) and incubated overnight at 37°C. A number of the resulting colonies were picked, and prepared for screening PCR as well. The remaining screening steps were carried out equally as already described above.

4.6.3 Preparation of the modified BACs

In the next step, the correctly modified BAC-DNA was prepared endotoxin-free according to the given protocol (3.2.9.2) to be transfected into porcine kidney cells (pKC) or primary fetal fibroblasts (pFF). Prior nucleofection, the constructs have been linearized, if necessary, with AscI as restriction enzyme of choice. The restriction digest was carried out according to the standard protocol for BAC digestion given in 3.2.4 using adequate amounts of enzyme for the amount of

DNA to be digested. The number of BAC-DNA isolations per construct and the resulting amount of DNA is listed in table 4.12.

Table 4.12: Overview of endotoxin-free BAC isolations

p248lacZ		p21F3lacZ	
# isolation	**DNA amount [µg]**	**# isolation**	**DNA amount [µg]**
1	17.7	1	12.3
2	55.0	2	11.0
3	10.8		
	83.5		**23.3**

4.6.4 Introduction of BAC-DNA into target cells

In order to replace *CFTR* and *GGTA1* by introducing the *lacZ* reporter gene into the porcine genome under the control of the respective endogenous promoter, additive gene transfer was the method of choice. An overview of the experiments carried out with the constructs p248lacZ (*CFTR*) and p21F3lacZ (*GGTA1*) respectively is given in table 4.13.

Table 4.13: Overview of nucleofection experiments using p248lacZ and p21F3lacZ.

p248lacZ	# epos	cell line	DNA	DNA [µg]	# cells (10^6)
200609	1	pKC	circular	9.6	1
220609	2	pKC	circular	9.6/4.8	1
300709	1	pKC	circular	10	1
241009	1	pFF	linear	5	1
p21F3lacZ	**# epo**	**cell line**	**DNA**	**µg DNA**	**# cells (10^6)**
200609	1	pKC	circular	0.52	1
300709	1	pKC	circular	10	1
241009	1	pFF	linear	5	1

The transfected cells were cultured for 48 hours prior the selection using antibiotic containing medium (geneticin; G418) was started. Overall the selection was done for 9 days including one passaging step and media change every other day. After reaching 60-80% confluence the cell clones were mixed and frozen in cryopreservation medium and stored in liquid nitrogen. For nuclear transfer the cells were thawed and cultured in starvation medium for 48 hours.

4.6.5 Nuclear and embryo transfer experiments

After thawing cell aliquots and culturing in starvation medium NT was performed as described in 4.5.1. The results of the additive NT/ET experiments are shown in table 4.14.

Table 4.14: Overview of NT/ET experiments for the *lacZ*-constructs.

p248lacZ	cell type	epo	maturation rate	# NT embryos	fused oocytes	embryos transferred	# piglets
240709 (c)	pKC	200609	182/265	157	128	112	0
140809 (c)	pKC	300709	183/318	162	140	134	0
041209 (l)	pFF	241009	132/156	131	120	119	**3** (#9975-9977)
150910 (l)	pFF	241009	102/194	92	87	87	0
160910 (l)	pFF	241009	114/161	105	99	99	0
141010 (l)	pFF	241009	216/325	201	191	178	**5** (#1180-#1184)
181110 (l)	pFF	241009	189/259	98	91	91	**6** (#1202-#1207)

p21F3lacZ	cell type	epo	maturation rate	# NT embryos	fused oocytes	embryos transferred	# piglets
210809 (c)	pKC	300709	194/267	172	142	129	0

(c): circular; (l): linear

4.6.6 Outlook

A total number of three established pregnancies resulted in the delivery of 14 piglets. Further characterization and proceeding of the resulting outcome was not part of this doctoral thesis, because the main focus was addressed to the establishment of targeted gene knock-outs. The aim of this experiment was to get an insight into the spatial expression of the respective genes throughout the body, never been observed before. Therefore genotyping of the respective piglets and expression studies by RT-PCR will have to be performed. Organ sections for *lacZ* staining, to determine the expression will be performed by our cooperation partners at the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Freie Universität Berlin.

5 DISCUSSION

Different strategies to produce large transgenic animal models have been established within the last decades, as PMI (Hammer *et al.*, 1985), SMGT or viral transgenesis (Hendrie *et al.*, 2005; Smith *et al.*, 2005). With the development of vectors derived from the adeno-associated virus (AAV) it became possible to introduce also targeted mutations into the host genome. The very low possible packaging size of only 5 kb (Muzyczka, 1992; Hirata *et al.*, 2000) and licensing requirements and orders to be allowed to work with AAV vectors make their use inappropriate for this project. Additionally, ZFNs have already been applied to introduce targeted mutations into the porcine genome (Watanabe *et al.*, 2010; Hauschild *et al.*, 2011). Pigs in particular represent ideal tools for the generation of various disease models, such as cystic fibrosis and could serve as organ donors in xenotransplantation. With this project both areas were linked by the establishment of a new technology enabling the targeted knock-out of two distinct genes, *CFTR* and *GGTA1*. As a side-project also additive gene transfer, utilizing BAC-based vectors for the introduction of the *lacZ* reporter gene into the porcine genome at the given loci, was used.

5.1 Generation of modified BACs for cell transfection

In previous studies, several strategies to overcome the low targeting efficiency in porcine primary cells, such as gene trapping, negative selection and adeno-associated viral vectors have been reported (Lai *et al.*, 2002; Jin *et al.*, 2003; Rogers *et al.*, 2008). It was stated that random integration of DNA constructs occurs 1000 to 100000 times more frequently than by HR and in particular the targeting efficiency of somatic cells is about two orders of magnitude lower than in murine ESCs (Schwartzberg *et al.*, 1990; Arbones *et al.*, 1994). An appropriate way to increase the targeting efficiency is represented by an increase of the homologous regions of the vector construct (Scheerer *et al.*, 1994). Generally, conventional targeting vectors are prone to carry 20 kbp as a maximum genomic insert size. In contrast, BACs are reported to be capable to carry large, regularly 200 to 300 kbp, genomic regions of interest (Shizuya *et al.*, 1992). It is said that inserts cloned and maintained in BACs show low frequency of chimerism and much higher stability compared to YACs (Monaco *et al.*, 1994). Additionally, BACs have also been used to increase the HR frequency in targeting approaches using ESCs to establish various mouse models (Valenzuela *et al.*, 2003; Yang and Gong, 2005; Barakat *et al.*, 2011). Effective targeting efficacies of up to 28% have been observed (Yang and Seed, 2003). Several different modification protocols to introduce desired alterations into BACs, independent of cloning strategies based on restriction enzymes, driven by homologous recombination have been reported (Yang *et al.*, 1997; Jessen *et al.*, 1998; Zhang *et al.*, 1998). In this project BAC modification was carried out according to the defective λ-phage-mediated recombineering protocol established by Copeland and colleagues (2001). Several different BACs have been purchased and screened by restriction digests, whether they really carry the genomic region of interest. BAC RP44-360A14, assumed to contain the *CFTR* gene, had to be rejected after comparison of the restriction digest pattern and the *in silico* pattern. Two additional BACs carrying the *CFTR* gene have been purchased and verified. BAC CH242-248P18 was used for further experiments, due to the higher yield of DNA obtained when prepared according to the regular plasmid isolation protocol (PCiA), compared to PigI-170I3. For the *GGTA1* locus BAC CH242-372F22 was also confirmed by restriction digest. In order to target the desired locus and introduce the alteration by homologous recombination the design and construction of an adequate DNA vector was necessary. This study aimed to obtain a complete knock-out of two distinct genes (*CFTR* and *GGTA1*), meaning that by the manipulation of the transcription or translation start, RNA or protein synthesis are eliminated. In a first step plasmid-based vectors have been generated to modify the respective BACs by recombineering. In case of *CFTR* both, RNA transcription and the protein translation start are localized in exon I. In order to knock-out the *GGTA1* gene, exon IV was targeted. For this reason, modification vectors, containing a STOP-box, comprising a HIS3 yeast protein termination sequence and a SV40 pA signal (Sauer, 1993) to be introduced downstream of the respective start codon, have been constructed. The STOP-box is coupled to a floxed neomycin/kanamycin resistance cassette under the control of a mPGK and T7 promoter (to enable the switch between prokaryotic and eukaryotic expression systems) also containing a bGH polyadenylation site. This vector component assures the possibility for an antibiotic-mediated pre-selection for construct integration into target cells. The neomycin/kanamycin resistance cassette is often used for positive selections and is well established in our lab (van der Weyden *et* *al.*, 2002). Possible interference of the selection cassette with the expression of the surrounding genes is prevented by the addition of both-sided loxP sites, which enable the removal of the cassette via arabinose-induced Cre recombinase-mediated excision (Sauer *et al.*, 1988). In case of the reporter gene approach, the *lacZ* gene was linked to the described neomycin/kanamycin resistance cassette. Another strategy to enrich targeted cell clones, positive-negative selection, usually achieving a two- to ten-fold enrichment of targeted cells, was also considered. Due to the fact, that the overall efficiency of this method might be reduced, because of damage or loss of the negative selection cassette, which becomes even more likely with increasing length of homologous arms provided by BACs, this idea was condemned (Hanson and Sedivy, 1995). Promoter-trap as an additional enrichment method, where the transcription of a selection cassette is driven by the endogenous promoter of the target gene, requires a transcriptionally active target gene in somatic cells (Marques *et al.*, 2006). Hence, the promoter-trap strategy was not suitable for this project, as it is known that *CFTR* is described as a silent locus in porcine fetal fibroblasts (Williams *et al.*, 2003). Homologous arms of 0.5 kb length were added on both sides to the STOP-box- or *lacZ*-antibiotic resistance region to enable HR, although they needed to be as short as 40-50 bp using the recombineering methodology according to the Copeland-protocol. However, it was reported that 0.1-0.3 kb homologous arms increase efficiency and specificity. Single arm homologous recombination, as it was used in GENSAT projects, is possible but adding a second homologous arm to the construct enables a targeting without including the vector backbone (Hollenback *et al.*, 2011). The modification rate of the BACs with the respective modification vector complied with the expectations, as they ranged from 12.5% to 58.3% correctly modified BAC clones. This implicates that on average only a limited number of colonies (less than 10) has to be screened for positive integration to obtain one correctly modified clone (Sparwasser *et al.*, 2004). One additional advantage of BAC vectors, especially if randomly integrated, is that the positional effects, where the host sequences surrounding the location of transgene integration are able to influence the expected expression pattern, are overcome by their large cloning capacity. Moreover, the inclusion of all necessary regulatory elements, as present on BACs, guarantees optimal expression levels in the produced transgenic animals, independent of the integration position (Giraldo *et al.*, 2001). The introduction of the *lacZ*-constructs in both approaches yielded a lower amount of colonies and also a lower amount of positively modified BAC clones after PCR screening. This might be due to a little higher salt-concentration of the *lacZ* modification vector DNAs, compared to STOP-approaches, used for electrotransformation into the BAC-containing SW106 *E. coli* cells, implicated by a shorter, but still sufficient, time constant (Hollenback *et al.*, 2011).

5.2 BAC-DNA isolation and cell transfection

The DNA of the correctly modified BACs was isolated according to a protocol using several components of two different commercially available kits. The buffer set and the QIAGEN-tips 500 of the Endofree plasmid Maxi Kit and the ETR-solution of the E.Z.N.A.® Endo-free Plasmid Midi Kit in combination achieved the best results regarding DNA yield, purity and transfection efficiency, although it was reported elsewhere that column-based BAC-DNA isolation should be avoided due to detrimental influence to the integrity of large BACs (Sparwasser *et al.*, 2004). Nevertheless, in this approach the impact of column-based purification seemed not to affect the DNA quality adversely, as it was possible to generate transgenic pigs subsequently. An endotoxin-free preparation strategy was chosen, because it is stated that endotoxins, known as cell-membrane components of gram-negative bacteria such as *E. coli*, are released during the lysis step of plasmid purification. They are known to be extremely toxic, as potent stimulator of the mammalian immune system, and therefore they are held to be responsible to significantly reduce transfection efficiencies (Budryk *et al.*, 2001). The amount of DNA obtained from several isolations varied from 10.0 µg to 42.8 µg for p248STOP, from 4.0 µg to 33.5 µg for p21F3STOP (table 4.1), from 10.8 µg to 55.0 µg for p248lacZ and from 11.0 µg to 12.3 µg for p21F3lacZ (table 4.12). All in all, the DNA yield of BAC-DNA was satisfying but this relatively high variability among different isolations, of course might be ascribed to the initial volume of BAC overnight culture (100 or 200 ml), but also might be explained by the binding capacity of the column. In some cases an overload of the column was quite obvious, implicated by the very slow entering of the DNA solution to the resin and the subsequent elution, indicating that utilizing a column-based isolation method didn’t affect DNA quality but, in some cases, might affect DNA yield. Additionally, it was observed that it has been important to start the BAC-DNA isolation with the inoculation of a 3 ml pre-culture, leaving it at least four hours on the shaking incubator before an overnight culture was inoculated. Omitting this pre-culture step on account of time problems always resulted in a lower BAC-DNA yield. Initially, it was tried out to resuspend the DNA pellet directly in a suitable amount of AMAXA nucleofection buffer, not to influence the transfection procedure by changing the nucleofection components using regular T-buffer. But this did not work very efficiently, as the pellet did not dissolve in AMAXA buffer. Furthermore, the DNA pellet was dissolved in an appropriate volume of T-buffer. In general, it really took very long until the pellet completely was dissolved, promoted by incubation at 42°C in the water bath. Due to time reasons, the DNA concentration might have been measured in some cases without complete dissolution of the DNA pellet also explaining the variable amounts of DNA obtained. Prior to nucleofection of the pFFs or pKCs with the endotoxin free prepared BAC-DNA, construct linearization was performed. An initial experiment was set up to determine the transfection efficiency in pFFs using whether circular (supercoiled) or linearized p248STOP DNA. The results (data not shown in the results) were in accordance with the literature, as with linearized DNA a higher amount of cell clones with stable construct integrations could be obtained (linearized 1 and 2 resulted in 31 and 74 cell clones compared to circular 1 and 2 resulting in 11 and 12 cell clones). It is also reported that the initial uptake of linear DNA, usually used if stable construct integration is needed, is lower compared to supercoiled DNA, which is mainly used for transient gene transfer (according to the handbook of the AMAXA nucleofector kit).

Since until now true ESCs have only been isolated from mouse, cultured somatic cells are most commonly used as donor cells in SCNT. Viable offspring have been produced utilizing fetal fibroblasts (Hyun *et al.*, 2003), adult fibroblasts (Brunetti *et al.*, 2008) and somatic cumulus cells (Polejaeva *et al.*, 2000). In this project, porcine fetal fibroblasts (pFFs) were used as donor cell source for SCNT for initial approaches, as they are routinely used in our lab. Also additive gene transfer approaches have been performed using pFFs. Additionally, in the meantime another promising donor cell line isolated from kidney samples of three month old male pigs (Niere m) has been established. These primary kidney cells (pKCs) feature comparable proliferation properties to fibroblasts, convenient isolation and modification characteristics and senescence is evolved at a later time point compared to pFFs (personal communication with Dr. Annegret Wünsch). BAC-DNAs were routinely linearized, if needed, with AscI and nucleofected with the same program and nucleofection solutions established by the provider for primary mammalian fibroblasts. The transfection itself and the generation of single cell clones are not further discussed, because they were carried out by Dr. Annegret Wünsch and her team.

5.3 Isolation of genomic DNA from cell clones

Due to the low expected targeting efficiency, numerous single cell clones have to be generated. In order to limit extensive cell culture work and to be aware of long-term cultures of primary cells, as the target cells do have a finite lifespan (Jeon *et al.*, 2011), we aimed to isolate DNA from only small amounts of cells for qPCR application. The generated single cell clones have been expanded in 96-well plates. Therefore a maximum amount of 8000 to 10000 cells per clone, limited by the growing surface of one well, could be obtained for subsequent DNA isolation. The isolation methods should meet pre-determined demands, such as applicability for very small amounts of available cells, achieving acceptable yields of high quality DNA to be suitable for subsequent qPCR, in a time-saving and not too cost-extensive manner, because once established, this method is going to be adapted to the routinely performed lab protocols, to be reproducibly used by anyone. Additionally, the possibility for an automated sample proceeding is aspired. Firstly, in particular due to the time factor and the reproducibility, commercially available kits based on DNA isolation via binding to silica membranes offered by different providers (Macherey & Nagel, Dueren; Peqlab, Erlangen; Qiagen, Hilden; Omega Bio-tek, Norcross) have been considered. Comparing the standard protocols of five different column-based commercial kits (Nucleospin® Tissue Kit, peqGOLD MicroSpin Tissue DNA Kit, Qiagen DNeasy Blood & Tissue Kit, QIAamp® DNA Micro Kit, E.Z.N.A® Tissue DNA Kit) indicated relatively similar proceeding steps. The kits were selected due to different reasons. Because the peqGOLD and E.Z.N.A® kit have been used in previous experiments, they have already been available in the lab. Nucleospin® and both QIAGEN Kits (DNeasy and QIAamp®) have been selected because they were told to be applicable even with very small amounts of cells for isolation (presumable amount from 10^2 to 10^7 cells). All kits started with a lysing step of the cell pellet, primarily suggested to be washed in PBS, whereas the incubation time with the respective lyse buffer, in all cases except the E.Z.N.A® Tissue DNA Kit (lysing protease is called OB protease) supplemented with Prot K varied from as short as 10 minutes to a maximum of 1-3 hours. Cultured cells are reported to have high levels of RNA, particularly liver and kidney cells due to their transcriptional activity. Using column-based kits a RNase A digestion in all cases except in the QIAamp protocol was suggested, avoiding co-purification of RNA with DNA. In the QIAamp protocol adding RNase A is omitted because the addition of carrier RNA is intended to achieve better binding properties of the DNA to the column membrane (Shaw *et al.*, 2009). In principle, the cells are lysed by Prot K to gain access to the DNA. By the addition of a chaotropic salt, in all cases guanidinium chloride or guanidine thiocyanate, a hydrophobic environment is created, promoting the DNA binding to the silica membrane of the column and the denaturation of proteins, therefore inactivating nucleases. Proteins, metabolites and other contaminants do not bind to the membrane and are removed in the subsequent washing steps. The DNA, bound to the silica membrane, is eluted applying low salt buffer (T-buffer). The supplied elution buffers had not been used due to possible inhibitory effects of the supplemented EDTA in subsequent qPCR applications as it was described by Huggett and colleagues (Huggett *et al.*, 2008). The elution volume was also one critical point, because elution from the silica membrane requires in general a higher volume (50-200 µl in the tested kits), accompanied by an undesired dilution effect of the inherently small amount of DNA, keeping in mind, that the qPCR mastermix is restricted to the addition of 2 µl sample. Only in the QIAamp and peqGOLD kit a satisfying elution with only 20 µl of T-buffer has been promised. Due to the small amount of starting material, a DNA concentration determination using a spectrophotometer was not possible. After isolating five trial samples of 7500 and 3750 pFF cells, respectively according to the given protocols per each kit, the obtained DNA was tested in qPCR. In all cases, C_t-values (cycle threshold; point at which fluorescence crosses the defined threshold) of 27 to 33 have been measured, indicating too less amounts of DNA to obtain determinable measuring results. Afterwards, a semi-automated purification system provided by Promega (MAXWELL® 16 DNA purification Kit) was tested. Samples are purified using paramagnetic particles, providing a mobile solid phase that optimizes capture, washing and elution of DNA. The isolation procedure is carried out by the MAXWELL® 16 Instrument, containing magnetic particle handlers, required for the processing of liquid and solid samples, for the transport of the magnetic particles through the purification reagents and for mixing during the processing.

The cell lysis is also achieved by Prot K and DNA binding to the magnetic particles and protein denaturation is promoted by guanidine thiocyanate. Initially, also five pFF samples (7500 and 3750 cells) as already tried out with the column-based kits, were proceeded. A critical point of the MAXWELL system was the predefined elution volume of 300 µl T-buffer. Hence, several different elution amounts have been tried out, ending up with 35 µl T-buffer. As satisfying results could be achieved by subsequent qPCR tests, indicated by C_t-values ranging between 23 to 25, it was decided to use this system for the isolation of generated single cell clones (pFF) transfected with the p248STOP construct. Finally, after isolation of 230 clones and evaluating the qPCR results (data not shown) the disadvantages of this method became quite obvious. The qPCR results exhibited high standard deviations in between the duplicate samples, maybe as a result of a varying binding capacity of the DNA to the particles or due to the downscaling of the elution volume. In many eluted samples residues of the paramagnetic particles remained, assumed to be responsible for the detected variability by influencing the SYBR green detection, concordant with literature (Cheong *et al.*, 2008). Furthermore, this system would have been very cost extensive, because not only the kit reagents but also the instrument itself would have to be bought. As a consequence, the MAXWELL purification system was condemned. Moreover, a filtration-based method, the nexttec^{TM} isolation system was evaluated. This method promised a one-step purification procedure in which proteins, detergents and low molecular weight compounds are retained by the nexttec^{TM} sorbent, whereas the DNA is so to speak filtered and passed through the clean column. Cell lysis is also achieved by Prot K digestion. The nexttec system allows a really fast proceeding and facilitates high throughput isolation due to the possibility of adapting it to a 96-well format. Although the amount of the obtained DNA was not that low compared to the column-based isolation methods mentioned, the reproducibility between different samples was insufficient. Finally, six conventional methods, two of them commercially available kits provided by Qiagen and Promega, have been compared. The protocol of the Gentra Puregene Kit allows cell lysis in a very short centrifugation step of only 10 sec in the presence of an anionic detergent (such as SDS), Prot K and a DNA stabilizer (such as EDTA), which limits the activity of intracellular DNases. An optional RNase step can also be added. Contaminants like proteins are removed by salt precipitation, and DNA is precipitated with alcohol (isopropanol) and dissolved in

T-buffer. The salt precipitation step is suggested to be supported by a five minutes incubation step on ice. Because a low DNA yield was expected, glycogen as DNA carrier was added during isopropanol precipitation, as suggested. The dissolving volume is recommended to be 50 µl, but as there is no elution from any DNA-binding material required, it is somehow freely adjustable, depending on the requirements. Hence, 35 µl have been chosen as in all the other conventional isolation methods, to obtain comparable results. In general 1-2 x 10^6 cells are used as starting material for DNA isolation using this Kit, but it is indicated that also small numbers of cells (100-10000) can be proceeded. The Wizard isolation Kit, designed for cell amounts of 1-8 x 10^6 works in a very similar way but there are slight differences: according to the protocol, there is no DNA stabilizer supplemented, there is no Glycogen addition suggested, cell lysis is carried out without Prot K and the salt precipitation is not carried out while incubating the sample on ice. All other steps, including salt precipitation of the proteins and contaminants, RNase digestion and DNA precipitation by isopropanol are identically performed to the Puregene Kit. In this case, the DNA is suggested to be dissolved in 100 µl T-buffer, but as already mentioned, 35 µl have been applied. In both kits the rehydration step of the cell pellet should be carried out for at least one hour at 65°C. The isolated DNA samples of both kits (again five times 7500/3750 pFF cells) have been tested in qPCR. Surprisingly, although the proceeding was very similar, the Wizard kit achieved very low DNA yield, insufficient for the following approaches. This might be due to an insufficient cell lysis, according to the Wizard protocol, in which Prot K addition is omitted. Using the puregene kit, sufficient amounts of DNA could be obtained, in particular evaluating the 7500 cell aliquots. Nevertheless, the qPCR-tests of the isolated 3750 cell aliquots were evidently accompanied by raised standard deviations in between the different samples. However, this method was considered to be used for further proceeding. The Kawasaki-buffer isolation method was tried out according to a protocol obtained from Dr. Marc Boelhauve, generally applied to isolate DNA from blastomeres of bovine embryos. Though, the isolated DNA subsequently was only used for conventional PCR tests. Cells are lysed in the presence of ProtK, KCl and Tween as a detergence, promoted by incubation at 55°C for one hour. After centrifugation the supernatant was directly used for qPCR testing. This method yielded insufficient amounts of DNA, varying in between the distinct isolated samples. The spermidine method, routinely used in the lab for genomic DNA isolation, particularly for isolation from mouse tail or porcine ear tips, was evaluated next whether to be suitable for small amounts of starting material. In order to lyse the cells a cutting buffer containing EDTA, DTT, NaCl and spermidine was mixed with Prot K and SDS. DNA was precipitated by isopropanol. The qPCR tests demonstrated the spermidine method to be a potential candidate for further isolations, as the DNA yield was sufficient and standard deviations were not that high. Due to the very fast and efficient DNA isolation an additional test has been performed. A pre-defined standard curve, as it was used subsequently for the LOWA-assay, was tested in qPCR with DNA isolated by the spermidine method. As a result, a kind of inhibitory effect to the SYBR green I-mediated qPCR test was observed, because the more sample DNA was added, the less fluorescence, indicated by very low C_t-values, was detectable. This effect might be related to the reported inhibitory effect of polyamines, such as spermidine, to DNA polymerases (Ahokas *et al.*, 1993). Although the method was promising at a first sight it had to be abolished. The PCiA method is also used in daily lab routine, applied for the isolation of genomic DNA of mouse tails or porcine ear tips. Lysing the cells is also achieved by Prot K digestion in combination with SDS. DNA was stabilized by EDTA. Contaminants are extracted using PCiA and DNA is precipitated using isopropanol. The high-salt precipitation originally was employed for the DNA extraction of spermatogonial cells (protocol provided by Dr. Marc Boelhauve). Cells are lysed by a buffer combination containing EDTA, SDS, DTT and Prot K. Proteins and contaminants were removed through a precipitation step using highly concentrated NaCl, supported by incubation on ice. DNA was precipitated with isopropanol. The qPCR tests with isolated pFF-DNA resulted in sufficient DNA amounts for both isolation methods, but better results in DNA yield have been achieved by the PCiA method. Though, higher standard deviations, mainly observed with the 3750 pFF cell aliquots, occurred with PCiA extraction, probably due to phenol residues, proved to influence qPCR adversely (Cankar *et al.*, 2006). In conclusion, the puregene kit, the PCiA method and the high-salt precipitation seemed to meet the demands regarding DNA yield. The PCiA method was mainly rejected, because it is quite laborious, the phenol extraction is a quite critical, error-prone step, as transferring just little phenol to the aqueous, DNA containing phase, might result in undesired qPCR variability. Not to forget, if possible, working without toxic reagents (like phenol) is always preferable. In a final decision step, although the puregene kit achieved sufficient results regarding DNA yield and showed a convincing time-saving proceeding, the high-salt precipitation was pointed out as method of choice, combining a not too laborious, easy to handle and comparably low-priced method with sufficient DNA yield, isolated from starting material as less as 3000 cells, and reproducible results in between distinct samples.

5.4 Targeted clone verification via qPCR

The decision to use BACs as targeting vectors was accompanied by one hurdle. Due to the large regions of homology provided by BACs, making them a very promising tool for HR-based targeting, the screening of the transfected cell clones was quite difficult. Conventional long-range PCR or Southern blotting were not applicable. It is described that the targeting efficiency increases with the length of the homologous vector arms, but in practice, homologous arms longer than 5 kb represent a problem for Southern blot analysis (Hofemeister *et al.*, 2011). In order to detect the number as well as the chromosomal localization of BAC integrants, FISH analysis could be used (Cao *et al.*, 2011). For both methods, which are known to be quite laborious and time-consuming, the amount of DNA obtainable from the generated cell clones would be insufficient. Hence, the ‘loss of wild-type allele’-assay was utilized for determining targeted cell clones (Valenzuela *et al.*, 2003). The resulting targeting efficiencies, produced by a non-viral vector system, of 1.35% in case of *CFTR*, and 3.91% for *GGTA1*, are competitive compared to literature (Dai *et al.*, 2002; Lai *et al.*, 2002; Jin *et al.*, 2003; Klymiuk *et al.*, 2010), although effective targeting efficacies of up to 28% have been observed, using murine ESCs (Yang and Seed, 2003).

5.5 Evaluation of fetuses and piglets

Using candidate clones transfected with the p248STOP construct, three NT/ET experiments have been performed. A total number of 264 embryos have been transferred resulting in the establishment of two pregnancies comprising an outcome of seven fetuses, obtained after pregnancy termination at day 59, and five piglets. Furthermore, for the establishment of *GGTA1*$^{+/-}$ pigs, three NT/ET experiments, using candidate clones, transfected with the p21F3STOP construct have been performed. For this purpose, 271 embryos have been transferred, two pregnancies have been established, one of them terminated to obtain ten fetuses, the other delivered three alive and one stillborn piglet at term. The obtained fetuses all looked normally developed, although obviously fetus #1 and fetus #7 in the *CFTR* approach and fetus #4 and fetus #5 in the *GGTA1* approach appeared smaller compared to the other respective littermates. This growth variation is described in literature as accessory phenomenon of SCNT (Cho *et al.*, 2007). Evaluation of the *CFTR*-fetuses revealed that fetus #1 could not be determined as correctly targeted, also reflected by the calculated copy number ratio related to the whole plate mean value of 1.27 (G/C) and 1.24 (H/C) shown in figure 4.11. In case of the *GGTA1*-fetuses, fetus #6 (cnr/MV 1.79 (H/G) and 1.29 (C/G)), fetus #8 (cnr/MV 1.80 (H/G) and 1.46 (C/G)) and fetus #9 (cnr/MV 1.79 (H/G) and 1.24 (C/G)) have not been correctly targeted (summarized in figure 4.15). This results were surprising, as with SCNT in general 100% of the offspring is transgenic (Wolf *et al.*, 1998). One possible explanation could be the aberrant transfer of a cumulus cell instead of the candidate fibroblasts or kidney cells (personal communication with Dr. Barbara Keßler), which might be true for the *CFTR* approach with only one negative fetus out of five. In contrast, to explain three untargeted *GGTA1*-fetuses another possibility has to be taken under consideration. Candidate clone GGTA250 was previously described as a mixed clone, indicated by an elevation of the copy number ratio change as required to be determined as correctly targeted clone, but compared to the other candidate clones the increase was not that convincing. One reason could be found in cell culture, where a cell could be wrongly identified as single cell clone although it was a mixed colony of targeted and non-targeted cells. Furthermore, the cloning team is choosing the donor cells by hand, deciding by morphological characteristics, regarding for example size and surface structure, which might lead to the transfer of untargeted donor cells not differing from targeted cells in their shape (personal communication with Dr. Annegret Wünsch and Dr. Mayuko Kurome). The obtained piglets, in both approaches, all have been ratified as correctly heterozygously targeted. *CFTR*$^{+/-}$pigs all established malformations of the forelegs, described in literature as contracted tendons, sometimes occurring with SCNT. It was also described that this kind of malformation is neither transferred to the offspring derived from affected founder animals (Prather *et al.*, 2004) nor cloning this animal resulted in 100% affected clones (one of 4 pigs suffered from contracted tendon as well)(Park *et al.*, 2002), indicating that the obtained affected *CFTR*$^{+/-}$ pigs nevertheless could be utilized for further mating or for re-cloning after targeting the second allele to obtain homozygous knock-out pigs. Pig #9978 suffered from anal atresia, representing a rarely recognized congenital disorder in neonatal pigs, with an estimated incidence of 0.1 to 1.0% (Hori *et al.*, 2001). Although atresia ani was also reported to occur in cloned pigs in another study, it is rarely seen in other reports related with pig somatic cell cloning (Walker *et al.*, 2002). For this reason, it is hard to estimate the possibility that anal atresia is directly related to SCNT, as it also occurs in normal piglets (Lee *et al.*, 2005). The *GGTA*$^{+/-}$ pigs didn't show any abnormalities. The achieved pregnancy rates (66% in both cases) and delivery rates (100% in both cases) are regularly observed in our lab, also being competitive regarding to literature (Vajta *et al.*, 2007).

5.6 Outlook

Especially in cystic fibrosis, one of the most common, genetically inherited disorders with recessive outcome, which is caused by mutations in the *CFTR* gene, adequate models mirroring the human phenotype are required. Although a *CFTR*-lacking porcine model already has been generated by Rogers and co-workers (Rogers *et al.*, 2008), which might not be available to be internationally used due to sanitary restrictions, it was decided to generate a second *CFTR*$^{-/-}$ knock-out pig, evolving another genetic background. In mice, it was shown that differences in their genetic background, accompanied by phenotypical changes, are providing insight to mechanisms of the disease (Wilke *et al.*, 2011). In the context of this doctoral thesis, an alternative method, utilizing modified BACs for targeting *CFTR* heterozygously, has been established. Further, but not as a part of this project, it was possible to apply the established technique to target the second *CFTR* allele, in order to generate homozygous knock-out pigs, resembling the pathological aberrations in human CF. The generated pigs demonstrated tight phenotypic similarities to the CF pigs produced via AAV-mediated gene transfer in the group of Rogers, with one exception: the meconium obstruction was located in the large intestine, compared to a localization oral and aboral to the ileocaecal junction described by Meyerholz and colleagues (2010). This newly generated porcine CF model can be applied for investigating CF disease mechanisms and for the development of novel treatment strategies (Klymiuk *et al.*, 2011). Additionally, *CFTR*$^{-/-}$ animals can also be produced by breeding.

Xenotransplantation represents a way out of the availability imbalance of donated human organs and the demand for transplantation. One major obstacle, xenograft rejection, could be overcome by the genetic modification of the porcine genome. In a first step hyperacute rejection, mainly mediated by natural antibodies directed against the α1,3-Gal epitope, synthesized by the α-1,3-galactosyltransfrease, encoded by *GGTA1*, has to be circumvented. For this purpose several different porcine models with targeted deletions of the *GGTA1* gene have been produced (reviewed in Klymiuk *et al.*, 2010). Among this porcine models, different strategies to inactivate *GGTA1* have been pursued, including the targeting of exon IV, which contains the endogenous translation initiation codon or exon IX, which comprises the majority of the coding region for the catalytic domain (Katayama *et al.*, 1998). The novel BAC-based targeting method, established in this project, was reproducibly adapted to generate *GGTA1*$^{-/-}$ pigs, inactivating *GGTA1* by targeting exon IV. With the transient transfection of a Cre recombinase containing plasmid, it is possible to remove the floxed neokan resistance, not being used in any other *GGTA1* knock-out model before. Further, the *GGTA1*$^{-/-}$ pigs have already been used in further experiments, being not a part of this thesis, to target the second *GGTA1* allele. This was achieved by the exchange of the neokan resistance by a blasticidin resistance. In the near future, after generation of homozygous knock-out clones in cell culture, both resistance cassettes, which are reported to possibly interfere with the surrounding genes (Pham *et al.*, 1996), are going to be removed through Cre-mediated cassette excision. Furthermore, *GGTA*$^{-/-}$ pigs without any antibiotic resistance, required for preclinical and clinical xenotransplantation, also preferable for further animal care and treatment, can be produced by SCNT. This reflects a time saving strategy compared to conventional breeding, which can be used to produce additional *GGTA1*$^{-/-}$ animals as well.

In order to evaluate if BAC vectors are suitable tools for additive gene transfer, knowing that they comprise all necessary regulatory elements (Giraldo and Montoliu, 2001), a side project was pursued in the context of this doctoral thesis. The *lacZ* reporter, terminating the transcription by a bGH-pA signal, introduced behind the ATG codon of exon I and IV respectively, is transcribed instead of the respective gene. Until now, it was possible to generate 14 piglets (*CFTR*), still needed to be determined by genotyping and regarding their *lacZ* expression. Sections of different organs, mainly relevant for CF-phenotypes, such as lung, pancreas, kidney and many more, can be investigated for *CFTR* distribution by *lacZ* staining.

6 SUMMARY

Establishment of BAC-targeting in porcine primary cells

The establishment of large animal models is of increasing interest, due to the inadequacy of rodent model organisms regarding special requirements, in particular their suitability as models for human diseases. Above all, the pig has become an important resource in biomedical research, covering many areas as expedient model. Advantageous characteristics, such as similarities to human in size, physiques, anatomy, physiology, metabolism, organ development and disease progression as well as beneficial properties like a standardized environmental situation, the well established reproductive technology and advanced techniques of genetic modification of the porcine genome, represent ideal prerequisites to be used as animal model for both, human diseases and xenotransplantation. The aim of this study was the establishment of a method enabling the introduction of targeted genome alterations, mediated by BAC-based vector constructs, so far not described being applied in large animal mutagenesis. For this purpose the targeted knock-out of the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, responsible for the development of cystic fibrosis if mutated, and the *GGTA1* (α-1,3-galactosyltransferase) gene, playing a major role in hyperacute rejection of xenografts, were pursued. BACs (bacterial artificial chromosomes) are described as circular molecules that are capable to carry large (up to 300 kb) genomic regions of interest, offering all necessary regulatory elements and an increased region of homology to elevate the targeting efficiency. Plasmid based modification vectors, containing a STOP-box and a neokan resistance cassette for positive selection, have been constructed to modify the respective BACs by recombineering. The DNA of the resulting correctly altered BAC clones, was prepared endotoxin-free, linearized, and used for nucleofection into porcine fetal fibroblasts (pFFs) or primary porcine kidney cells (pKCs). The propagated single cell clones provided only small amounts of cells for subsequent qPCR evaluation through the ‘loss of wild-type allele’-assay. Therefore 13 different isolation methods, regarding DNA yield and quality, have been compared, pointing out high-salt precipitation as the most suitable method among them. In case of *CFTR* a targeting efficiency of 1.35%, for *GGTA1* 3.91%, has been achieved. Correctly targeted candidate clones were used for nuclear transfer (NT) followed by embryo transfer (ET) to synchronized gilts. Three NT/ET experiments were carried out, resulting in two established pregnancies in each targeting approach. One pregnancy was terminated after 59 (*CFTR*) and 58 (*GGTA1*) days respectively, and fetuses were recovered for qPCR validation of the correct heterozygous targeting. For *CFTR* six out of seven, in case of *GGTA1* seven out of ten could be ratified as correctly targeted. The other pregnancies delivered five correctly targeted *CFTR*$^{-/+}$ piglets (#9978-#9982) and three correctly targeted *GGTA1*$^{-/+}$ animals (#9987-#9989). All *CFTR*$^{-/+}$ animals established contracted tendons of the forelegs, but not influencing the welfare of these animals, maybe as side effect of SCNT, and one out of these suffered from a congenital anal atresia. The *GGTA1*$^{-/+}$ animals could be characterized as vital and normally developed. As a side project, in order to evaluate the suitability of BACs for additive gene transfer as well, the *lacZ* reporter gene was introduced to be transcribed, under the control of the endogenous promoter, instead of the respective gene, allowing the determination of *CFTR* and *GGTA1* expression and localization in the pig. Seven NT/ET experiments resulted in a total number of 14 piglets, still needed to be determined by genotyping and regarding their *lacZ* expression.

These results indicate that the use of BAC vectors combined with SCNT provides a suitable strategy for the production of genetically modified pigs, represented by the successful targeting, achieving competitive targeting efficiencies, of two distinct genes and the additive introduction of the reporter gene *lacZ* into the given loci.

7 ZUSAMMENFASSUNG

BAC-Targeting Etablierung in porcinen Primärzellen.

Die Etablierung von Großtiermodellen gewinnt immer mehr an Bedeutung, da Nager als Modellorganismen in Bezug auf gewisse Anforderungen, vor allem ihre Eignung als Krankheitsmodelle, nicht immer ausreichend geeignet sind. Das Schwein im Besonderen stellt mittlerweile eine wichtige Ressource in der Biomedizin dar, indem es eine Vielzahl von Bereichen als zweckdienliches Modell abdeckt. Vorteilhafte Charakteristika, wie etwa die große Ähnlichkeit zum Menschen in Größe, Körperbau, Anatomie, Physiologie, Stoffwechsel, Organentwicklung und Krankheitsverlauf, aber auch günstige Eigenschaften einer standardisierten Umgebungssituation, die gut etablierte Reproduktionstechnologie und die weiterentwickelten Techniken zur genetischen Modifikation des Schweinegenoms, stellen ideale Voraussetzungen zur Nutzung, sowohl als Krankheitsmodell als auch als Modell für die Xenotransplantation, dar. Diese Studie verfolgt das Ziel eine Methode zur Einbringung gezielter Genomveränderungen zu entwickeln. Dies soll mittels BAC (bakterielle artifizielle Chromosomen) Vektoren erreicht werden, deren Anwendung in der Großtiermutagenese bisher noch nicht beschrieben wurde. Deshalb wurde ein gezielter Knockout des *CFTR* (cystic fibrosis transmembrane conductance regulator) Gens, dessen Mutation für die Entstehung von Mukoviszidose verantwortlich gemacht wird, und des *GGTA1* (α-1,3-Galactosyltransferase) Gens, das eine grundlegende Rolle in der hyperakuten Abstoßungsreaktion von Xenotransplantaten spielt, angestrebt. BACs sind zirkuläre Moleküle, die große genomische Regionen von Interesse (bis zu 300 kb) tragen können und somit gleichermaßen alle benötigten regulatorischen Elemente beinhalten, sowie eine größere homologe Region zur Erhöhung der Targetingeffizienz mittels homologer Rekombination verfügbar machen. Plasmid-basierte Modifikationsvektoren, die eine STOP-Box und eine Neokan Resistenzkasette zur positiven Selektion tragen, wurden konstruiert, um BACs mittels Recombineering-Technologie entsprechend zu verändern. Die DNA der ordnungsgemäß veränderten BAC-Klone wurde frei von Endotoxinen vorbereitet, linearisiert, und zur Nukleofektion in porcine fetale Fibroblasten (pFF) oder porcine primäre Nierenzellen (pKC) verwendet. Aus den hochgezogenen Einzelzellklonen konnte nur mit geringen DNA-Mengen

gerechnet werden, die folglich mittels des qPCR-basierenden „Verlust des Wildtypallel“-Assays ausgewertet werden sollten. Deshalb wurden 13 verschiedene DNA-Isolationsmethoden, bezüglich Ausbeute und Qualität, verglichen, wobei die Hochsalz-Fällung als Methode der Wahl ermittelt werden konnte. Targetingeffizienzen von 1.35% für *CFTR* und 3.91% für *GGTA1* wurden erzielt. Die ordnungsgemäß veränderten Kandidatenzellklone wurden für den Kerntransfer verwendet, gefolgt von Embryotransfers auf synchronisierte Jungsauen. Drei NT/ET Experimente resultierten in jeweils zwei Trächtigkeiten pro Targetingansatz, wobei jeweils eine davon nach 59 (*CFTR*) und 58 (*GGTA1*) Tagen abgebrochen wurde. Die gewonnen Föten wurden in weiterer Folge mittels qPCR auf ein korrektes heterozygotes Targeting hin untersucht. Im Falle des *CFTR*-Ansatzes konnten sechs aus sieben und für *GGTA1* sieben aus zehn Föten als korrekt getargetet bestätigt werden. Die verbleibenden Trächtigkeiten ergaben fünf korrekt veränderte *CFTR*$^{-/+}$ Ferkel (#9978-#9982) und drei korrekt getargetete *GGTA1*$^{-/+}$Schweine (#9987-#9989). Bei allen *CFTR*$^{-/+}$ Ferkeln konnte eine Sehnenkontraktion der Vorderläufe festgestellt werden, die aber keine weiteren Einschränkungen in der Lebensqualität der Ferkel brachte und möglicherweise als Nebenerscheinung des Kerntransfers mit somatischen Zellen gewertet werden muss. Eines von diesen Ferkeln litt zusätzlich an einer Analatresie. Im Falle der *GGTA1*$^{-/+}$ Schweine konnten alle als vital und normal entwickelt beschrieben werden. Als Nebenprojekt, um die Eignung von BACs auch im additiven Gentransfer zu ermitteln, wurde das Reportergen *lacZ* in die beschriebenen Genloci eingeführt, um unter der Kontrolle des jeweiligen endogenen Promoters transkribiert zu werden, und so Rückschlüsse auf die Expression und Lokalisierung des *CFTR* beziehungsweise *GGTA1* Gens ziehen zu können. Aus sieben NT/ET Experimenten konnten insgesamt 14 Ferkel gewonnen werden, die allerdings noch bezüglich ihrer *lacZ*-Expression genauer untersucht werden müssen.

Anhand dieser Ergebnisse konnte gezeigt werden, dass BAC-Vektoren in Kombination mit SCNT eine geeignete Methode zur Erstellung genetisch modifizierter Schweine bieten. Dies konnte durch das erfolgreiche Targeting, im Rahmen wettbewerbsfähiger Targetingeffizienzen, zweier verschiedener Gene und der additiven Einbringung des *lacZ*-Reportergens in die vorgegebenen Genloci, hervorgehoben werden.

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