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Genome Editing for the Generation of Immunodeficient Pigs

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Contents

Abstract

Genome editing comprises the use of customizable engineered nucleases for the genetic modification of cells. Two commonly used systems are transcription activator-like effector nucleases (TALEN) and the CRISPR/Cas system. While TALENs are two-domain proteins similar to the established zinc finger nucleases (ZFN), differing only in the DNA-binding domain, the CRISPR/Cas system is composed of the endonuclease Cas9 and a short RNA molecule (crRNA) which guides Cas9 to the site of interest. Like ZFNs, TALENs and the CRISPR/Cas system can be used for both directed mutagenesis and targeted insertion of an exogenous DNA donor. During this study, genome editing tools were applied for the modification of three genes playing crucial roles in the immune system, with the aim of generating immunodeficient pigs. Pigs share many similarities with humans in terms of genetics, metabolism, diet and life span, and could prove valuable tools in biomedicine in areas where murine models fail. The porcine equivalent to SCID mice (severe combined immunodeficiency), immunodeficient pigs could be used for cancer research or in regenerative medicine for the verification of stem cell therapies.

Suitable target sites within *JAK3, RAG1* and *RAG2*were chosen and TALENs and crRNA molecules for these sites generated. Subsequently, they were introduced into primary cell lines derived from various porcine tissues and clones screened for the presence of desired mutations. For *RAG1*, up to 50% of analysed clones showed indel mutations around the target site. Transfection with a crRNA targeting a nearby site did not yield any mutated clones.

JAK3 was modified both via directed mutagenesis and insertion of a targeting vector. A targeting vector replacing part of exon 2 with a neomycin resistance cassette was introduced together with the respective pair of TALENs; an insertion rate of 6% was observed following selection. A crRNA molecule targeting the same gene proved to be even more efficient; around 30% of unselected clones showed mutations and 50% of these had mutations on both alleles. Deletions of up to 107 bp were observed in these clones at the expected site, while no mutations could be found at possible off-target sites. The obtained cell clones were used for the generation of genetically modified

animals via somatic cell nuclear transfer (SCNT). While no live piglets from the first rounds of SCNT were born, further trials should yield the expected offspring.

In summary, both TALENs and the CRISPR/Cas are suitable for the genetic modification of porcine cells with efficiencies greatly surpassing those observed with conventional gene targeting.

Zusammenfassung

Unter dem Begriff Genome Editing versteht man die Verwendung von modifizierbaren Endonukleasen für die genetische Veränderung von Zellen. Zwei oft genutzte Systeme sind Transkriptions-Aktivator-ähnliche Effektor-Nukleasen (TALEN) und das CRISPR/Cas-System. Während TALENs als Proteine mit zwei Domänen den etablierten Zink-Finger-Nukleasen (ZFN) ähneln und sich von diesen nur durch die unterschiedliche DNA-Bindungsdomäne unterscheiden, besteht das CRISPR/Cas-System aus der Endonuklease Cas9 und einem kurzen RNA-Molekül (crRNA), welches Cas9 zum gewünschten Locus führt. Wie ZFNs können beide Systeme für gerichtete Mutagenese und die gezielte Insertion eines exogenen DNA-Donors verwendet werden. Im Rahmen der vorliegende Studie wurden Werkzeuge des Genome Editing für die Modifizierung von drei Genen verwendet, die eine wichtige Rolle im Immunsystem spielen. Ziel dabei war die Generierung eines immundefizienten Schweins. Schweine sind dem Menschen in Bezug auf Genetik, Metabolismus, Diät und Lebensspanne sehr ähnlich und könnten sich als wichtige Werkzeuge in der biomedizinischen Forschung erweisen, wo Mausmodelle oft nicht gewünschten Ergebnisse erzielen. Ein immundefizientes Schwein wäre das Äquivalent zu SCID-Mäusen (schwere kombinierte Immundefizienz) und könnte unter anderem in der Krebsforschung oder in der regenerativen Medizin zur Verifizierung von Stammzelltherapien Anwendung finden.

Geeignete Erkennungssequenzen in den Genen *JAK3*, *RAG1* und *RAG2* wurden ausgewählt und TALENs und crRNA-Moleküle für diese generiert. Anschließend wurden sie in primäre Zelllinien eingebracht, die aus einer Vielzahl von porcinem Gewebe entstanden waren, und die Klone schließlich auf das Vorhandensein der gewünschten Mutation hin gescreent. Bei *RAG1* zeigten bis zu 50% der anaylsierten Klone zeigten Indel-Mutationen rund um die Zielsequenz. Transfektion mit einer crRNA, die eine Stelle ganz in der Nähe erkannte, führte nicht zu mutierten Klonen.

JAK3 wurde sowohl über gerichtete Mutagenese als auch Insertion eines Targeting Vectors modifziert. Ein Targeting Vektor, der einen Teil von Exon 2 mit einer Neomycin-Resistenzkassette ersetzt, wurde zusammen mit dem entsprechenden Paar

von TALENs in Zellen eingebracht; dabei wurde eine Insertionsrate von 6% nach Selektion beobachtet. Ein crRNA-Molekül für das selbe Gen war sogar noch effizienter; ohne vorherige Selektion zeigten rund 30% der Klone zeigten eine Mutation, 50% davon auf beiden Allelen. Deletionen von bis zu 107 bp an der erwarteten Stelle im Genom wurden beobachtet, wobei keine Mutationen an verwandten Off-Target Loci detektiert werden konnte. Die isolierten Zellklone wurden für die Generierung von genetisch modifzierten Tieren via somatischem Zellkerntransfer (SCNT) verwendet; zwar wurden in den ersten Versuchen keine lebenden Tiere zur Welt gebracht, aber weitere Runden sollten die gewünschten Ferkel bringen.

Insgesamt konnte diese Studie zeigen, dass sowohl TALENs als auch das CRISPR/Cas-System für die genetische Modifizierung von porcinen Zellen geeignet sind und Mutationen mit einer wesentlichen höheren Effizienz als herkömmliches Gene Targeting induzieren.

PART I

INTRODUCTION

While basic medical research is constantly discovering new drugs and therapeutic concepts, the translation of these research findings into potent therapies is still inefficient and costly. The key in enhancing the transition "from bench to bedside" lies in the application of valid animal models. Rodents have been widely used for testing novel pharmaceuticals, but their practical value is often limited. Large animal models, on the other hand, often represent the specifics of human diseases better than their murine counterparts. Pigs especially have been established as models for complex conditions because of their similarities in size, life span and metabolism. Thanks to the arrival of gene editing tools such as zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and RNA guided endonucleases (RGENs), the generation of genetically defined pig models has been greatly facilitated. As the equivalent for NOD/SCID mice, the creation of an immunodeficient porcine model ranks high on the priority list. Lacking most functional immune cells and therefore unable to reject xenotransplants, such a model could be used for the verification of stem cell therapies as well as for tumour graft models.

In the following, the genetic and molecular background of severe combined immunodeficiency (SCID) will be discussed, at the same time highlighting possible genomic targets. This will be followed by a review of different types of customizable nucleases and their application in the genetic modification of large animals.

1 Severe combined immunodeficiency

SCID is a hereditary form of primary immunodeficiency, characterised by lack of cellular immunity and severely impaired humoral immunity (Gaspar et al., 2014). In humans, the disease is described as a paediatric emergency with a mortality of 100% if left untreated (Buckley et al., 1999). A variety of conditions is summarised under the term SCID, each of them caused by specific genetic defects. These include mutations in the janus kinase 3 gene (*JAK3*), in the gene coding for the common gamma chain (*IL2Rg*) (X-linked SCID) and in the recombination activating genes (*RAG1/2*) (Omenn syndrome). Other genes where mutations may cause SCID are adenosine deaminase (ADA), CD45 and various components of the CD3 receptor. **Fig.1** gives an overview over different mutations found in SCID and their frequency. While all of these mutations prevent the formation of functional T cells, they act on different levels of lymphocyte proliferation and can be classified accordingly. **Tab.1.1** lists various molecular defects with regard to their immunophenotype.

SCID has an overall incidence rate of 1:50,000 to 1:100,000 among newborns, with much higher rates in certain ethnic populations (Kwan et al., 2013; van der Burg and Gennery, 2011; Verbsky et al., 2012). Because of the requirement for timely therapy, several states in the US have started pilot newborn screening programmes. These screenings are based on quantitative PCR of T cell receptor excision cells - a by-product of normal T cell receptor development - and can be performed on dried blood spots collected at birth (Gaspar et al., 2014). Once diagnosed, the state of the art treatment for SCID consists of hematopoetic stem cell transplantation (HSCT) from an HLA-identical sibling (Buckley, 2004, 2011). If such is not available, haploidentical parental HSCT may be considered, although survival rates are lower than for HLA-identical HSCT (75% vs. 90%) (Antoine et al., 2003). X-linked SCID is also a suitable candidate for gene therapy. The most common approach includes gamma-retroviral transduction of CD34+ bone marrow cells. To date, several successful trials have been conducted with restoration of normal T cell leves, but in all of them severe adverse events such as leukemia occurred as well (Candotti, 2014; Gaspar et al., 2011; Hacein-Bey-Abina et al., 2010). This was later attributed to aberrant activation of oncogenes

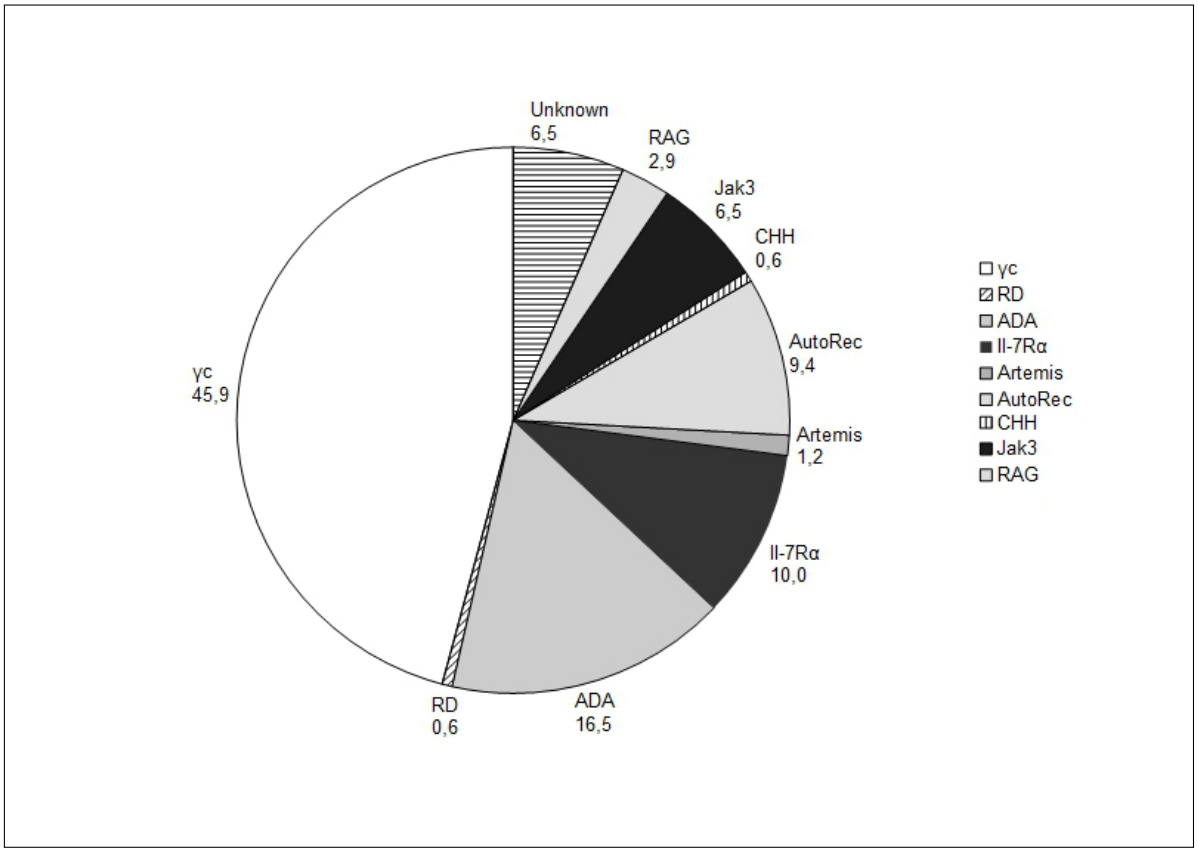

Figure 1: Genetic types of SCID and their frequency. ADA: adenosine deaminase, AutoRec: autosomal recessive of unknown molecular type, CHH: cartilage hair hypoplasia, RD: reticular dysgenesis. Adapted from Buckley (2004).

by the enhancer element of the retroviral vector (Candotti, 2014). By omitting the enhancer, it is hoped that negative effects can be minimised (Thornhill et al., 2008, Hacein-Bey-Abina et al., 2013).

In the next chapters, a closer view will be paid to selected molecular mechanisms causing SCID. The focus will be on those gene defects that are also exploited for the generation of immunodeficient animal models.

T/B/NK	*Gene*	*Protein*	*Disease*
Defects in cytokine signaling			
T- B+ NK-	*IL2Rg*	Common γ-chain	X-linked SCID
T- B+ NK-	*JAK3*	Janus Kinase 3	
T- B+ NK+	*IL7RA*	IL-7 and TSLP receptor α chain	
Defect in V(D)J recombination			
T- B- NK+	*RAG 1+2*	*RAG1*, *RAG2*	Omenn syndrome
T- B- NK+	*DCLRE1C*	Artemis	
Impaired signaling through the pre-T cell receptor			
T- B+ NK+	*CD3D*	CD3δ	
T- B+ NK+	*CD3E*	CD3ε	
T- B+ NK+	*CD3Z*	CD3ζ	
T- B+ NK+	*CD3G*	CD3γ	
T- B+ NK+/NK-	*PTPRC*	CD45	
Increased lymphocyte apoptosis			
T- B- NK-	*ADA*	Adenosine deaminase	Reticular dysgenesis
T- B- NK-	*AK2*	Adenylate kinase 2	
Other mechanisms			
T- B+ NK+	*RMRP*	RNA of RNase MRP complex	Cartilage hair hypoplasia (CHH)

Table 1.1: Classification of SCID based on the immunophenotype. Various immunophenotypes found in SCID and their genetic sources. Based on Buckley (2004); Cossu (2010).

1.1 Deficiencies in cytokine signalling

Defects in cytokine signalling can be caused by mutations in either the genes encoding for cytokine receptors (*IL2RG, IL7-RA*) or the kinases involved in signal transduction (*JAK3*) (Macchi et al., 1995; Noguchi et al., 1993; Russell et al., 1995).

1.1.1 Mutations in *IL2Rg*

One of the most common forms of SCID in humans is X-linked recessive SCID (T- B+ NK-). It is caused by a mutation in *IL2RG*, which is localised at Xq13.1 and encodes for the common γ-chain. Because of its location on the X chromosome, only males are affected by this form, with their mothers being silent carriers of the mutation. Firstly discovered as part of the IL2 receptor, the common γ-chain is in fact part of several cytokine receptors, namely IL2, IL4, IL7, IL9, IL15 and IL21 (Leonard, 2001; Russell et al., 1993). The receptors for these cytokines all show a common structure, consisting

of three subunits – α, β and γc – which are not covalently linked. Only upon stimulation of the α-chain via the respective cytokine, a stable heterotrimer is formed (Malek and Bayer, 2004) (**fig.2**). *JAK3* molecules bound to the γc-chain and Jak1 molecules bound to β-chain then phosphorylate key tyrosine residues in themselves and the β-subunit, thereby leading to an amplification of the signal. Next, members of the STAT (signal transducer and activator of transcription) pathway are phosphorylated, causing their dimerization and migration into the nucleus. Here they act as gene regulators controlling numerous steps of T cell proliferation (Sponzilli and Notarangelo, 2011). Thus, a mutation in *IL2RG* leads to impairment of several cytokine signalling pathways at once. IL7 plays a major role in T cell development (see tab.1.2), so that blocking of its signalling induces the T- phenotype found in X-linked SCID. The same phenotype is observed in patients with *IL7RA* mutations (Puel et al., 1998). Faulty IL15 signalling, on the other hand, induces the NK- phenoytpe, with IL15 being responsible for NK cell development (Kennedy et al., 2000).

Cytokines relying on γc mediated signal transduction	
IL2	T cell proliferation
	Antigen induced cell death
	Boosting of cytolytic activity of NK cells
IL4	B cell proliferation
	TH2 cell development
	Ig class switching
IL7	T and B cell development in human and mice
	B cell development in mice
IL9	Mucus production
	Mast-cell proliferation
IL15	NK cell development
	CD8 memory T cell homeostasis
IL21	Potential actions on T cells, NK cells, B cells

Table 1.2: Based on Leonard (2001); O'shea (2004).

1.1.2 Mutations in *JAK3*

Hindering the same pathways, only on a different level, are mutations in *JAK3*, i.e. Janus kinase 3. As mentioned above, *JAK3* is responsible for cytokine signal transduction (**fig.2**). Thus, patients suffering from this autosomal recessive form of SCID show the same T- B+ NK- phenotype as X-linked SCID patients. In both cases, the presence of B cells might be considered surprising, since IL7 is also known to influence

pro-B cell differentiation. This indicates a redundancy in IL-dependent B cell development in humans (Puel et al., 1998). However, B cells found in SCID patients do not undergo class switching (Buckley, 2004). Surprisingly this deficiency cannot be cured even by bone marrow transplantation, with treated patients still lacking NK cells and functional B cells.

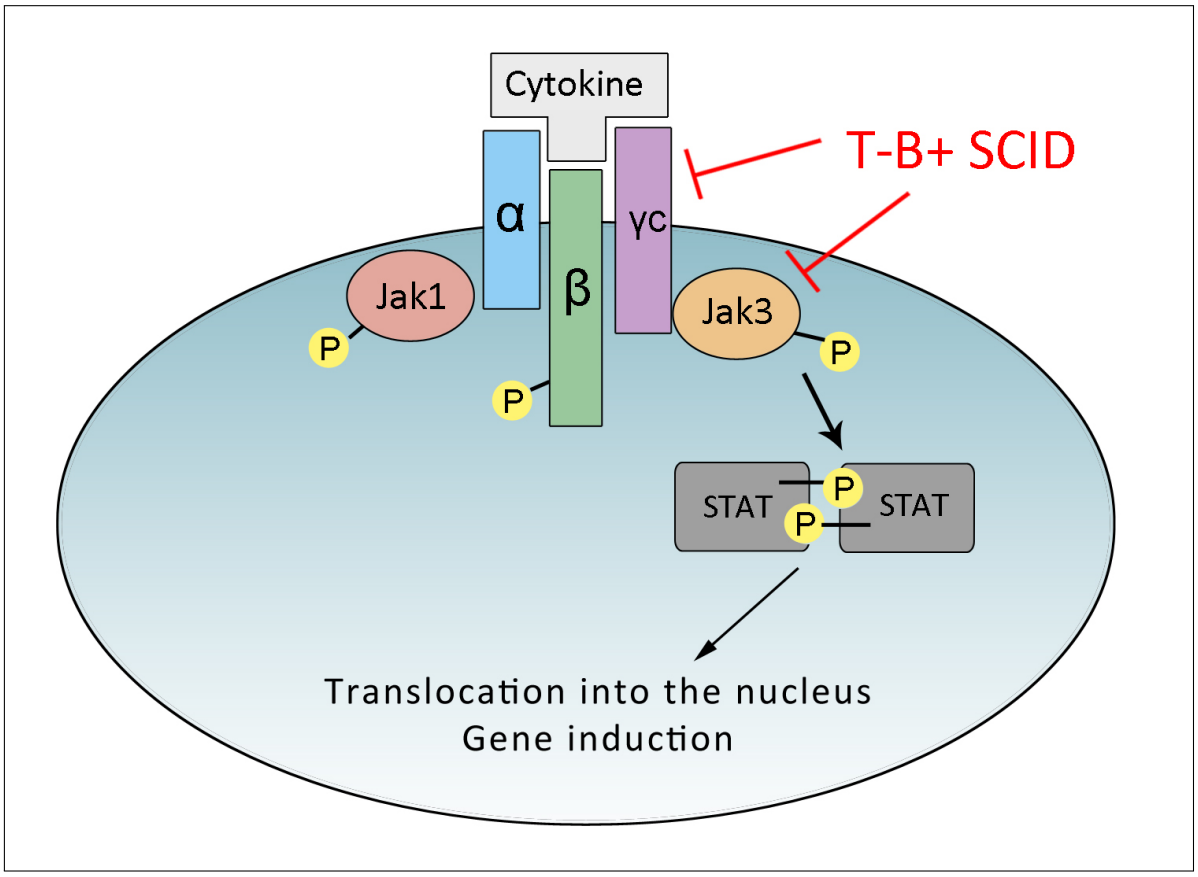

Figure 2: γc mediated cytokine signalling. Roles of *JAK3* and γc in cytokine signalling. Binding of an interleukin molecule to its respective receptor brings the subunits α, β and γc in close proximity to each other, resulting in phophorylation and subsequent activation of Jak1 and *JAK3*. Phosphorylation of STAT members leads to their dimerizaton and translocation into the nucleus, where they regulate genes involved in T cell maturation. Adapted from Malek and Bayer (2004); O'shea (2004).

1.2 Defective V(D)J recombination

Another type of SCID is characterised by the absence of both T and B cells (T- B-). This phenotype is caused by impairment of the recombination of antigen receptor genes, most often based on mutations in the recombination activating genes *RAG1*

and *RAG2*. The great diversity of T cell receptors (TCR) and immunoglobulins (Ig) is ensured by V(D)J arrangement occurring in developing T and B lymphocytes. During this process, a heterodimer consisting of *RAG1* and *RAG2* protein cleaves first one, then the other strand of DNA, yielding terminal hairpins (Oettinger et al., 1990; van Gent et al., 1996)(**fig.3**). This initiation step is followed by the processing phase, during which the DNA-protein kinase complex (DNA-Pkc) binds to the the hairpins and phosphorylates Artemis (gene product of *DCLRE1C*). Activated Artemis finally cuts open the hairpins, so that two coding structures from different gene clusters can be ligated in a coding joint. The ligation is based upon the mechanism of non-homologous end joining (NHEJ). Given the imprecise nature of this DNA repair mechanism, small insertions and deletions may occur, thereby increasing the variability of generated receptor molecules. DNA cleavage by the *RAG1/RAG2* complex is triggered by recombination signal sequences (RSS), which vary in size between the different regions - 23RSS for V (Variable) and J (Joining) and 12RSS for D (Diversity). This ensures that recombination will result in a functional gene containing segments of all three clusters in the right order (Sadofsky, 2001). Mutations in any of the genes involved in V(D)J rearrangement lead to a similar phenotype, namely T- B- NK+. Most commonly, mutations are found in *RAG1* and *RAG2*, but have also been detected in *DCLRE1C*.

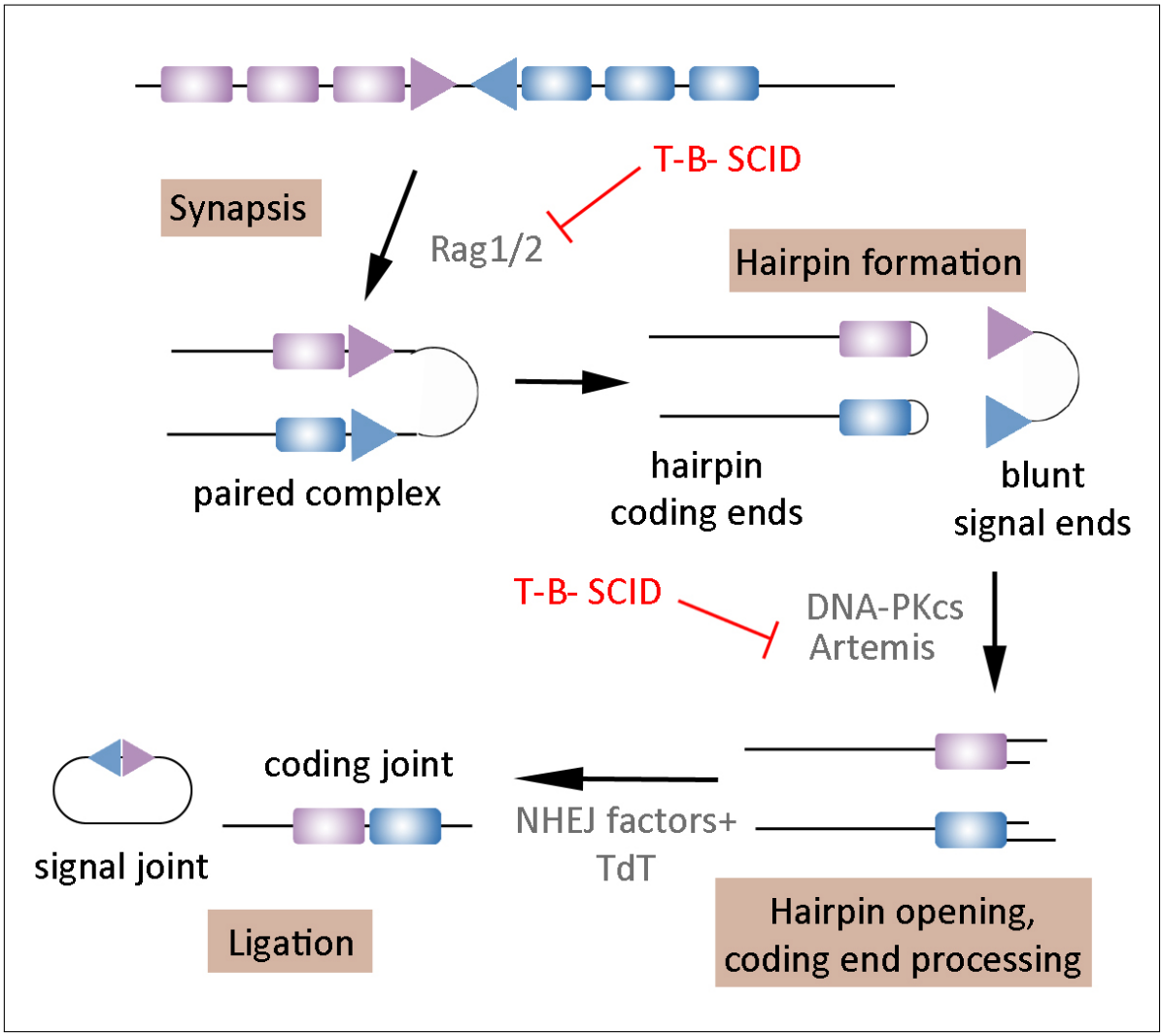

Figure 3: V(D)J recombination depends on *RAG1/RAG2*. A complex of *RAG1/RAG2* binds to and cleaves V, D and J segments, triggered by the respective RSS sites (triangles). The resulting hairpins are opened by concerted action of DNA-PKc and Artemis and can be ligated to form a coding joint, a process involving NHEJ-related enzymes and terminal deoxynucleotidyl transferase (TdT). Adapted from de Villartay et al. (2003); van der Burg and Gennery (2011).

2 Immunodeficient animal models in biomedical research

The same genetic defects that cause SCID in humans can be introduced into animals to induce immunodeficiency. This yields not only animal models for SCID, but, more importantly, creates valuable tools for immunology, cancer research and transplant studies. Unable to reject foreign cells and tissues, these models allow for engraftment of tumorigenic material to generate tumour graft models; stem cells to assess gene therapy safety; and human lymphocytes to model the human immune system *in vivo*. Mice are the prime animal model, but more recently, efforts have also been directed to generate immunodeficient models in larger animals.

2.1 Murine models

Characteristically, most SCID mice carry the *Prkdc*^{scid} mutation, which results in an almost complete lack of mature T and B lymphocytes (Greiner et al., 1998). This mutation was firstly discovered in C.B-17 mice (Bosma et al., 1983); suppressing expression of functional "protein kinase, DNA activated, catalytic polypeptide" (*Prkdc*), it interferes with V(D)J recombination and therefore formation of TCR and Ig. This leads to an impairment of T and B cell development (see 1.2).

Because it does not completely block lymphocyte formation (Bosma et al., 1988; Nonoyama et al., 1993), the *Prkdc*^{scid} genotype is often combined or replaced with other mutations influencing the innate and adaptive immune system, so to further permit xenografts. These include mutations of *Il2rg* (Cao et al., 1995; Ito et al., 2002), *Lyst/bg*^{J} (Christianson et al., 1996), *RAG1/2* (Mombaerts et al., 1992; Shinkai et al., 1992; Traggiai et al., 2004) and *JAK3*(Thomis et al., 1995).

Currently, a multitude of different immunodeficient mouse models exists, differing not only in genetic mutations, but also in background strain. All faithfully produce a phenotype with very low T and B cell counts, often in combination with low levels of innate immunity (Shultz et al., 2007). Most allow engraftment of human cells

and tissue; transplantation with human hematopoetic stem cells and peripheral blood monocytes has been used for the generation of several models of the human immune system (Ishikawa et al., 2005; Lapidot et al., 1994; Mosier et al., 1988; Shultz et al., 2005). SCID mice are also extensively used for cancer studies (Pearson et al., 2008; Tentler et al., 2012), either in individualised tumourgraft models (Kelland, 2004) or for testing of therapies such as tumour-growth inhibitors (Dewan et al., 2003), humanised antibodies (Flavell et al., 2006) or angiogenesis inhibitors (O'Reilly et al., 1996). However, it has become clear that immunodeficient mice models have limitations. Notably, the phenotype caused by *IL2RG*$^{-/-}$ or deficient IL7 signalling differs between mouse and human; the former show complete absence of B lymphocytes, whereas in humans, B cells are still present, albeit poorly functional due to lack of T cell help. Also, humanised immunodeficient mouse models, while producing a diverse repertoire of B cells (Kolar et al., 2004), are unable to form human T cells (Greiner et al., 1998) and therefore do not show T cell mediated responses such as delayed-type hypersensitivity (Shultz et al., 2007). Besides, human allograft rejection has not been observed in these models, either (Shultz et al., 2007). Mice in general also show different responses to inflammation (Seok et al., 2013) and sepsis (Fairbairn et al., 2011).

2.2 Other animal models

Therefore, different immunodeficient animal models are needed, especially in regard to long term studies or research into complex diseases. Looking for an alternative organism with a more suitable lifespan and physiology, immunodeficient animal models in rats and rabbits have been generated. In rats, *Prkdc* and *Il2rg* have been knocked-out with ZFNs, either alone or in combination (Mashimo et al., 2012, 2010). Double knock-out animals showed a superior immunodeficient phenotype in comparison to similar mice models, with no kind of T cells and no B cells and only NK cells being detected. *RAG1* knock-out rats have also been generated with the help of engineered nucleases, but unlike the respective mouse models, these rats show residual T and B cells, resulting in eventual rejection of allotransplants (Ménoret et al., 2013). In rabbits, *RAG1* and *RAG2* have been knocked-out with the help of TALENs (Song et al., 2013). Here, no T and B cells could be detected in lymphoid organs and peripheral blood, showing once more that the same knock-out may lead to different outcomes in different species due to innate disparity in immunology (Haley, 2003). To account for these differences, it is therefore desirable to develop immunodeficient animal models in a variety of species. Large animals are of particular interest in this context, as they provide a suitable platform for longitudinal studies needed for example for the evaluation of stem cell

therapies. Since few naturally occurring immunodeficient animal strains have been reported, these models have to be generated by gene targeting techniques. Thus, in the next chapter, conventional gene targeting methods and recent developments in genome editing will be reviewed.

3 Gene Targeting

Long before the Nobel prize was awarded to Capecchi, Evans and Smithies in 2007, their discovery of gene targeting in embryonic stem cells (ESCs) (Thomas and Capecchi, 1986) had been honoured by the scientific community by making it one of the most widely used techniques in modern life sciences. The targeted introduction of genomic alterations such as mutations, deletions and insertions is not only at the base of every animal model used in fundamental and medical research, but is also the key for gene therapy. But while conventional gene targeting is highly efficient in ESCs, frequencies in somatic cells are considerably lower, making time-consuming screening processes of large numbers of clones a prerequisite for organisms where ESCs are not available. As somatic cells show a finite lifespan in culture and often do not survive the screening procedure, this has substantially impeded development of and research in model organisms other than mouse. With the advent of customizable engineered nucleases (CENs), efficient gene targeting in almost any cell type has become attainable for every standard molecular biological laboratory. CENs have given rise to genome editing, by which multiple specific knock-outs and/or knock-ins can be introduced in a fraction of the time previously needed. In the following chapter, the development of gene targeting, from conventional gene targeting to genome editing, will be highlighted, with special focus on TALENs and RGENs.

3.1 Conventional gene targeting

Conventional gene targeting is the stimulation of crossover between an exogenous targeting vector and a cognate genomic sequence by homologous recombination. Since the vector is generated *in vitro*, it can be altered at will to reflect the experimental goal — introduction of point mutations or addition and deletion of exons or whole genes are commonly used in reverse genetics. By providing homologous sequences on the targeting vector flanking the cassette, homologous recombination (HR) is triggered between the donor vector and the genomic locus. HR is a conserved repair mechanism; in the context of gene targeting, it leads to the seamless insertion of the DNA fragment

of interest, similar to the chromosomal exchange between sister chromosomes during meiosis.

Targeting efficiency for conventional gene targeting is low with about one positive event per 10^5–10^7 transfected cells. Since random integration, i.e. random insertion of the targeting construct into the host genome, occurs with 1000-fold frequency, concerted positive/negative selection is indispensable to identify correctly targeted clones. This screening process is laborious and time-consuming and often yields only few correctly targeted clones, especially when working outside the well established mouse model. Thus, researchers have been looking into possibilities to activate the HR pathway in gene targeting.

3.2 Genome editing with customizable nucleases

In the 1990s, experiments with rare-cutting homing meganucleases showed how introduction of a double strand break (DSB) at a site of interest could stimulate HR (Rouet et al., 1994; Segal and Carroll, 1995). While it was later shown that homing endonucleases could also be engineered to target a specific site of interest (Ashworth et al., 2010; Smith et al., 2006), time-consuming statistical analysis was required to modify the protein-DNA interaction, which considerally hindered broad application. It was with the discovery of ZFNs that genome editing gained real momentum. Since then, TALENs and RGENs have only added to the success story of site-specific nucleases as tools for genetic engineering. All of these approaches are based on the same principle, namely combining a customizable DNA-binding domain — or, in the case of RGENs, a short RNA molecule — with an (unspecific) DNA cleavage domain. This allows introduction of small random mutations or integration of a specified modification. While these techniques are certainly helpful in established model organisms, they are invaluable for organisms where gene targeting is difficult due to lack of embryonic stem cells. When introduced into the cell, customizable engineered nucleases (CENs) bind to their target site where their cleavage domain usually causes a DSB. To repair the damage, the cell possesses two mechanisms, namely homology-directed repair (HDR) or non-homologous end joining (NHEJ)(**fig.4**).

In NHEJ, DSBs are repaired by simple ligation of the free ends. Due to the error-prone nature of this mechanism, small insertions or deletions (indels) may occur, often resulting in a frameshift and thus a knock-out of the gene of interest. This process is also termed directed mutagenesis.

When an exogenous DNA donor containing homologous regions is supplied, DSBs will stimulate the HDR pathway, which will lead to the targeted insertion of the

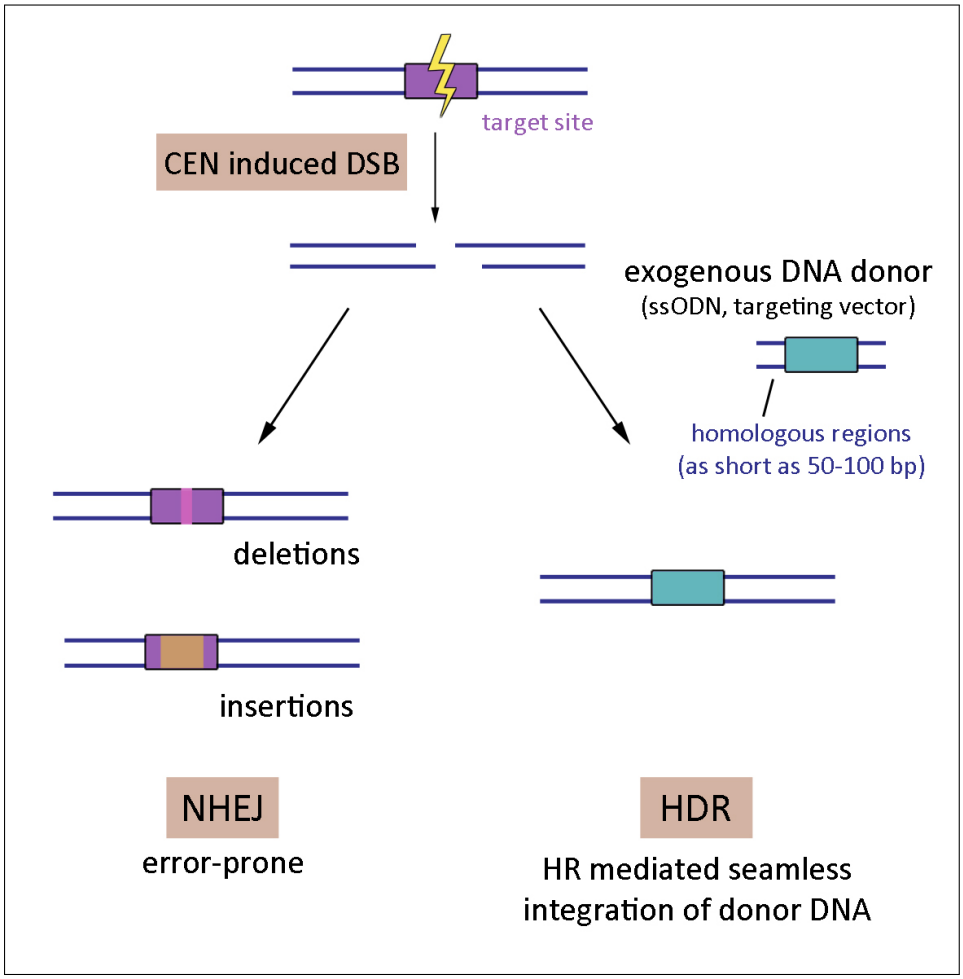

Figure 4: Schematic overview over possible outcomes of CEN induced DSBs. CEN induced DSBs can be repaired either via NHEJ or HDR. NHEJ is an error prone mechanism and often yields small insertions or deletions. HDR occurs in the presence of an exogenous DNA donor and leads to the seamless insertion of the cassette between the homologous regions. ssODN: single stranded oligonucleotide. Adapted from Wright et al. (2014).

exogenous DNA. After resection by an enzyme repair complex, during which the 5' ends near the DSB site are chewed back, one 3' strand of the damaged DNA invades the donor DNA. There, it is used as a primer for amplification of the exogenous DNA. After another round of polymerisation, this time starting from the second damaged strand, the two Holliday junctions are resolved, which mostly results in chromosomal crossover. Thus, the sequence of the exogenous DNA, which may carry SNPs or mutational cassettes, is integrated error-freely into the host genome. Consequently, CENs offer four editing possibilities: simple NHEJ-mediated knock-outs; induction of larger deletions or inversions by simultaneous introduction of two (pairs) of CENs; introduction of a defined mutation or corrected allel via HR; and gene addition via

either pathway. Apart from functioning as nucleases, the DNA-binding domains found in CENs can also be fused to other catalytic domains, so to activate transcription or simply visually localise a certain chromosomal segment.

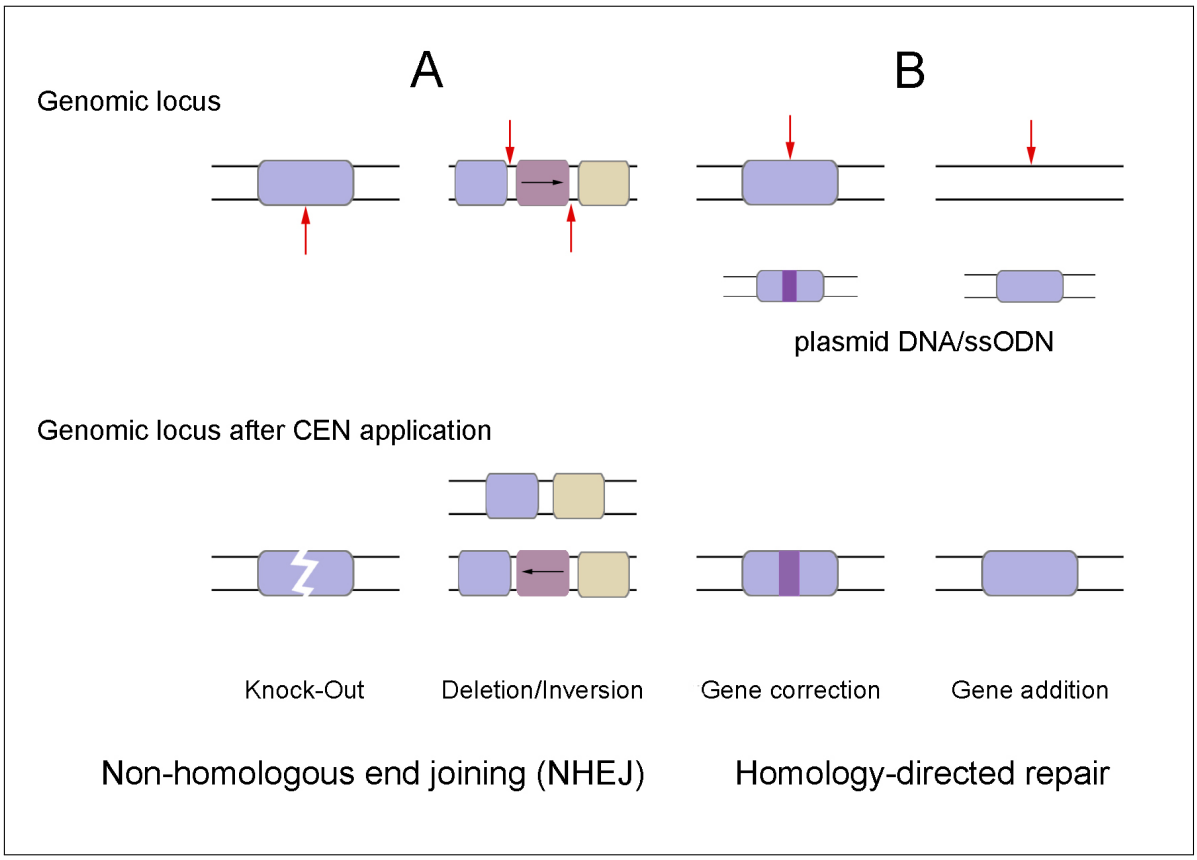

Figure 5: Genome editing with CENs. Introduction of a DSB by CENs can activate two different pathways: A) NHEJ and B) HDR. In the absence of an exogenous DNA donor, NHEJ will cause indels or can result in large inversions and deletions (A). When cotransfected with an exogenous DNA donor (such as a targeting vector or ssODN), CENs can be used for gene correction or addition. Adapted from Gaj et al. (2013).

3.3 Zinc finger nucleases

The first CENs to be widely applied, ZFNs are based on the common DNA-binding scaffold Cys_2His_2-zinc fingers (ZF). ZF motifs, one of the most common DNA binding motifs in eukaryotes, feature 20-30 aa in a ββαconformation and recognise a base triplet via selected residues at the surface of the so called recognition helix. In ZFNs, three to four ZFs are fused to the catalytic domain of the FokI endonuclease, so that a complete ZFN specifies a recognition site of 9-12 bp (Beerli and Barbas, 2002) (**Fig.6A**). Since

the FokI domain requires dimerization, ZFNs have to be used in pairs, with their target sites in tail-to-tail orientation and a spacer sequence of 5-6 bp between them (Miller et al., 2007). Consequently, the total recognition site of a pair of ZFNs is 18-24 bp long, thus enabling specific targeting within the human genome (Gaj et al., 2013). After the first successful application in *Drosophila melanogaster* (Bibikova et al., 2002), several groups established assembly and screening protocols to facilitate the production of customised ZFNs (Beerli and Barbas, 2002; Gonzalez et al., 2010; Hurt et al., 2003; Kim et al., 2011b; Maeder et al., 2008). Because combinatorial selection-based methods are very labour intensive and modular assembly alone not reliable in terms of binding affinitiy and toxicity (Pruett-Miller et al., 2008; Ramirez et al., 2008), most approaches nowadays apply a combination of the two methods. Thereby, individual ZF motifs are picked from preselected libraries and the generated ZFNs are then selected for high affinity. Since they have become readily available, ZFNs have been widely utilised to generate mutant zebrafish (Doyon et al., 2008a), mice (Meyer et al., 2010; Perez-Pinera et al., 2012a), rats (Geurts et al., 2009; Zschemisch et al., 2012), rabbits (Flisikowska et al., 2011), pigs (Hauschild et al., 2011; Li et al., 2013c; Yang et al., 2011) and cattle (Yu et al., 2011). They are also used for gene editing in human cells (Bobis-Wozowicz et al., 2011; Hockemeyer et al., 2009; Lombardo et al., 2007; Zou et al., 2009) and are investigated in clinical trials for gene therapy for HIV/AIDS (Sangamo).

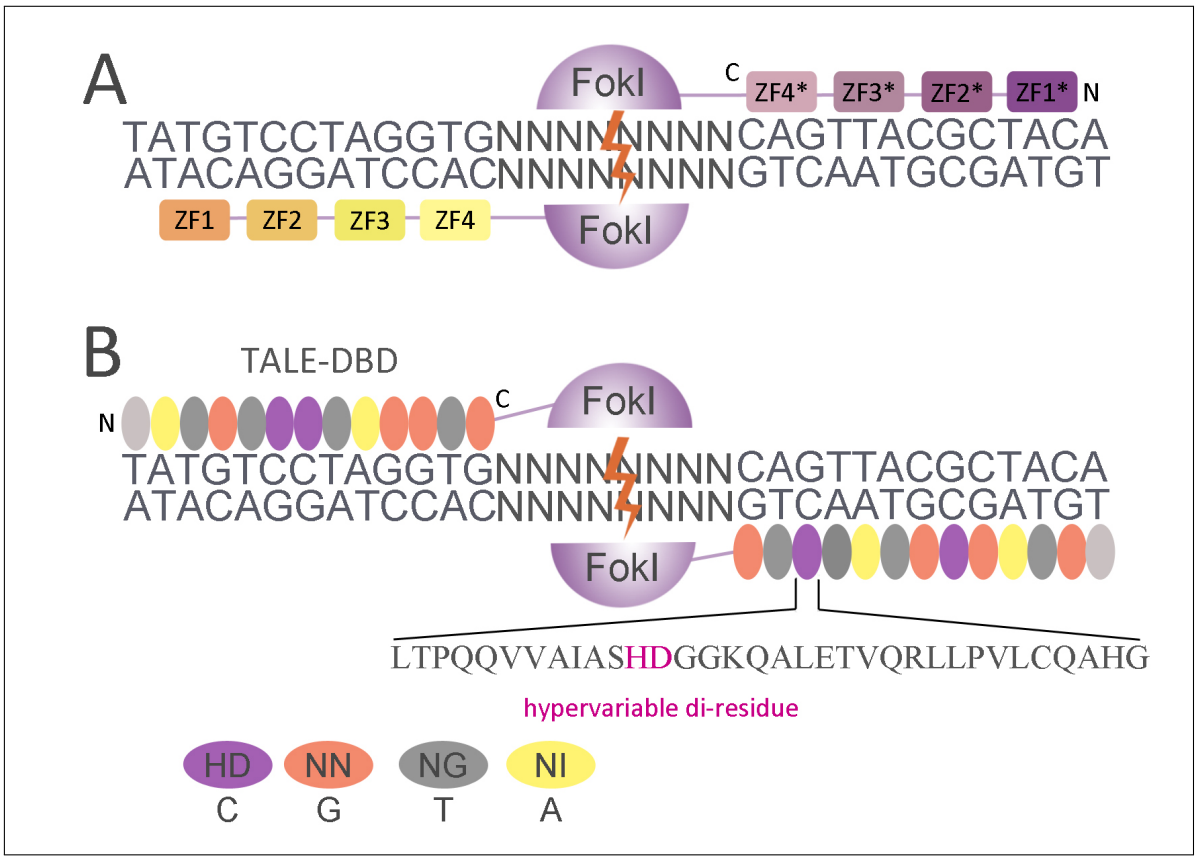

Figure 6: Schematic overview of TALEN and ZFN structures. Binding to the target site and cleaving after dimerization of A: ZFNs and B: TALENs. DBD: DNA binding domain; FokI: FokI catalytic domain.

3.4 TAL effector nucleases

TAL effectors (transcription activator like effectors (TALE)) were first discovered in phytopathogenic bacteria such as *Xanthomonas spp.*, where they are secreted into host cells via the type III secretion system (Kay and Bonas, 2009). Once transported into the nucleus, they act - as their name suggests - as transcription factors, activating certain sets of host genes (Kay et al., 2007). But it was not until the deciphering of the surprisingly straight forward binding code of TALEs that their potential as customisable DNA binding proteins became clear (Boch et al., 2009; Moscou and Bogdanove, 2009).

3.4.1 TALE DNA binding domain

AvrBs3 was the first identified TALE and its structure is canonical for all TALEs (Bonas et al., 1989). It contains an N-terminal bacterial secretion and translocation sequence, a central DNA-binding domain with 17.5 repeat units, a nuclear localization sequence (NLS) and an acidic transcriptional activation domain (Schornack et al., 2006; Van den Ackerveken et al., 1996). The number of repeat units in the DNA-binding domain varies between 1.5 and 33.5 in natural occurring TALEs and around 14.5.-20.5 in artificial ones. Each of the repeats is 33-35 aa long and highly conserved for all but two amino acids — positions 12 and 13 are hypervariable and thus termed repeat-variable diresidues (RVD) (Boch and Bonas, 2010). It is via these RVDs that individual basepairs of the target site are bound in a strikingly simple code. Each RVD specifies one target base in a completely modular and context-independent fashion (Christian et al., 2012). The two available crystal structures (Deng et al., 2012; Mak et al., 2012) show that aa 13 is responsible for binding the sense strand, while aa 12 stabilises the loop by forming hydrogen bonds with the protein backbone (Wright et al., 2014). The RVD, together with an invariable Gly, are located in a loop between the two left-handed α-helices that make up each repeat unit. The most common RVDs and the basepairs that they recognise are depicted in **fig.6B**. Although the code is slightly degenerated with for example NN recognizing both G and A, artificial TAL binding domains have been successfully generated using the most basic RVDs (Cermak et al., 2011; Li et al., 2011b; Morbitzer et al., 2011; Sakuma et al., 2013; Streubel et al., 2012). While DNA-binding specificity of TALEs is solely conveyed by the DNA-binding domain, certain parts of the N- and C-terminus still seem to be necessary for DNA binding (Christian et al., 2010; Kay and Bonas, 2009). In order to ease engineering and enhance attachment of the fused catalytic domain, various trials to

trim extraneous peptide have been made. Commonly used architectures now include a Δ152 N-terminal segment and a shortened C-terminal segment with a length between 14 and 63 bps (Bedell et al., 2012; Carlson et al., 2012; Cermak et al., 2011; Ma et al., 2012; Miller et al., 2011; Mussolino et al., 2011; Sanjana et al., 2012; Zhang et al., 2011). More recent findings indicate that the C-terminal domain can be further optimised by replacing cationic lysine and arginine residues with glutamine. These engineered C-terminal segments have less binding energy and are therefore more specific for binding the correct target sites, thus greatly reducing off-target cleavage (Guilinger et al., 2014).

3.4.2 FokI domain

Based on previous experiences with ZFNs, TALE DNA-binding domains are fused to the 196 aa endonuclease domain of FokI to generate TALENs. FokI is a type II restriction endonuclease that cleaves DNA upon dimerization (Bitinaite et al., 1998). Unlike other restriction enzymes of the same class, FokI is monomeric. This is especially important with regard to TALENs and other programmable nucleases because it means that one FokI monomer, which is bound to the DNA via the corresponding DNA-binding domain, can also dimerise with another FokI monomer still in solution. This greatly increases the risk of off-target cleavage. Off-target activity can be reduced by using obligate heterodimeric FokI domain variants (Miller et al., 2007) or nickases (Ramirez et al., 2012).

3.4.3 TALEN design and assembly

The readiness with which the scientific community has embraced the TALEN technology can be explained by the simplicity of design, assembly and application of TALENs. Determining a suitable target site is indeed straightforward, since only few requirements are known. Because of their fusion with the FokI endonuclease domain, TALENs have to be designed in pairs with a tail-to-tail orientation. The length of the spacer between the two recognition sites may vary; reports have shown that spacers of 12–22 bps work best (Li et al., 2011a; Mussolino et al., 2011). The optimal length of the spacer also depends on the length of the C-terminus which serves as an interdomain linker between the central repeat unit and FokI domain (Miller et al., 2011). In natural occurring TALEs, target sites mostly start with an initial T which is probably bound by a signal in the nonrepetitive N-terminus. While exceptions have shown that this is not a definite requirement (Meckler et al., 2013; Miller et al., 2011; Sun et al., 2012), TALENs with a preceding T seem to be more robust (Jankele and Svoboda, 2014). RVD composition has also been discussed as a factor to influence binding affinity of

TALEs. While it seems to be hard to classify individual RVDs definitely as strong or weak (Meckler et al., 2013; Streubel et al., 2012), long stretches of the same RVD will destabilise DNA binding.

Careful selection of the target site includes searching for possible off-target sites. Depending on the used FokI domain, both hetero- and homodimeric off-target sites with various spacer lengths have to be considered (Kim et al., 2013). TALENs seem to be more sensitive for mismatches in the 5'-region in comparison with the 3'-end (Meckler et al., 2013). Several algorithms have been developped to facilitate TALEN design, incorporating many of the above rules (Cermak et al., 2011; Heigwer et al., 2013; Kim et al., 2013; Neff et al., 2013; Sander et al., 2010; Sanjana et al., 2012). When it comes to assembly, it is possible to establish one's own protocol, but the easy availability of validated kits renders this virtually unnecessary. The basic principle for all of these kits lies in hierarchical ligation of the repeat units in a so called Golden Gate reaction (first described by Engler et al. (2008)). Individual repeats are obtained either by PCR or from a set of plasmids. Through introduction of restriction sites for type IIS endonucleases, which cut outside their recognition site, adaptor-like overhangs are generated at the end of each individual repeat unit. Thus, the repeats can assemble only in the desired order. Ligation into recipient vectors containing the last half-repeat plus the FokI domain then results in the specified TALEN plasmids. This process is usually subdivided into two or more cycles and can be accomplished in any molecular biological laboratory in one week (Cermak et al., 2011; Ma et al., 2013; Sander et al., 2011; Sanjana et al., 2012). Besides small scale setups, high-throughput protocols for TALEN assembly have been described, notably FLASH (fast ligation-based automable solid-phase high-throughput), LIC (ligation-independent cloning) or REAL (restriction enyzme and ligation)-Fast, which mostly rely on preassembled multimers instead of single repeat monomers (Reyon et al., 2012a,b; Schmid-Burgk et al., 2012; Zhang et al., 2011). These robust assembly methods recently made it possible for a single group to produce a library of TALENs targeting more than 18,000 human genes (Kim et al., 2013), showing how easily TALEN libraries can be generated.

3.4.4 Application

TALENs have been used in a variety of organisms with different aims. So far reports have shown TALEN activity in human (Ding et al., 2012), mouse (Panda et al., 2013), rat (Tesson et al., 2011), zebrafish (Cade et al., 2012; Huang et al., 2011), silkworm (Ma et al., 2012), nematodes (Wood et al., 2011), *Xenopus laevis* (Lei et al., 2012) and livestock such as pig (Huang et al., 2014) and cow (Carlson et al., 2013). Efficiency

varies depending on delivery method and cell type, but is mostly in the range of 10–30% of analysed clones for NHEJ-mediated mutagenesis. Notably, biallelic mutations also occur quite frequently (up to 50%). For HDR, efficiency decreases, but is still easily detectable with a frequency of 2–20%. TALENs have been applied in somatic cells, iPSCs (induced pluripotent stem cells) and ESCs , but also directly in zygotes and embryos (Lillico et al., 2013; Sander et al., 2011; Wefers et al., 2013b). The microinjection of TALEN-mRNA offers an interesting possibility to directly generate modified animals, circumventing cloning and all of the associated problems (see 4). Taking the example of the pig, with a mutation efficiency of around 30% of screened clones and 11–30% of these showing biallelic mutations (Carlson et al., 2012; Lillico et al., 2013), this could prove a fast and powerful tool to obtain simple knock-out animals (Wefers et al., 2013b). Another interesting prospect is the simultaneous application of two pairs of TALENs to induce chromosomal rearrangement. Using two pairs of TALENs targeting the same chromosome, Carlson et al. were able to obtain both deletions and inversions of a 6.5 kb fragment after selection (Carlson et al., 2012). Similar observations have been made in silkworm (800 bp of deletion) (Ma et al., 2012) and mouse (700 bp of deletion) (Flemr et al., 2013). Inversion of DNA fragments is also possible with one pair of TALENs in certain regions of the genome: Park et al. inverted a 140 bp cassette causing hemophilia A in human iPSCs with the help of of TALENs, thereby proposing a novel gene therapy (Park et al., 2014). When aiming for integration of exogenous DNA, TALENs tolerate a large span in length. Successful integration of short ssODNs (single stranded DNA oligo nucleotides) with homologies of only $\tilde{5}0$ bp has been reported repeatedly (Strouse et al., 2014; Wefers et al., 2013b), but interestingly, also integration of a 15 kb fragment via an NHEJ-based pathway (Maresca et al., 2012). Thus, TALENs can facilitate both the introduction of small, precise mutations as well as the addition of large gene constructs.

3.5 RNA guided endonucleases

While ZFNs and TALENss hare a common structure, RNA guided endonucleases (RGENs) differ in that they depend on RNA-guidance for DNA binding instead of protein-DNA interaction. The most prominent example of an RGEN is the CRISPR/Cas system. Derived from the acquired immune system in prokaryotes (Wiedenheft et al., 2012), CRISPR stands for clustered, regularly interspaced, short palindromic repeats. These repetitive arrays integrate foreign DNA as spacers (usually 20-50 bp) between conserved repeat sequences with a similar length, thus forming a genetic memory of infection (Bolotin et al., 2005; Marraffini and Sontheimer, 2010;

Terns and Terns, 2011). Upon transcription, CRISPR are processed into precursor (precrRNA) and later mature crRNAs (CRISPR-derived RNAs) and function similar to RNAi. Assembled with one or more Cas (CRISPR associcated) molecules to a patrolling complex, they monitor the intracellular space for invading foreign DNA and RNA with matching protospacer sequences, which are inactivated upon detection (Bhaya et al., 2011; Brouns et al., 2008; Hale et al., 2009). This type of adaptive immune system can be found in 40% of all bacteria and 90% of archaea (Grissa et al., 2007); but while the basic principle remains the same, the processing mechanisms for CRISPR arrays differ greatly, as do the mechanistically extremely diverse Cas proteins involved (Haft et al., 2005; Kunin et al., 2007). Three distinct systems have been described (Makarova et al., 2011); the platform used for genome editing is based on the type II CRISPR/Cas9 system found in *Strep. pyogenes*.

3.5.1 CRISPR/Cas9 system

The CRISPR/Cas9 system found in *S. pyogenes* consists of four elements: precrRNA; a trans-activating crRNA (tracrRNA) that is complementary to the repeat sequence and triggers crRNA maturation and later DNA cleavage in the presence of crRNA; the double-stranded RNA-specific ribonuclease RNase III which processes precrRNA; and the signature protein Cas9 (formerly Csn1 or Cas5) that acts as a molecular anchor bringing together CRISPR/crRNA and tracrRNA and also inactivates target DNA by introduction of a DSB (Deltcheva et al., 2011; Jinek et al., 2012; Sapranauskas et al., 2011). After precrRNA has been transcribed from an CRISPR array, it pairs with tracrRNA via their complimentary repeat sequences. This tracrRNA:precrRNA complex then stimulates processing by recruiting both RNase III and Cas9 (Deltcheva et al., 2011). After maturation of crRNA is concluded, tracrRNA stays paired with crRNA within the Cas9 scaffold, forming a binary guide RNA (gRNA):Cas9 complex. This probably enables correct orientation of the crRNA for recognition of the target sequence (Jinek et al., 2012). For binding of the gRNA:Cas9 complex to its target site, an additional consensus sequence is required at the 3' end of the target site — the so called protospacer adjacent motif (PAM). PAM sequences vary between different CRISPR/Cas systems (Mojica et al., 2009); in *S. pyogenes*, they consist of a three bp NGG consensus sequence. In the absence of a PAM, the Cas9:gRNA complex rapidly dissociates from the DNA. When a PAM is present on the complimentary strand, it will license unwinding of the target DNA. Subsequent pairing of crRNA and the target DNA is then initiated from the seed region at the 3' end of the crRNA (Jinek et al., 2012; Sternberg et al., 2014). Finally, the heteroduplex, formed by 20

nt of gRNA and protospacer, is subjected to cleavage by Cas9. This 1100–1400 aa multidomain nuclease contains two endonuclease domains, one homologous to HNH and the other one homologous to RuvC. Furthermore, it possesses a REC domain responsible for recognition of the gRNA:target DNA complex. Upon stabilization of the gRNA:DNA complex,the RuvC domain cleaves the target strand of the DNA, while the HNH domains cuts the complimentary, non-target one (Garside and MacMillan, 2014; Jinek et al., 2012; Nishimasu et al., 2014).

This whole system can be further simplified by fusing functional parts of tracrRNA and mature crRNA together to yield a single guide RNA (sgRNA) of $\tilde{1}00$ nt in length (Jinek et al., 2012)(**fig.7**). Additionally, the function of RNase III can be completely replaced by Cas9 (Cong et al., 2013). For efficient targeting with the modified CRISPR/Cas9 system, it is thus sufficient to introduce the sgRNA containing the 20 bp target site and Cas9, either encoded by a plasmid or directly as mRNA.

3.5.2 Applications

Despite its novelty, this two-component RGEN platform has already proven to be extremely efficient for targeted genome editing in a variety of cell types and organisms. After first applications in cultured hunan and murine cells (Cong et al., 2013; Mali et al., 2013b), the system has been utilised in bacteria (Jiang et al., 2013), yeast (DiCarlo et al., 2013), *Drosophila* (Gratz et al., 2013), zebrafish (Hwang et al., 2013), goat (Ni et al., 2014), rabbit (Yang et al., 2014), pig (Whitworth et al., 2014) and plants (Li et al., 2013b; Shan et al., 2013). It can also be used for the direct generation of knock-out animals via RNA microinjection into zygotes or embryos (Bassett et al., 2013; Li et al., 2013a; Yu et al., 2013). A possibility unique to RGEN is multiplexing, i.e. the simultaneous introduction of different sgRNAs to target several genes at once. The feasiblity of this approach has been shown by targeting 3–5 genes in rat, zebrafish, human cells and murine ES cells (Jao et al., 2013; Li et al., 2013b; Mali et al., 2013b; Wang et al., 2013). As for TALENs, huge libraries of gRNAs and cell pools harbouring copies of these have been generated for both the human and the murine genome, enabling examination of genetic functions by positive and negative phenotypic screening (Koike-Yusa et al., 2014; Shalem et al., 2014). In an attempt to even further streamline the process of generating precise knock-out animals, a mouse strain carrying a Cre-dependent Cas9 cassette has been established (Platt et al., 2014). Application of the CRISPR/Cas9 system is not limited to genome editing. Catalytically inactive variants of Cas9 (dead Cas9, dCas9) offer a platform for a myriad of fusion proteins (Sander and Joung, 2014). Thus, dCas9 can act as a repressor (Bikard et al., 2013; Qi et al.,

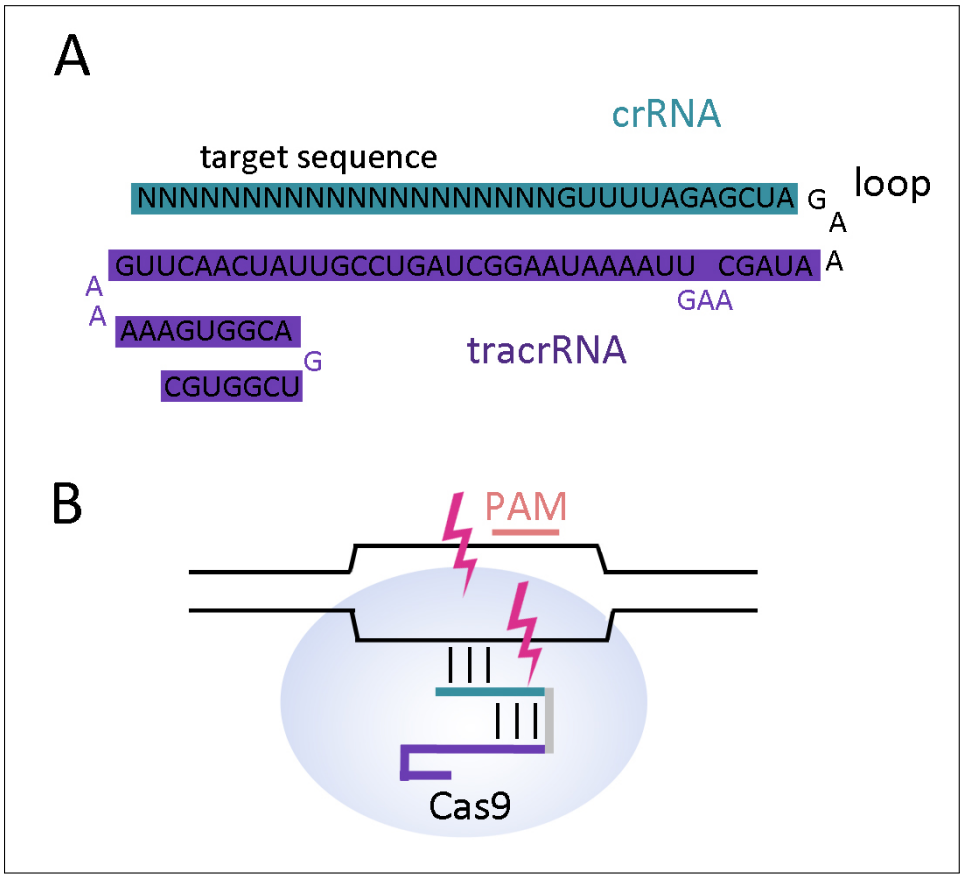

Figure 7: Schematic overview of sgRNA and Cas9 mediated cleavage. (A) In sgRNAs, crRNA (green) and tracrRNA (purple) are fused together and connected by a loop. (B) Upon recognition of a PAM at the non-complimentary strand, Cas9 starts interrogating the adjacent sites for crRNA complimentarity; after binding and formation of an R-loop, DNA is cleaved by the two nuclease domains of Cas9. Based on Hsu et al. (2013); Sander and Joung (2014).

2013) or be paired with effector domains to yield a transcriptional activator (Cheng et al., 2013; Gilbert et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013; Qi et al., 2013) when directed to a promoter by a suitable sgRNA. Yet another possibility is the fusion of a fluorescent domain to allow visualization of DNA loci to enable studies of chromosome dynamics (Anton et al., 2014; Chen et al., 2013a).

4 Porcine models for medical research

After decades of extensive studies of mouse genetics, metabolics and pathophysiology, there is hardly any mouse disease, be it natural or artificially inflicted, that modern science cannot cure. But translation of the insights into validated medical treatments for humans remains difficult. Because of differences in anatomy, life span and nutrition, to name but a few, many clinical studies for anti-cancer drugs fall short of their promising tests in mice (Sausville and Burger, 2006). Furthermore, due to their smaller size, mice do not provide a platform for testing diagnostic and surgical methods. And for complex, multifactorial conditions such as cardiovascular diseases or inflammatory responses, mice models often fail to show the full range of associated symptoms found in humans (Tan et al., 2012). Thus, the need arises for a better animal model and while pigs (*Sus scrofa*) are mostly seen as an important source for protein, they also offer a host of benefits over mice as scientific animal models. In terms of genetics, pigs are closely related to humans, even more so than mice, as shown by the latest published porcine genome sequence (Groenen et al., 2012). At the nucleotide level, the identity between human and porcine genome is three times higher than between human and murine genome (Prather, 2013); and although pigs and humans diverged at the same time as mice and human, the pig sequence is more similar to the human sequence than the mouse one and shares more ultraconserved regions (Wernersson et al., 2005). With regard to metabolism, pigs are omnivorous like humans and many of their physiological and pathophysiological responses are the same as in humans (Flisikowska et al., 2014). Furthermore, the porcine immune system is more similar to human than the murine one (Schook et al., 2005). And aside from their utility as disease models, pigs can also be genetically engineered to provide a source of xenotransplants (Bendixen et al., 2010; Lai et al., 2002; Phelps et al., 2003). Short gestation time and early sexual maturation, larger litter size plus a relatively long life span further favour the use of pigs in biomedical research. Due to the long tradition of domestication, housing and feeding conditions for pigs are standardised and they can be easily kept in designated

pathogen free facilities (Rehbinder et al., 1998). Lastly, ethical concerns regarding pigs are very low. Several porcine models for complex diseases such as Diabetes mellitus, cystic fibrosis or cancer have been established (rev. by Flisikowska et al. (2014)). An immunodeficient pig model could function similar to NOD/SCID mice and would be helpful for the verification of stem cell therapies, the establishment of primary tumour graft models and, given the similiarities of porcine and humane immunome (Dawson et al., 2013), as a model for the humane immune system.

Generation of genetically defined disease models

Research with pigs has substantially benefited from recent progress in genome editing. Until a few years ago, generation of genetically engineered pigs consisted of conventional gene targeting in porcine cells with subsequent somatic cell nuclear transfer (SCNT). And while this approach yielded some promising disease models, both conventional gene targeting and SCNT are tedious and labour-intensive and require considerable tweaking before satisfactory efficiency is reached. The drawbacks of conventional gene targeting have already been discussed in 3.1; as for SCNT, its efficiency is low, with about 1-5 %, and influenced by a complex interplay between multiple factors that can only partly be controlled (Huang et al., 2013; Kurome et al., 2013). The success of nuclear transfer is highly dependent on the donor cells used, with a poor donor cell preparation resulting in failure to establish or complete gestation. Thus, the application of CENs can accelerate the process of generating porcine disease models in two ways (**fig.8**). First, targeting with CENs substantially shortens the time needed to obtain correctly targeted cell clones (Tan et al., 2013). Secondly, CEN-mRNA can be, with or without mutagenic ssODNs, directly injected into zygotes and embryos, where it efficiently introduces the desired mutation (Bedell et al., 2012; Carlson et al., 2012; Tesson et al., 2011; Wefers et al., 2013b). This concept has also been applied with TALENs and ZFNs in porcine zygotes (Hauschild et al., 2011; Lillico et al., 2013; Yang et al., 2011).

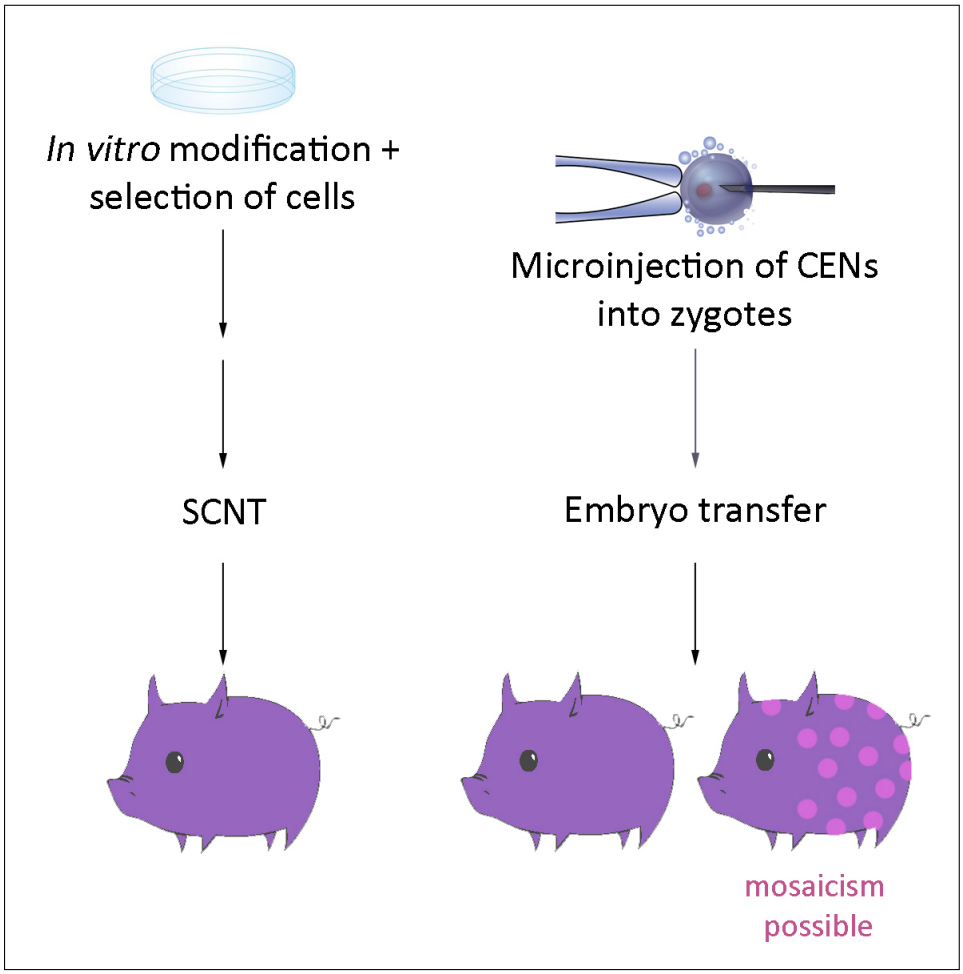

Figure 8: Possibilities for the generation of genetically modified pigs utilizing CENs. CENs can be used either for *in vitro* modification of suitable cells and subsequent SCNT or directly injected into zygotes. While the latter pathway is faster, it includes the possibility of mosaicism, depending on the stage during which mircoinjection occurs. Pictures modified from Generalic (2014); Schroeder (2013).

5 Aim of the study

The aim of this study was the application of novel CENs, mainly TALENs and RGENs, for the generation of an immunodeficient pig model. Focus was placed on three genes known to play an important role in the adaptive immune system: *RAG1*, *RAG2* and *JAK3*. Since CENs are relatively new tools for genetic engineering in pigs, various factors had to be optimised. First, it had to be established how to deliver CENs with maximum efficiency at minimum toxicity; delivery as plasmid DNA and as mRNA with and without a polyadenylation signal plus various transfections methods and kits were tested. Next, it had to be determined which cell type would tolerate CEN-induced mutagenesis; to this extent mesenchymal stem cells from different tissue as well as porcine fetal fibroblasts were isolated. Lastly, a feasible method to select for positive mutation events had to be determined; cells were cotransfected with conventional targeting vectors, marker plasmids and ssODNs.

PART II

MATERIAL

1 Cell culture

1.1 Cell lines

pADMSC 110111	Mesenchymal stem cells isolated from adipose tissue
poFF 251113	Fetal fibroblasts
pBMMSC 071210	Mesenchymal stem cells isolated from bone marrow

Cell lines were isolated by various members of the Chair for Livestock Biotechnology.

1.2 Cell culture media and components

Accutase	PAA, Pasching, Austria
Advanced Dulbecco's Modified Eagle's Medium (DMEM)	PAA, Pasching, Austria
Amino acids, non-essential (100x)	PAA, Pasching, Austria
Amphotericin B (250 µg/ml)	PAA, Pasching, Austria
Cell culture water	PAA, Pasching, Austria
Dimethyl sulfoxide (DMSO)	Sigma, Steinheim, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	PAA, Pasching, Austria
Dulbecco's Phosphate buffered saline (PBS), w/o Ca, Mg	PAA, Pasching, Austria
Fetal calf serum (FCS)	PAA, Pasching, Austria
G-418 sulfate (geneticin) (50 mg/ml)	PAA, Pasching, Austria
Hank's buffered salt solution (HBSS), w/o phenol red, with Ca, Mg	Biochrom, Berlin, Germany
Heparin sodium salt	Sigma, Steinheim, Germany
Human fibroblast growth factor (FGF-2)	Genaxxon, Biberach, Germany
Hypoosmolar buffer	Eppendorf, Hamburg, Germany
L-Glutamine (GlutaMAX)	Gibco BRL, Paisley, UK
Lymphocyte separation medium LSM 1077	PAA, Pasching, Austria
Opti-MEM reduced serum	Gibco BRL, Paisley, UK
Penicillin/Streptomycin	PAA, Pasching, Austria
Sodium pyruvate	PAA, Pasching, Austria

Media

pBMMSCs	Advanced DMEM 10% FCS 1x GlutaMAX 1x NEAA 10 mM beta-Mercaptoethanol optional: 50 ng\ml FGF-2
poFF	Advanced DMEM 15% FCS 1x GlutaMAX 1x NEAA 10 mM beta-Mercaptoethanol optional: 50 ng\ml FGF-2

Media components other than basic medium and FCS were filtered through 0.22 µm filter.

1.3 Cell Culture Kits

Basic Primary Fibroblasts Nucleofector®Ki	Lonza, Basel, Switzerland
Human MSC Nucleofector®Kit	Lonza, Basel, Switzerland
Stemfect^{T}M RNA Transfection Kit	Stemgent, Cambridge, MA
MACSselect K^{k}	Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany

2 Bacterial culture

2.1 Bacterial strains

Escherichia coli ElectroMAX™DH10B™	Invitrogen, Karlsruhe, Germany
Escherichia coli Stbl3 ™	Invitrogen, Karlsruhe, Germany

2.2 Bacterial culture media and plates

Lysogeny Broth, Difco	Becton Dickinson, Heidelberg, Germany
Ampicillin (100 mg/ml)	Sigma, Steinheim, Germany
Chloramphenicol	Sigma, Steinheim, Germany
Spectinomycin	Sigma, Steinheim, Germany
Bromo-chloro-indolyl-galactopyranoside (x-Gal) (100 mg/ml)	Carl Roth, Karlsruhe, Germany
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	Biomol, Hamburg, Germany

Ampicillin was used at a concentration of 100 µl/ml, Chloramphenicol at a concentration of 50 µl/ml.

3 Chemicals

Bromphenol blue	Serva, Heidelberg, Germany
Bovine serum albumine (BSA)	PAA, Pasching, Austria
Ethanol absolute	Riedel-de-Haen, Seelze, Germany
Ethidiumbromide (10 mg/ml)	Sigma, Steinheim, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Steinheim, Germany
GenAgarose LE	Genaxxon BioScience, Ulm, Germany
Glacial acetic acid	Fluka, Seezle, Germany
Isopropanol	Roth, Karlsruhe, Germany
Propidium iodide (PI)	Sigma, Steinheim, Germany
Quick Extract Buffer	Biozym, Oldendorf, Germany
Sodium acetate	Carl Roth, Karlsruhe, Germany
Sodiumdodecylsulfate (SDS)	Omnilab, Bremen, Germany
Sucrose	Fluka Chemie, Buchs, Suisse
TRIS Trizma base	Sigma, Steinheim, Germany
Trizol	Invitrogen, Karlsruhe, Germany

4 Solutions and buffers

10x TBE	0.9 M Tris, 0.9 M boric acid, 20 mM EDTA, pH 8.3
50x TAE	2 M Tris, 50 mM EDTA, 2 M acetic acid, pH 8.0
5x Gel loading buffer	6.0% sucrose, 0.075% EDTA, 0.0025% bromphenol blue
Lysis buffer with Igepal	50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-EDTA, 0.5% Tween-20, 0.5% Igepal, NP 40, pH 8.8
DNA minipreparation	
Solution I	5 mM sucrose, 10 mM EDTA, 25 mM Tris, pH 8.0
Solution II	0.2 mM NaOH, 1% (w/v) SDS
Solution III	3 M Sodium acetate, pH 4.8

5 Enzymes

Antarctic Phosphatase (5000 U/ml)	New England BioLabs, Frankfurt, Germany
Antarctic Phosphatase Buffer 10x	
GoTaq®DNA Polymerase	Promega, Madison,WI
5x Green GoTaq®Reaction Buffer	
Phusion®High Fidelity Polymerase (2 U/µl)	Finnzymes, Espoo, Finland
5x Phusion®High Fidelity Buffer	
Restriction Enzymes	New England BioLabs, Frankfurt, Germany
10x NEB Buffer 1-4	
RNase A Solution (20 mg/ml)	Sigma, Steinheim, Germany
T4 DNA Ligase (3 U/µl)	Promega, Madison,WI
10x T4 Ligation Buffer	
Proteinase K	Sigma, Steinheim, Germany
Kleenow polymerase (5 U/µl)	New England BioLabs, Frankfurt, Germany

6 Kits

CloneJET™PCR Cloning Kit	Fermentas, Burlington, Canada
DualGlo Luciferase Assay	Promega, Madison, WI
MEGAclear™	Applied Biosystems, Darmstadt, Germany
MEGAShortScript™	Applied Biosystems, Darmstadt, Germany
mMESSAGE mMACHINE®SP6/T7 Kit	Applied Biosystems, Darmstadt, Germany
Miniprep Kit	Sigma, Steinheim, Germany
NucleoBond®PC Kit	Machery-Nagel, DÃ¼ren, Germany
pGEM®-T Easy Vector System	Promega, Madison,WI
Poly(A) Tailing Kit	Applied Biosystems, Darmstadt, Germany
Qiagen EndoFree Plasmid Maxi Kit	Qiagen, Hilden, Germany
GenElute™Mammalian Genomic DNA Wizard®SV Gel and PCR Clean-Up System	Promega, Madison, WI

7 Recognitions sites of TALENs and crRNAs

RAG1 TALENs	ttcagggtgagatcctttgaaaaggcacctgaaaaggctcaaacgga
RAG2 TALENs	accttcctcctctccgctacccagccacttgcacattcaaaagcagcttag
JAK3 TAL HH	tgtcctgttggttccccccaagccacatcttctccgtggaggatgca
JAK3 TAL TZ5	tgatccctcagcgctcctgcagcctctcctcttcagaggctggtgccctgca
JAK3 TAL TZ6	tgaagagacacccttgatccctcagcgctcctgcagcctctcctcttcaga
RAG1 crRNA	gctggagattgctccagcgaggg
JAK3 crRNA 1-20	ctgcagcctctcctcttcagagg
JAK3 crRNA 1-18	gcagcctctcctcttcagagg
JAK3 crRNA 2	tgcatgttctgctgccccctcgg

8 Primers and Oligonucleotides

Primers were ordered salt free from Eurofins Genomics, Ebersberg, Germany. Oligonucleotides of more than 80 bp were ordered from biomers.net, Ulm, Germany.

Oligos

JAK3 TAL TZ5 ssODN	tccaagtgaagagacaccctgatcagcgctcctgcaggatcctctcctcttcagaggc tccctgctgttctgctgccccctcggg
JAK3 TAL HH ssODN	ctctggccacggaggacctgccctgttccccccaaggatccacatcttctccgaggat gcgggcacccaagtcctc
RAG1 crRNA	cacctaataatacgactcactatagGCTGGAGATTGCTCCAGCGA
	aaacTCGCTGGAGCAATCTCCAGCctatagtgagtcgtattatta
JAK3 crRNA 1-18	cacctaataatacgactcactatagGCAGCCTCTCCTCTTCAG
	aaacCTGAAGAGGAGAGGCTGCctatagtgagtcgtattatta
JAK3 crRNA 1-20	cacctaataatacgactcactatagGCTGCAGCCTCTCCTCTTCAG
	aaacCTGAAGAGGAGAGGCTGCAGctatagtgagtcgtattatta
JAK3 crRNA 2	cacctaataatacgactcactatagTGCATGTTCTGCTGCCCCCT
	aaacAGGGGGCAGCAGAACATGCActatagtgagtcgtattatta
JAK3 TAL HH Screening Construct	cggccaccatggtcgtgtcctgttggttccccccaagccacatcttctccgtggaggat gcatg
	catgcatcctccacggagaagatgtggcttgggggggaaccaacaggacacgacc atggtggc
JAK3 TAL TZ5 Screening Construct	cggccaccatggtcgtgatccctcagcgctcctgcagcctctcctcttcagaggctg gtgccctgcatg
	catgcagggcaccagcctctgaagaggagaggctgcaggagcgctgagggatcacg accatggtggc
JAK3 TAL TZ6 Screening Construct	cggccaccatggtcgtgaagagacacccttgatccctcagcgctcctgcagcctctc ctcttca gcatg
	catctgaagaggagaggctgcaggagcgctgagggatcaagggtgtctcttcacg accatg gtggc
JAK3 crRNA 1 Screening Construct	cggccaccatggtcgCTGCAGCCTCTCCTCTTCAGtg
	caCTGAAGAGGAGAGGCTGCAGcgaccatggtggc
JAK3 crRNA 2 Screening Construct	cggccaccatggtcgTGCATGTTCTGCTGCCCCCTtg
	caAGGGGGCAGCAGAACATGCAcgaccatggtggc

Primer

Name	Sequence	used for	Tm [°C]	Product length [bp]
JAK3-HA1_F2_HindIII	tgtaagcttCCAGTGCCCATCTGC TAGAAA	Cloning of targeting vector	65	3134
JAK3-HA1_R2_SacII	tccgcggCTCGAGGGACCTAATAACTT CGTA			
JAK3-HA2_F2	tatgattcgcgaCCAAGTCCTCGTCTAC AGGCTCCG	Cloning of targeting vector	63	1057
JAK3-HA2_R2	tatgtcgacGGGACAGGCACCGGTAGGGT			
JAK3_TALENs_F	GCTGCACTCATGGCACCTCCA	Screening JAK3 TALENs	60	917
JAK3_TALENs_R	TCCCTGGGACACCCACCAGGA			
J3 CRISP Scr1 F1	CCCTGGGCATCAACAAGAGT	Screening JAK3 crRNA	60	742
J3 CRISP Scr1 R1	CTCCCTCTGGCCAATCCTTC			
J3 OT1 F2	GCGACCTGACGTTAGCTGTT	Screening Off-target sites JAK3 crRNA 1	60	952
J3 OT1 R2	CAGGTGCTCTACTATTAGCCATCA			
J3 OT2 R	CCCAAAGACCTAATGCCCTGA		60	404
J3 OT2 R	TCTCTGACAGTGAGAAACAACACA			
J3 OT3 F	AAGTGTTGACTGCTCCGTGA		60	339
J3 OT3 R	GGCAAGAAAACTGAGCTTCCC			
J3 OT4 F	ACCAATGGGGAAGCTTCAGA		60	304
J3 OT4 R	TATCTGGGTGGAGTCGCTGG			
RAG1 T7E1 F	GGGACTCAGTTCCGCCCCAGA	Screening RAG1 TALENs	57	902
RAG1 T7E1 R2	GCTTGCAGCTGGTCTCCACCG			
RAG2 TAL F	CCCAGCTCGCCTGGATTTTTGC	Screening RAG2 TALENs	60	663
RAG2 TAL R	CCGTCCTCCAAAGAGAACACCC			
Scr JAK3 F	GACATAGCGTTGGCTACCCG	Screening JAK3 NTV	60	2022
Scr JAK3 R	CGTACCTCTTCTCCTGGGCT			
JAK3 endo F2	CCACTCCCTCTTTGCTCTGG	Endogenous control JAK3	60	1576
JAK3 endo R	ACTCACCAAGTCGTTGCGAT			
J3 Scr ssODN TZ5 F	GGTGAGAATAGGGGTGGGAC	Screening JAK3 TAL TZ5 ssODN	60	589
J3 Scr ssODN TZ5 R	GAGGGGAGAACGTGGAATGG			
FokI F	CACCTGGGCGGATCTCGCAA	Screening Integration of FokI domain	60	313
FokI R	GCACGGCGCCATTGCAGTTT			

9 Consumables

1.5/2.0 ml microcentrifuge tubes	Zefa Laborservice, Harthausen, Germany
15/20 ml centrifuge tubes	Corning, New York, USA
14 ml round-bottom tubes	Becton Dickinson, Heidelberg, Germany
T25/T75/T150/T220 cell culture flasks	Corning, New York, USA
6-/12-/24/96-well plates	Corning, New York, USA
100/150 mm cell culture dishes	Corning, New York, USA
1.8 ml CryoTubes	Nunc, Wiesbaden, Germany
1/2/5/10/25 ml plastic pipettes	Corning, New York, USA
Filter pipette tips	Zefa Laborservice, Harthausen, Germany
Glass pasteur pipettes	Brand, Wertheim, Germany
10/25/50 ml plastic syringes	Becton Dickinson, Heidelberg, Germany
0.22/0.45 µm filter	Sartorius, Göttingen, Germany

10 Software and Websites

Vector design and analysis	
Everyvector	www.everyvector.com
Vector NTI	Invitrogen, Karlsruhe, Germany
Agarose gel documentation	
GeneSnap	Syngene, Cambridge, United Kingdom
Design of CRISPRs and TALENs	
TAL Plasmids Sequence Assembly Tool	http://bit.ly/assembleTALsequences
CRISPR Design Tool Zhang Lab	crispr.mit.edu
ZiFIT (Sander et al., 2010)	http://zifit.partners.org/ZiFiT/
TALEN*designer*	http://www.talen-design.de

11 Devices

Thermocycler DNA Engine®DYAD PCR reaction tubes	Bio-Rad Laboratories, Hercules, CA
5100 Cryo 1°C Freezing Container, "Mr. Frosty"	Nalgene, Rochester, USA
Amaxa®Nucleofector®	Lonza, Basel, Switzerland
Biophotometer 6131 Cuvettes UVette®	Eppendorf, Hamburg, Germany
Clean Bench HERASafe®	Heraeus Instrument, München, Germany
Gene Genius Bio Imaging System	Syngene, Cambridge, United Kingdom
Heating block	Gefran, Seligenstadt, Germany
Incubator BD 115	Binder, Tuttlingen, Germany
Membrapure	Membrapure, Bodenheim, Germany
Multiporator®Electroporation cuvettes (2/4-mm gap)	Eppendorf, Hamburg, Germany PeqLab, Erlangen, Germany
Nanodrop Lite	Thermo Scientific, Waltham, Germany
Orbital Shaker 420	Thermo Scientific, Waltham, Germany
pH meter Cyberscan 510	Eutech Instruments, Singapore, Singapore
Steri-Cycle CO2 Incubator	Thermo Electron, Dreieich, Germany
Transjector 5246 InjectMan	Eppendorf, Hamburg, Germany
Vortex-Genie®2	Scientific Industries, Bohemia, NY
Centrifuges	
Eppendorf MiniSpin®	Eppendorf, Hamburg, Germany
Sigma 1-15K (Rotor 12024)	Sigma, Steinheim, Germany
Sigma 4K15 (Rotors 11150, 13350)	Sigma, Steinheim, Germany
Sigma 3-16 (Rotor 12024)	Sigma, Steinheim, Germany
Balances	
Kern 440-33N	Kern & Son, Balingen, Germany
APX-1502	Denver Instrument, G´ottingen, Germany
Microscope and accessories	
AxioCAM Mrc	Zeiss, Oberkochen, Germany
AxioCAM MRm	Zeiss, Oberkochen, Germany
Axiovert 25	Zeiss, Oberkochen, Germany
Axiovert 40 CFL	Zeiss, Oberkochen, Germany
Axiovert 200M	Zeiss, Oberkochen, Germany

Axiovert 10	Zeiss, Oberkochen, Germany
HBO 100	Zeiss, Oberkochen, Germany

PART III

METHODS

1 Molecularbiological work

1.1 Preparation of plasmid DNA

Plasmid DNA can be isolated from over night *E.coli* cultures by alkaline lysis. This method is based on the protocol by Birnboim and Doly (1979) and uses SDS to disrupt phopholipid bilayers and sodium hydroxide to denature released protein. If purified plasmid DNA was needed, DNA was extracted from samples using affinity chromatography.

Minipreparation

Single clones were picked from over night plates and incubated in 3-5 ml LB_{Amp} over night at 37 °C under shaking. 2 ml liquid bacteria culture was centrifuged for 1 min at 18000 x g and the supernatant discarded. The cell pellet was then resuspended in 100 µl of Solution I. 200 µl of Solution II was added and samples mixed by inversion. After incubation for 3 min at room temperature 150 µl of Solution III was added and samples left for incubation on ice for 30 min. Cell debris was then pelleted for 5 min at 18000 x g and 1 ml of 95% ethanol added to the supernatant. After DNA precipitation at 18000 x g for 15 min the pellet was washed with 500 µl of 80 % ethanol for 10 min at 18000 x g, air dried and finally dissolved in 50 µl of ddH_2O with 20 µg/ml RNase A solution added.

Midi-/Maxipreparation

100-300 ml LB_{Amp} were inoculated from glycerol stocks and grown over night at 37 °C while shaking . Midi- and maxipreparations of plasmid DNA were then performed using NucleoBond PC Kit or Qiagen EndoFree Plasmid Maxi Kit and standard procedures.

1.2 Preparation of genomic DNA

Genomic DNA can be isolated from mammalian cells by lysing the cells first with an chaotropic salt which also ensures denaturation of the DNA and consequent precipitation with ethanol. For isolation of genomic DNA, a GenElute Mammalian Genomic DNA Miniprep Kit and standard procedures were used. For screening purposes, DNA from single cell clones was obtained by resuspending the cell pellet in 30 µl Quick Extract buffer and subsequent incubation for 15 min at 65°C. After inactivation by incubating at 95°C for 8 min, cell debris was pelleted for 10 min at 14 000 x g and the supernatant used for screening PCR.

1.3 Polymerase Chain Reaction (PCR)

DNA sequences with a length of up to several kilobasepairs (kb) and a known starting and ending sequences can be amplified using polymerase chain reaction (PCR). When using a proof-reading polymerase such as Phusion High Fidelity Polymerase, incorrect base pairs will be excised and replaced by the correct ones, resulting in error rates as low as 4.4 x 10^7. This can be useful when PCR amplified sequences are used for cloning. 50-200 ng of template DNA was amplified as specified in **tab.1.1**.

Component	Phusion High Fidelity Polymerase	GoTaq Polymerase
Template DNA	50-200 ng	50-200 ng
dNTP mix	200 µM each	200 µM each
Primer for/rev	0.5 µM each	0.5 µM each
Buffer	1x	1x
Polymerase	0.02 U µl	1.25 U µl
ddH_20 to	20 µl	50 µl

Table 1.1: PCR Setup for different polymerases.

	Temperature	Time
Initial denaturation	95-98 °C	30 s - 2 min
Denaturation	95-98 °C	10-30 s
Annealing	Primer specific	30 s
Elongation	72 °C	30 s - 1 min/kb
Final elongation	72 °C	5 min

Table 1.2: Thermocycler conditions. Conditions were adjusted to user manual of the respective polymerase.

	Temperature	Time
Initial denaturation	93 °C	3 min
Denaturation	93 °C	15 s
Annealing	45-55 °C	30 s
Elongation	68 °C	6 min
Repeat for 9 more cycles		
Denaturation	93 °C	15 s
Annealing	45-60 °C	30 s
Elongation	68 °C	6 min + 20 s every cycle
Repeat for 16 more cycles		

Table 1.3: Long range PCR using the 5 Prime polymerase

1.4 Restriction digest of DNA

Restriction enzymes specifically recognize short nucleotide sequences, mostly palindromes with a length between 4 and 12 bp, and cleave DNA molecules at these sites. While some restriction endonucleases produce two identical, i.e. blunt ends, others leave an overhang at the 3' or 5' strand. Restriction digests provide the foundation for cloning experiments, as vector DNA has to be linearised before it can be ligated with an insert. One can also remove unnecessary DNA sequences, e.g. plasmid backbone, by digesting the DNA preparation and isolating the fragment of interest from an agarose gel (preparative digest). Furthermore, restriction analysis is also a valuable tool for identification of DNA sequences, as the band pattern resulting from restriction digest with a certain restriction endonuclease is characteristic for any DNA sequence (analytical digest). Samples were incubated at 37 °C or as specified by the user manual for at least 45 min, longer for preparative digests.

Component	Final concentration
DNA	1-5 µg
NEB buffer	1x
Restriction endonuclease	5-20 U
ddH_20 to	20-30 µl

Table 1.4: Setup for restriction digest.

1.5 Dephosphorylation of DNA

In order to prevent self-ligation of digested DNA, 5' phosphate groups should be removed from DNA later serving as a vector. Phosphatases catalyse the hydrolysis of terminal phosphoric acid monoesters and are therefore commonly used to increase efficiency of ligation reactions. 5 U antarctic phosphatase was added to the digestion set up and buffered with 1x antarctic phosphatase buffer. Samples were then incubated for 30 min at 37 °C and the enzyme inactivated at 65 °C for 5 min.

1.6 Ligation of DNA

DNA fragments such as PCR products can be ligated into plasmids provided that the two DNA molecules have been digested with restriction enzymes producing compatible ends, i.e. both of them have either blunt ends or a complimentary 3'/5' overhang. DNA fragments were ligated using 3 U T4 ligase, buffered in 1x ligation buffer. Ligation set ups were left at room temperature for 1 h or at 4 °C over night.

1.7 Gel electrophoresis

Due to their negatively charged sugar-phosphate backbone, DNA fragments in a gel matrix migrate from the cathode to the anode when voltage is applied. Migration is hereby mainly influenced by the size of the DNA fragments with shorter fragments moving faster through the agarose matrix. This can be used to purify DNA fragments with a certain length. If the DNA was digested with restriction enzymes, it is also possible to identify a DNA sequence with the help of its characteristic band pattern of restriction fragments. 0.8-2.0 % agarose was dissolved in either 1 x TAE or 1 x TBE buffer by heating and ethidium bromide added to the gel solution to a final concentration of 0.6 µg/ml. Prior to loading, 5 x gel loading buffer was added to the samples to a final concentration of 1 x. For RNA samples, denaturing gels were

prepared by adding 400 µl formaldehyde to 50 ml agarose-buffer solution. RNA samples were denatured by mixing 1 µl sample + 4.5 µl loading buffer and heating for 10 min at 70 °C. Gels were run for 45-180 min at 80-120 V until bands of the marker were clearly separated. Visualization was achieved by illuminating gels with UV light (400 nm).

1.8 Transformation of *E.coli* by electroporation

When cells undergo an electric pulse, their membranes become permeable for a short time, which can be used to introduce new genetic material such as plasmid into the cells. For most experiments electrocompetent E.coli ElectroMAX DH10B cells were used, only for vectors with high probability of recombination, the recombinase-deficient strain Stbl3 was used. 2 µl plasmid DNA was added to 50 µl competent cells and the set up transferred into 2-mm electroporation cuvettes taking special care not to transfer any bubbles. Transformation was performed at 2500 V for 5 ms; after transformation cells were incubated in 700 µl LB0 for at least 30 min at 37 °C under shaking before plating on LB plates containing the appropriate antibiotic. If a vector system suitable for blue/white screening (e.g. pGEM-T Easy) was used, 40 µl X-gal and 20 µl IPTG were added on each plate. Plates were inoculated with various dilutions of transformed cells, ranging between 10 µl and 200 µl. For each transformation, three plates were incubated at 37 °C over nighµ

1.9 RNA *in vitro* transcription

RNA can be obtained from plasmids by in vitro transcription, which mimics the natural transcription process. For this, DNA templates had to be linearised and purified by phenol chloroform precipitation. For in vitro transcription, poly(A) tailing and RNA purification, commercially available kits were used according to the instructions provided by the manufacturer. mRNA from crRNA templates was transcribed using the MEGA Short Script kit without additional poly (A) tailing, while mRNA for TALENs and Cas9 was transcribed using mMessage Machine Kit, with optional poly (A) tailing.

1.10 Purification of DNA

Promega Wizard SV Kit

Purification of DNA from PCR set ups or after excision from agarose gel was performed using Wizard SV Gel and PCR Clean-Up System and standard procedures.

Exonuclease digestion

When PCR products with single bands had to be sequenced, an exonuclease digest was performed to free samples of primers. For this, a suitable amount of unpurified PCR sample (usually 10–20 µl) was digested with 4 U of each exonuclease I and antarctic phosphatase for 30 min at 37 °C, following heat inactivation for 15 min at 65°C.

Ethanol precipitation

To obtain sterile DNA after restriction digest, DNA was precipitated using 100% ethanol. First, 1/10 volume of 3 M NaCl was added to the set-up, followed by two volumes of 100% ethanol. Samples were then incubated at -20°C over night and the DNA pelleted by centrifugation at 18 000 x g for 30 min at 4 °C. After that, the pellet was washed with 1 ml 70% steril-filtrated ethanol and centrifugation for 10 min at 18 000 x g. DNA was finally dissolved in an appropriate amount of sterile H2O or low-TE buffer to a final concentration of 1-2 µg/µl.

Phenol chloroform precipitation

For subsequent RNA transcription, DNA samples had to be purified with phenol chloroform precipitation. For this, samples were filled up with ddH_20 to 150 µl and an equal volume of phenol chloroforme was added. Samples were then inverted and incubated at room temperature for 10 min. After a first centrifugation step at 18 000 x g at room temperature, the aequous phase was transferred under the hood into a fresh tube and 1\10 volume of 5 M sodium acetate and 2 volumes of ethnaol were added. Samples were incubated for at least 15 min at -20 °C and subsequently centrifuged at 4 °C for 15 min. Afterwards, the supernatant was discarded and the pellet left to dry. Finally, the DNA was dissolved in 20 µl RNase-free water.

1.11 Quantification of nucleic acids

Due to extinction of the double helix, concentration of DNA can be determined by measuring the extinction at 260 nm. Extinction at 260 nm was measured photometrically using ddH_20 as a blank. Based on the Beer-Lambert law with A = εx c x d, DNA concentration was then determined using the following equation: DNA [µg/ml] = (OD_{260} x 50 x dilution factor)/1000. RNA content can be measured using the absorption at 260 nm and 280 nm and using the following equation: RNA [µg/ml] = A x 40 µg/ml Â· dilution factor Alternatively, dilution series of the sample were run on agarose gels and intensity of the band compared to a commercial DNA ladder with known concentration.

1.12 Production of CENs

For the generation of CENs, a DBD targeting the respective site has to be fused into a suitable recipient vector. Because not all TALENs are functional, an activity screening can be performed after completion of the cloning process.

TALENs

TALENs were produced with the Golden Gate TALEN 2.0 kit described by the Voytas group (Cermak et al., 2011). Recognition sites and TALEN vectors were designed using TAL Effector-Nucleotide Targeter (TALE-NT) 2.0 (Doyle et al., 2012). RVD arrays were cloned according to the protocol established by Cermak et al. and finally inserted into pCAG-TAL3 trunc.

crRNAs

sgRNAs consisting of crRNA and tracRNA were produced using the vector pBS U6 chimaeric (Jinek et al., 2012). Oligonucleotides containing the recognition site and the T7 promoter sequence as well as suitable overhangs were cloned into pBS U6 chimaeric previously digested with BbsI. The obtained plasmids were sequenced; a correctly assembled vector was then used as a template for two subsequent rounds of PCR with primers T7 FW and Trac RV. The purified PCR product of the second PCR was then transcribed with the MEGAshortscript T7 kit.

Reporter plasmid and activity screening

In order to get a first impression of the activity levels of produced designer nucleases, a modified single strand annealing assay (SSA) was used . It is based on activation of β- galactosidase expression was used (Epinat et al., 2003; Townsend et al., 2009). For this, oligonucleotides containing the recognition site of the respective designer nuclease and suitable overhangs were cloned into pCMV Duplirep previously digested with BstBI. The finished construct contained the first 405 bp of the β- galactosidase cassette and, out of frame, a complete version of the same coding sequence, both separated by the recognition site. The backbone of the plasmid also features a luciferase cassette with its own promoter. The reporter plasmid and the respective nucleases were then co-transfected into either HEK293 cells or poFFs. Nuclease activity at the recognition site lead to a DSB and subsequent homologous recombination of flanking regions, resulting in expression of functional β- galactosidase. To measure this as well as luciferase expression, cells were lysed after 24 hrs and chemoluminiscent assays (β-gal Gene Reporter Assay (chemoluminiscent), DualGlo Luciferase Assay) performed. After normalization values could be used as a measurement for the functionality of the tested designer nuclease.

1.13 Detection of CEN induced mutagenesis

Mutation detection with mismatch specific nucleases

Mismatch specific nucleases are used for the detection of CEN induced mutagenesis. They recognize and cleave heteroduplexes that form when mutation-containing DNA fragments are denaturated and then renaturate with the other, mismatched species. Two examples for mismatch specific nucleases are T7E1 and Surveyor Nuclease. Both nucleases require the amplification of the respective DNA fragment with a proof-reading polymerase. Afterwards, reference (wild type) and mutant DNA are mixed, heated to 95°C and then slowly cooled down to room temperature to allow for rehybridisation. Finally, the nuclease is added and the sample left at 42°C for digestion for at least 60 min.

Single strand conformation polymorphism (SSCP)

Single strand conformation polymorphism (SSCP) employs a polyacrylamid gel with high resolution to visualize the conformational differences between matched and mismatched DNA samples. A XX% polyacrylamide gel was prepared and run for 2 hrs

at 50 mA, 200 V and 4 °C. Samples were mixed with formamide buffer (deionized formamide + bromphenole blue) in ratio of 1:5 to 1:10, denaturated at 95°C for 5 min and cooled down on ice for at least 10 min. The gel with the denaturated samples was then run for 18 hrs at 180-200 V, 50 mA and 4°C. For the silver staining, the gel was washed with 10% ethanol for 15 min and then three times with ddH_2O. After washing with 1% HNO_3 for 10 min and subsequent rinsing with ddH_2O, it was stained with 0.2% silver nitrate for 30 min in the dark. Development was carried out with 3% sodium carbonate and stopped by 10 % acetic acid for 15 min.

2 Tissue culture work

Cells were cultured with 5.0% CO2 at 37 °C in a humidified atmosphere. All experiments were conducted in a sterile environment. All cell lines used had been tested and were negative for mycoplasms.

2.1 Thawing, culturing and freezing of cells

Thawing of cells

Frozen cells were thawed in a water bath at 37 °C and immediately transferred into 5 ml of the respective medium. After centrifugation for 5 min at 300xg, the cells were resuspended in 0.5-1.0 ml medium and plated in an adequate vessel.

Passaging of cells

For normal cell culture, cells were passaged when they had reached 80-90% confluency. Cells were first washed with PBS and then incubated at 37 °C for 5-10 min with either prewarmed Accutase or Trypsin-EDTA. After addition of medium to inhibit the enzyme, cells were reseeded in appropriate flasks.

Freezing of cells

For freezing, cells were detached as usual and centrifuged for 5 min at 300xg. The pellet was resuspended in freezing medium and aliquots of 0.7-1.5 ml pipetted into cryo vials which were gradually cooled to -80 °C using a freezing device and finally transferred to liquid nitrogen tanks.

2.2 Transfection of cells

Nucleofection

Nucleofection is a transfection method based upon electroporation. It uses a specialized device called Nucleofector®and the appropriate kit to introduce foreign genetic material right into the nucleus. For nucleofection, 4–5 x 10^5 cells were detached as usual, pelleted for 5 min at 300 x g and then resuspended in 100 µl prewarmed Nucleofector Solution. After adding the desired amount of DNA cells were transferred bubble-freely into nucleofection cuvettes and nucleofected using programme C-17 for MSCs and U-12 for poFFs. Immediately after nucleofection, 500 µl of medium was added and the sample pipetted into a T25 flask using the supplied pipettes. Medium was changed after 24-48 hrs.

RNA transfection with Stemfect

RNA transfection was performed with the Stemgent Stemfect kit. Usually, 8 x 10^4 cells (poFF) were plated onto a 12-well-plate. Transfection was performed after 24 hrs according to manufacturers' instruction with approx. 0.5 µg of total mRNA and 2 µl of Stemfect transfection reagent in 50 µl tranfection reagent diluent. The DNA:transfection reagent solution was added dropwise to 1 ml of medium, which was exchanged the next day.

2.3 Isolation of single cell clones

In order to isolate single cell clones, cells were split very thinly onto 10 cm- or 15 cm-dishes. Once cell clones had reached an appropriate size, they were marked under the microscope. After aspirating the medium, a cloning ring made from 0.5 ml Eppendorf tubes was dipped into silicone grease and put over each clone. 100 µl Accutase were added into each ring and clones incubated for 5 min at 37°C. After addition of 100 µl medium, clones were transferred into either 12- or 24-wells and left to grow. Alternatively, poFFs were diluted to 150-200 cells/20 ml and plated onto 15 cm dishes. Frozen cells were thawed in a water bath at 37 °C and immediately transferred into 5 ml of the respective medium. After centrifugation for 5 min at 300xg, the cells were resuspended in 0.5-1.0 ml medium and plated in an adequate vessel. Usually cells were passaged when they had reached 80-90% confluency. Cells were first washed with PBS and then incubated at 37 °C for 5-10 min with either prewarmed Accutase or Trypsin-EDTA.

After addition of medium to inhibit the enzyme, cells were reseeded in appropriate flasks. For freezing, cells were also detached and then centrifuged for 5 min at 300xg. The pellet was resuspended in freezing medium and aliquots of 0.7-1.5 ml pipetted into cryo vials which were gradually cooled to -80 °C using a freezing device. Liquid nitrogen was chosen for final storage.

PART IV

RESULTS

1 Screening methods for CEN induced mutations

When using CENs for directed mutagenesis, clones can be screened by either loss of a nearby restriction site or by detection of mismatches. While the first approach is dependent on the respective target site, the latter can be applied universally. Several methods are used to visualise mismatches, two common techniques being mismatch specific nucleases and SSCP. The suitability of these was tested during this thesis using controls and later *RAG1* knock-out clones with known mutations.

1.1 Mismatch specific nucleases

Mismatch specific nucleases cleave heteroduplexes of mismatched DNA strands and can even detect single point mutations. Two mismatch specific nucleases were tested, Surveyor nuclease and T7E1. For the Surveyor nuclease, a positive control was generated following the manufacturer's instructions. Two PCR products of 633 bp differing by a single base pair were digested with Surveyor nuclease either mixed (GC) or alone (CC). In the case of GC, this should give rise to two bands at 217 and 416 bp. Digestion of the positive control yielded the expected bands (**fig.9**), as did digestion of the PCR product of a *RAG1* mutated clone. However, digestion of wild type PCR product, which should not contain any mismatches, yielded a smear with several bands. This was probably because pDNA was used as a template for PCR of the mutated clones, while gDNA was used for the wild type control.

T7E1 was also tested with the positive control of the Surveyor nuclease kit and showed the expected bands, although not as clear as the Surveyor nuclease (**fig.10**).

In the later course of experiments, T7E1 failed to detect mutations in clones that had already shown mutations by screening for a loss of restriction site (see **chapter 2.1**).

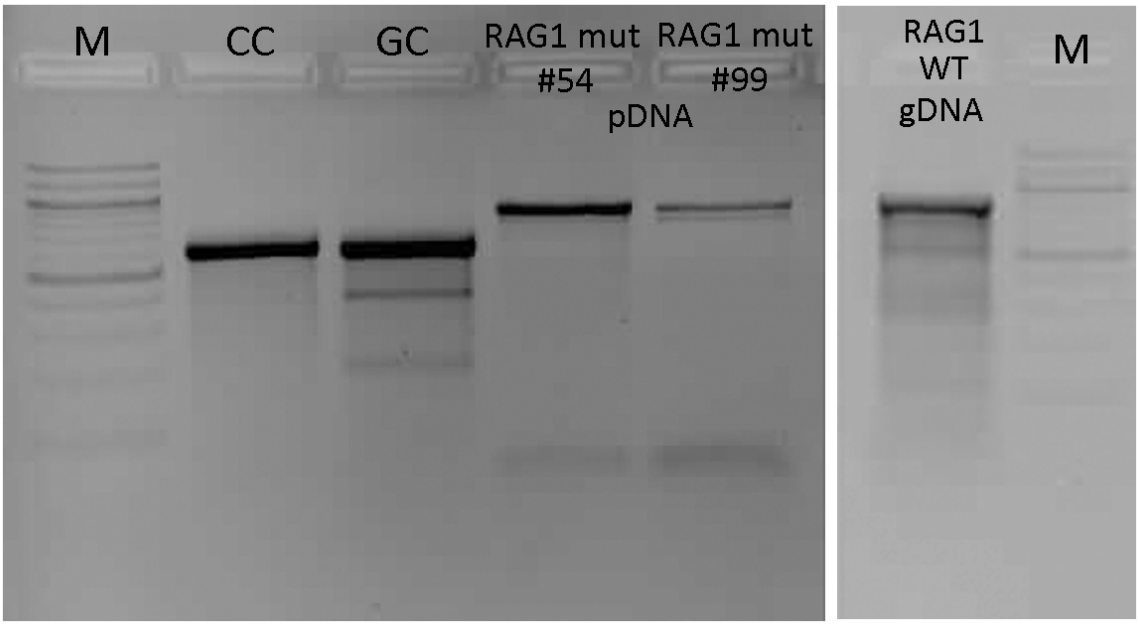

Figure 9: Establishing the Surveyor nuclease assay. Digestion with mismatch specific Surveyor nuclease yielded expected results for controls (CC: 633 bp; GC: 416/217 bp) and *RAG1* targeted clones (900/800/100 bp), but digestion of wild type control showed a smear with bands at 700 and 300 bp. 1.0% TBE agarose gel. M: 100 bp ladder.

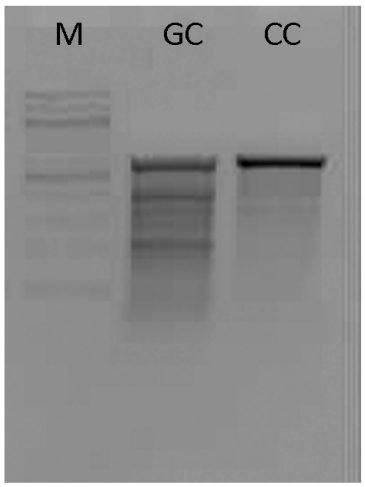

Figure 10: Establishing the T7E1 assay. Digestion of positive controls with and without a point mutation showed the expected bands (CC: 633bp; GC: 416/217 bp), albeit not as clear as the Surveyor nuclease digest. M: 100 bp ladder. 1.0 % TBE agarose gel.

1.2 SSCP

Single strand conformation polymorphism (SSCP) uses the fact that sequences with only a few base pairs difference take different conformations as single strands. These conformational differences can be detected via polyacrylamide gel electrophoresis (PAGE). SSCP was tested with short PCR products (< 500 bp) from wild type gDNA and gDNA from a mutated clone (see **chapter 2.1**). A difference in migration behaviour was detectable, but further optimization would be necessary.

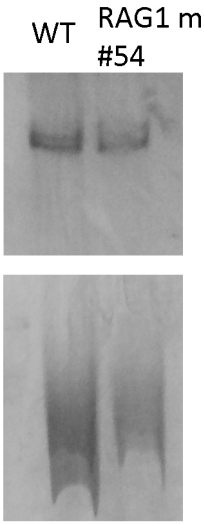

Figure 11: Establishing SSCP. A short PCR product spanning the *RAG1* TALEN target site was obtained from WT gDNA and a clone carrying a mutation confirmed by sequencing. A difference in migration behaviour was observed. 12% polyacrylamide gel.

2 Modification of porcine RAG1/2

Because of their role in V(D)J recombination, knock-out of *RAG1* and *RAG2* has been used in mice (Mombaerts et al., 1992), rats (Ménoret et al., 2013) and rabbits (Song et al., 2013) to generate immunodeficient animals. In pig, both genes are located on chromosome 2, with exon 1 of *RAG2* being homologous to exon 2 of human *RAG2*.

2.1 RAG1

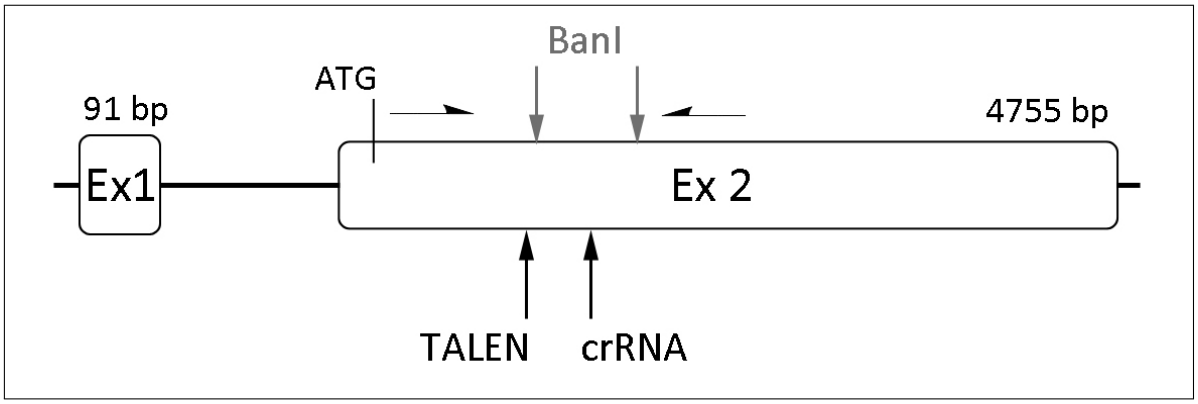

Figure 12: Porcine *RAG1* locus including TALEN and crRNA target sites used in this study. TALEN activity results in loss of a BanI site. Ex: exon. Not true to scale.

2.1.1 TALENs

TALENs were first tested for their ability to induce indel mutations within the second exon of *RAG1*. For this, TALENs were designed and generated with pCAG TAL as recipient vector, using the four most common RVDs for bp specificity (**fig.12**).

Initial experiments

Since cloning of a targeting vector for *RAG1* proved to be difficult (data not shown), a different approach was used to enrich and screen for positive transfection and targeting events. MSCs from porcine bone marrow (pBMMSC 071210) were simultaneously transfected with TALEN plasmid DNA (pDNA) and pMacs K^kII, a commercial vector encoding for truncated mouse MHC class I molecule H-$2K^k$. This allows for selection of transfected cells via the surface marker using magnetic beads. Cells were transfected with a mixture of TALEN and marker plasmid in a weight ratio of 10:1 (350 ng:35 ng), thus ensuring that most selected cells also contained the TALEN DNA. Transfection was performed with nucleofection solution for MSCs and program C-17. Selection was performed after 48 hours with two μ columns. For screening, a PCR spanning the TALEN target site was performed and the product subsequently digested with BanI. Loss of the BanI restriction site within the TALEN target site was detected by lack of cleavage of the 586 bp fragment. Thus, in addition to the 317 bp fragment, biallelic targeted clones should have a 585 bp fragment, monoallelic targeted clones 585 and 491 bp fragment and wild type ones one a 491 bp fragment (**fig.13**). The presence or absence of the 95 bp fragment was not taken as an indicator because it was often difficult to detect.

The PCR product of positive clones were then subcloned into the commercial cloning vector pGEM-T Easy and the sequence determined. The fact that mutations could often be detected in only 1 of 8 subclones indicated a mixed cell population.

Transfection of pBMMSCs with TALEN pDNA was then repeated with 500 ng of TALEN pDNA. Transfection efficiency was very high and 21 positive clones were obtained from 40 clones screened. A T7E1 digestion of the amplicons carrying mutations was also performed, but unlike in the above BanI digest, no positive clones could be detected; even the control with WT genomic DNA (gDNA) showed considerable smearing, but no defined bands (**fig.15**).

Clones were also screened for integration of the TALEN plasmids via a FokI specific PCR. All clones showed integration of the FokI domain, while no band could be detected in untreated wild type DNA (**fig.16**), indicating that one or both TALEN plasmids had indeed integrated in the majority of clones.

The marker plasmid pMACS K^kII, however, could not be detected via PCR, showing that plasmid integration is concentration-dependent (**fig.17**).

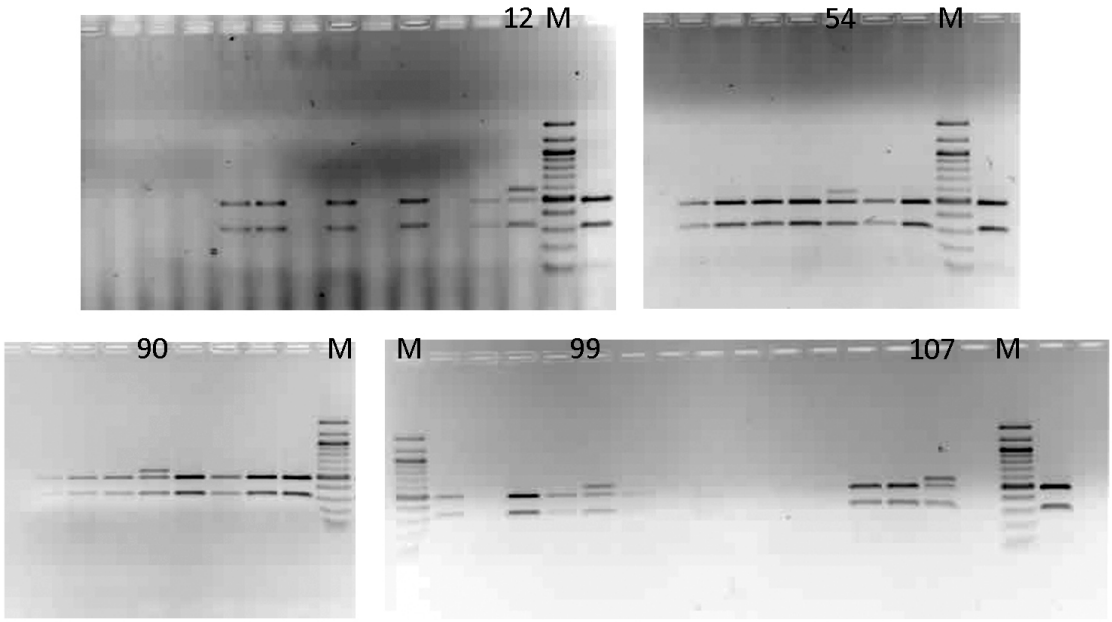

Figure 13: TALEN mediated mutagenesis of *RAG1*. TALEN activity resulted in loss of a BanI site. Monoallelic mutated clones showed three bands (585/491/317 bp), wild type clones two (491/317 bp). 95 bp fragment was not detectable. Positive clones are highlighted by numbers. 107 clones were screened. 2% TBE agarose gel. M: 100 bp ladder.

```
cl.12   ATTCAGGGTGAGATCCTTTG-AAAGGCACCT--GCCCATGATCAAACGGAAAAG
cl.54   ATTCAGGGTGAGATCCTT------------T--GAAAAGGCTCAAACGGAAAAG
   WT   ATTCAGGGTGAGATCCTTTGAAAAGGCACCT--GAAAAGGCTCAAACGGAAAAG
cl.90   ATTCAGGGTGAGATCCTTTGAAAAGGCACCT--GAA-TTTAAAAAACGGAAAAG
cl.99   ATTCAGGGTGA--T-CAAAG-GATCTCACCTTTGAAAAGGCTCAAACGGAAAAG
```

Figure 14: Sequence of selected clones with TALEN induced *RAG1* mutation. Base pair exchanges are marked in **bold**, additions in grey. TALEN target site is underlined, BanI site is marked in light grey.

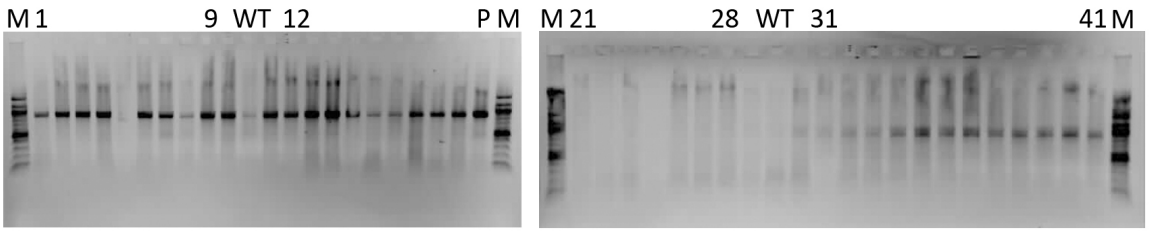

Figure 15: T7E1 digestion of *RAG1* mutated clones. Clones with a TALEN induced *RAG1* mutation were digested with T7E1, which should result in cleavage at the mutation site for heterozygous clones. Other than the band of the PCR fragment at 902 bp, no fragments were detected. 2% TAE agarose gel. M: 100 bp ladder, P: Pool

Circumventing integration of TALEN DNA

Integration of TALEN plasmids bears the risk of interruption of endogenous genes; ~~additionally, prolonged expression of TALENs can lead to a higher intracelullar con-~~

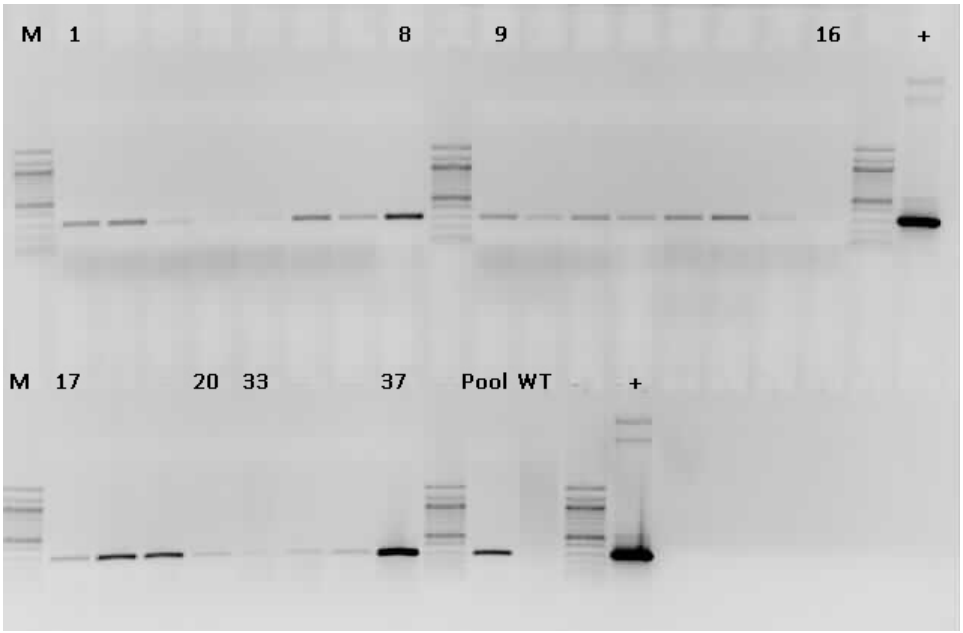

Figure 16: Screening for integration of TALEN plasmids. Clones with a TALEN induced *RAG1* mutation were screened for random integration of the TALEN plasmids by a PCR amplifying the FokI cassette. Expected fragment size: 313 bp. 1% TBE agarose gel. M: 100 bp ladder.

centration and thus increased off-target cleavage. Therefore, I tried to reduce integration levels by decreasing the amount of DNA used of transfection and by using mRNA rather than pDNA. Transfection with TALENs was repeated with reduced amounts of TALEN pDNA (50/100/200 ng), keeping the 1:10 ratio of TALEN and marker plasmid. 20, 20 and 46 clones were screened, but no positive clones were detected.

Next, *RAG1* TALEN mRNA was produced and pADMSCS (110111) were transfected with 200 ng of each TALEN mRNA. No further enrichment measures were taken; 75 clones were screened, but no positive clones detected.

Testing different cell types

Since it is quite possible that different cell types vary in their susceptibility to TALEN induced mutagenesis, transfection with pDNA was therefore repeated in porcine adipose MSCs (pADMSCS 110111), using electroporation, which had been shown to work efficiently in this cell type (see **chapter 3**). DNA amount was accordingly adjusted to 2.5 µg TALEN pDNA and 250 ng pMACS K^kII. 34 clones were screened, but no positive clones were detected.

The initial experiment (nucleofection with 350 ng of TALEN pDNA + 35 ng

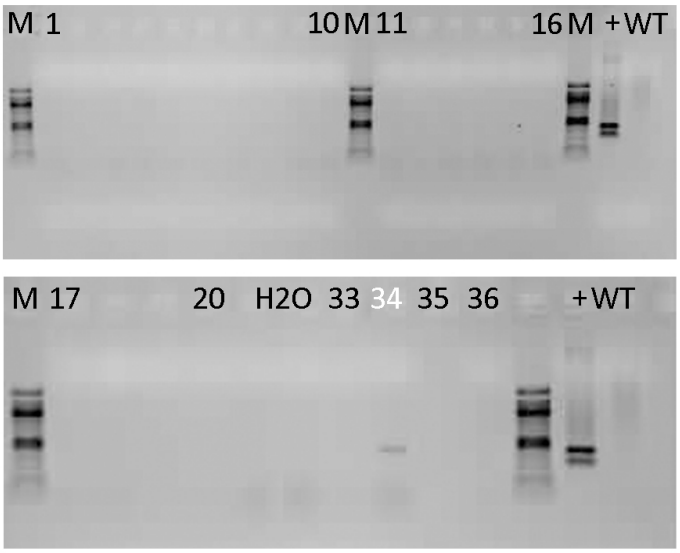

Figure 17: Screening for integration of marker plasmid pMACS K^{k}II Clones with a TALEN induced *RAG1* mutation were screened for random integration of the marker plasmid pMACS K^{k}II via PCR. Only one clone (34) had showed the expected band at 434 bp. 1% TBE agarose gel. M: 100 bp ladder.

of pMACS K^{k}II; subsequent selection with magnetic beads) was repeated once more repeated in porcine foetal fibroblasts (poFF 251113 # 4). Selection was performed after 24 hours or 72 hours when cells were treated with a cold shock (72 hrs at 30°C). 44 and 27 clones were screened, but no positive clones were obtained.

2.1.2 NHEJ based transgenesis

Recent reports have shown that long DNA constructs of up to 15 kb can be integrated into the genome via a TALEN mediated, NHEJ based mechanism (Maresca et al., 2012). This approach was tested using the *RAG1* TALENs. A ligation gated recombination (LiGaRe) vector was constructed, containing the target site of the *RAG1* TALENs, a 6x stop cassette (3x in both directions), a PGK-neo selectable cassette and loxP sites for transgene removal (Bromberger, 2013). pADMSCS (080812) were transfected via nucleofection with 350 ng of each TALEN and 1.3 µg of the LiGaRe donor plasmid. Following transfection, cells were subjected to a 72 hrs cold shock at 31 °C. Both single cell clones and cell pools were screened for integration of the donor plasmid. Screening was performed with a PCR spanning the whole vector (expected product size: 5.7 kb; wild type: 1 kb). Under standard conditions, only the wild type allele was amplified (**fig.18A**). Decreasing the annealing temperature to 55 °C yielded non-specific bands at sizes between 3 and 5 kb (**fig.18B**), but these could also be observed in wild type gDNA and were not more pronounced in any of the 38 single cell clones (**fig.18C**).

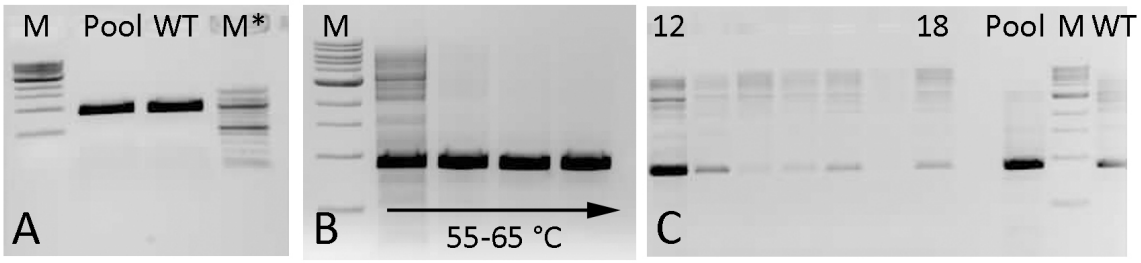

Figure 18: Ligation gated recombination at the *RAG1* locus. Screening of cells transfected with a LiGaRe donor plasmid gave only the wild type band (1 kb) or unspecific bands. A: PCR of pooled cells under standard condition. B: Decreasing the annealing temperature to 55°C yielded unspecific bands in wild type cells. C: Screening of single cell clones yielded the same unspecific bands as seen in the wild type. M*; 100 bp ladder; M: 1 kb ladder. 1.0% TBE gels.

2.1.3 CRISPR/Cas9

A sgRNA targeting the same region of the *RAG1* gene was designed and expressed using the expression vector described by Jinek et al. (2012) (Pham-Thi, 2014). 200 ng of this sgRNA was introduced either alone or in combination with *JAK3* crRNA 1-20 into poFFs (251114 # 4) in addition to 200 ng Cas9 mRNA. While *JAK3* mutations were found via PCR and subsequent sequencing (see **chapter 3.2**), no *RAG1* mutations were found.

2.2 RAG2

TALENs to disrupt *RAG2* were designed to target a region 200 bp 3' of the the translational start. These TALENs have been reported as efficiently inducing mutagenesis in porcine *RAG2* (Carlson et al., 2012). RVD assembly was performed using the Golden Gate 2.0 kit (**fig.19**); as a recipient vector, pCAG-TAL3 was chosen, which contains a Δ153 NTS and a 46 aa CTS.

mRNA with and without an added polyadenosine sequence was produced *in vitro* and used to transfect poFFs (poFF 251113 # 4) with the Stemfect kit. For easy identification of positive clones, cells were co-transfected by nucleofection with an ssODN containing the two slightly altered TALEN recognition sites as initiation sites for homologous recombination and an additional HindIII restriction site. Various amounts of TALEN mRNA (300/600 ng) and treatment with and without cold shock at 30°C for 48 hrs were tested, but screening of both single cell clones and cell pools did not yield any targeted clones.

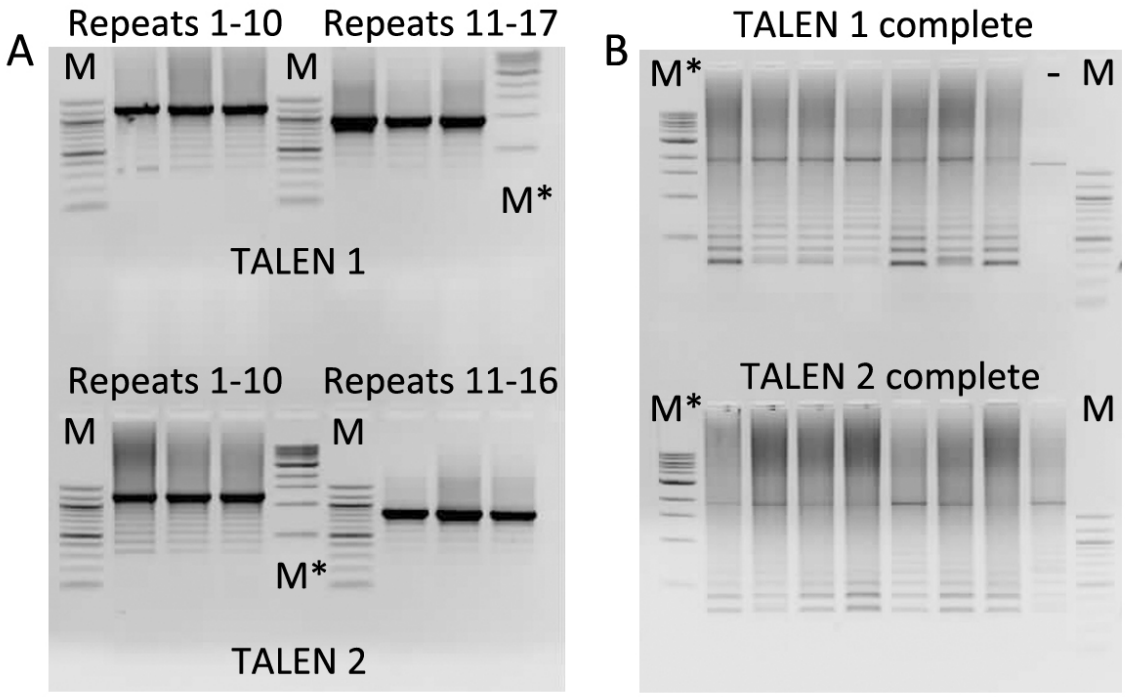

Figure 19: Construction of *RAG2* TALENs. *RAG2* TALENs were constructed using the Golden Gate 2.0 kit. In a first Golden Gate reaction, repeats 1–10 and 11–16/17 were assembled (A); in the second round, the corresponding repeat arrays plus the last repeat were cloned into the recipient vector pCAG-TAL3 (B). Correct assembly was detected by a laddering effect after PCR amplification; incorrectly assembled clones do not show this effect (denominated with (-) in B). M; 100 bp ladder; M*: 1 kb ladder. 1.0 % TBE gels.

3 Modificaton of porcine JAK3

Another molecular target for the generation of SCID models is *JAK3*. In pigs, the gene is located on chromosome 2 and consists of 23 exons, with the start codon in exon 1.

3.1 TALENs

Three pairs of TALENs were designed, two targeting exon 1 (*JAK3* TAL TZ 5+6) and one targeting exon 2 (*JAK3* TAL HH) (**fig.20**). These TALENs were kindly provided by Dr Ralf Kuehn and Dr Pavel Pelczar.

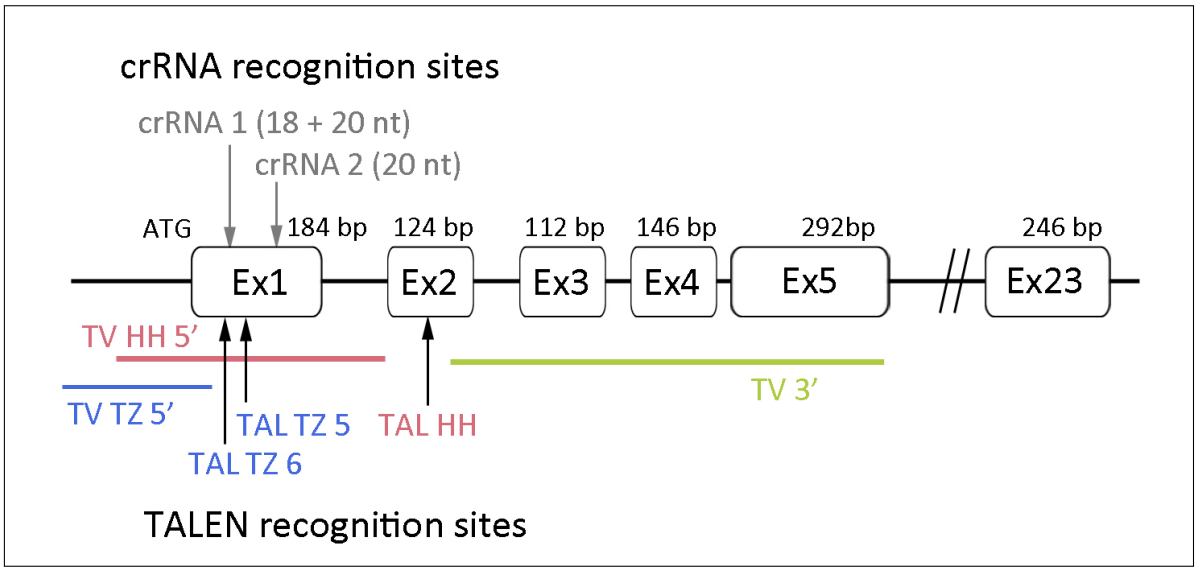

Figure 20: Porcine *JAK3* locus including TALEN and crRNA target sites used in this study. Target sites are indicated by arrows. The two targeting vectors differed only in their 5'-homologous arm. TV: targeting vector; 3'/5': 3'/5' homologous arm. Not true to scale.

3.1.1 Activity screening

The cleavage activity of all TALEN pairs was tested in a β- galactosidase based single strand annealing (SSA) assay (Wefers et al., 2014). For this, oligonucleotides carrying the respective target site were introduced into a screening vector. The finished vector contained aa 1-400 of LacZ and, further downstream and out of frame, the complete coding sequence of the LacZ gene; the TALEN target site was cloned between these two cassettes. TALEN activity induces a DSB which leads to homologous recombination generating a functional LacZ cassette in frame. Activity levels were measured as β-galactosidase activity. All three pairs showed sufficient activity (**fig. 21**.

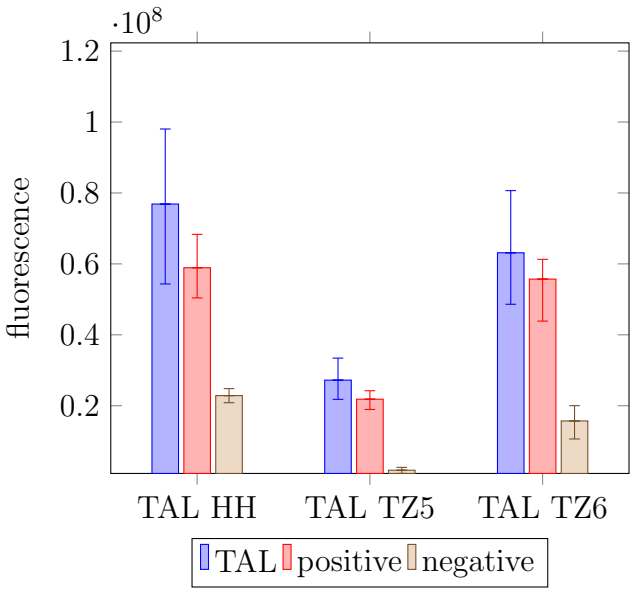

Figure 21: *JAK3* TALENs activity test. All TALEN pairs were tested in separate tests and showed sufficient activity levels. Positive: vector carrying a complete LacZ cassette; negative: cells transfected with a dummy plasmid. TALEN activity reconstituted beta-galactosidase activity. Assays were conducted in HEK293 cells and results not normalised.

First, I wanted to apply the same approach as for *RAG1*, namely transfection with TALEN HH pDNA and pMACS K^{k}II and subsequent enrichment of transfected cells with magnetic beads. After PCR amplification of a 900 bp fragment spanning the TALEN recognition site, clones could be screened by digestion with CviKI-1. This was first tested with WT gDNA. A band pattern with three bands at 569/217/138 bp for wild type was expected, but not found (**fig.22**).

Thus, surveyor nuclease digestion was used for screening of clones transfected with 350 ng of each *JAK3* TALEN HH pDNA. All clones showed the same bands at 300 and 700 bp, indicating that these are artefacts.

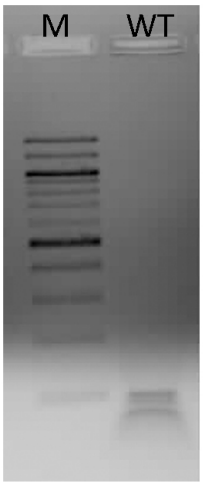

Figure 22: Screening for *JAK3* mutations with CviKI-1. Digestion with CviKI-1 of WT PCR product did not yield the expected pattern of 569/217/138 bp. 1.5% TBE agarose gel.

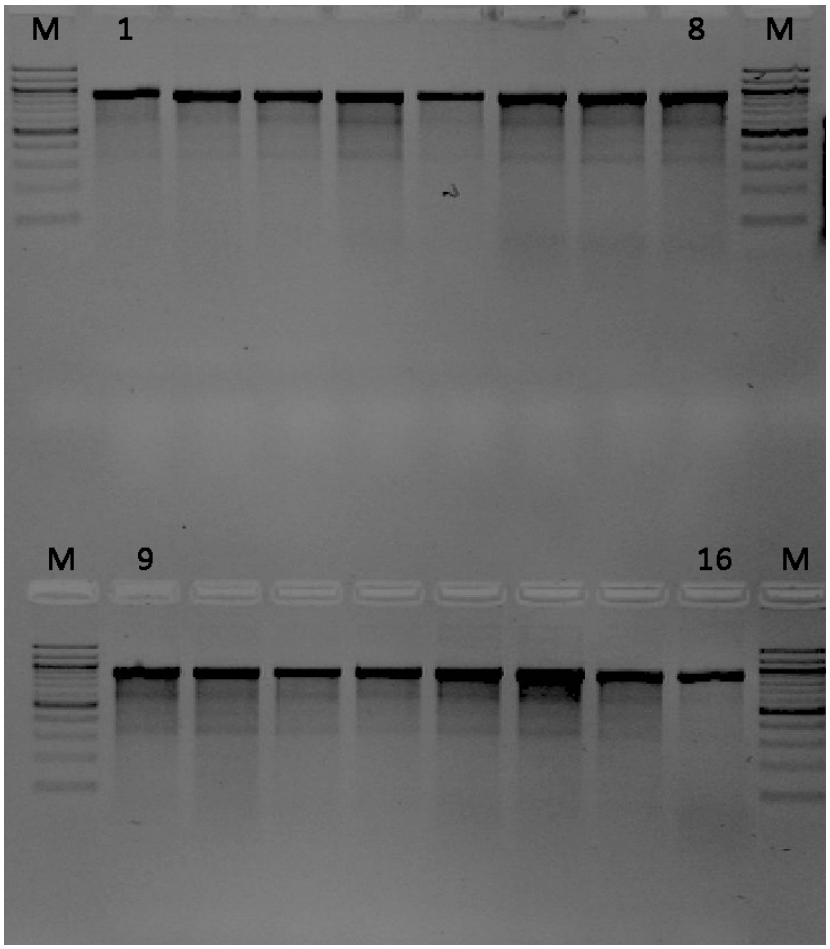

Figure 23: Screening for TALEN induced *JAK3* mutations by Surveyor assay. Digestion with Surveyor nuclease yielded the same bands at 300 and 700 bp observed before (compare **fig.9**). M: 100 bp ladder. 1.0 % TBE agarose gel.

3.1.2 HDR mediated introduction of a resistance cassette

Initial experiments

Because screening of indel mutations in *JAK3* proved to be difficult, a targeting vector containing two homologous regions of 1.2 kb each and a neomycin resistance cassette under the ubiquitously expressed phosphoglycerate kinase (PGK) promoter was constructed. The 5'-homology arm plus the PGK-neo cassette were amplified via PCR from a previously described construct (Durković, 2012); the 3'-homology arm was obtained via PCR with suitable primers from wild type gDNA. The target site for *JAK3* TALENs HH was not contained within either homology arm to avoid re-cleavage. The final construct was assembled into the commercially available psl1180 as vector (**fig.24**).

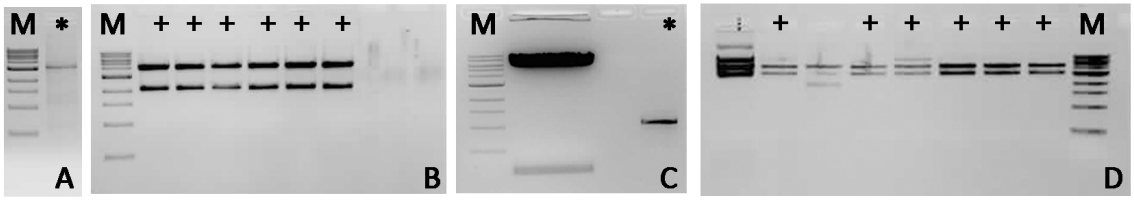

Figure 24: Construction of a targeting vector to be co-transfected with *JAK3* TALs HH. A: PCR amplification of the 5'-HA+ PGK-neo cassette, exp. length: 3134 bp. B: Cloning of 5'-HA + PGK-neo cass. into psl1180; restriction digest with EcoRI. Exp. bands: 4.3/2.1 bp. C: PCR amplification of the 3'-HA, exp. length: 1057 bp. D: Cloning of 3'-HA into psl1180 + 5'-HA. Restriction digest with EcoRI. Exp bands: 4.3/3.1. bp. Correct PCR fragments are denoted with *, correctly assembled vectors with +. HA: homology arm; M: 100 bp ladder. 1.0 % TBE agarose gels.

In a first experiment, pADMSCs (110111) were transfected with 2.5 µg of each TALEN pDNA and 10 µg of linearised targeting vector. Few cell clones survived transfection and subsequent picking, but 3 of 5 clones finally showed a band in the targeting PCR, albeit at 1.7 kb instead of 2.0 kb (**fig.25**). Sequencing confirmed an unexpected deletion of 300 bp in the 3'-homology arm that could not be detected in the targeting vector itself and might have been caused be incomplete homologous recombination.

For *JAK3* TAL TVTZ, a similar targeting vector was constructed, albeit with the 5'-homology arm 5' of the translational start to exclude the target sites (compare **fig.20**). p53 targeted pADMSCs (080812) were chosen for transfection via nucleofection; from experience in the laboratory, p53 mutated clones seem to be more suitable for SCNT (unpublished data). 350 ng of each TALEN plasmid and 1.3 µg of linearised

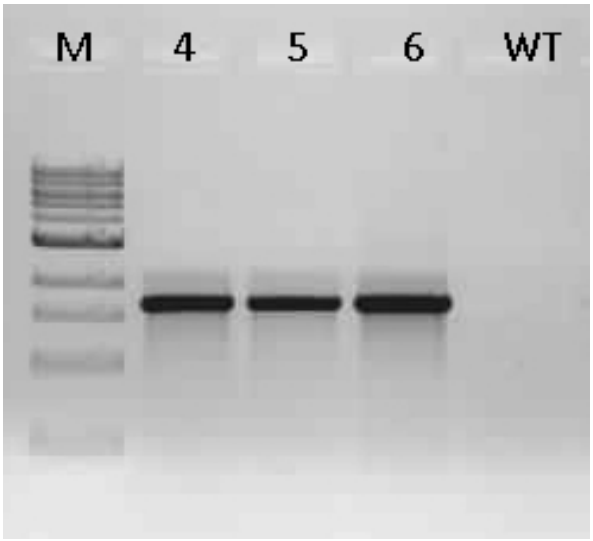

Figure 25: Targeted introduction of a resistance cassette mediated by *JAK3* TALENs HH. TALEN mediated HDR resulted in introduction of a resistance cassette into the *JAK3* locus, detectable by the presence of a PCR fragment in the targeting PCR. Clones showed a deletion in the 3'-homology arm, resulting in a 1.7 kb fragment instead of a 2.0 fragment. Targeting efficiency was 50% (other clones not shown). M: 100 bp ladder. 1.0 % TBE agarose gel.

targeting vector were introduced into the cells via nucleofection. 5 out of 77 clones were positive, as determined by PCR screening over the junction of vector and the genomic locus. Sequence determination confirmed theses reuslts. As positive control, the subcloned targeting PCR from one of the positive clones identified in the previous experiment was used.

Interestingly, two of these clones showed the same 300 bp deletion observed in the previous experiments with *JAK3* TALENs HH. Both targeting vectors contained the same 3'-homology arm, but in both cases, the TALEN target site was 750 bp upstream of the site where the deletion occurred. Thus, TALEN activity is unlikely to have caused this deletion. The two clones without the deletion were mixed and used for SCNT together with cell clones carrying a KRAS mutation. From that pregnancy, three piglets were born, all of which died within a few days. gDNA isolated from earclips was used for PCR analysis. None of piglets showed a targeted *JAK3* allele.

Optimization

Targeting with ssODNs instead of a targeting vector was tested with both *JAK3* TALENs HH and TZ5. For this, cells were transfected with pDNA of the respective TALENs plus an ssODN consisting of the slightly mutated target site plus an additional BamHI site. 91 and 39 clones were screened by PCR amplification and subsequent BamHI digest, but no positive clones could be detected (data shown for *JAK3* TALEN HH, **fig.28**).

To minimise off-target activity, *JAK3* TAL HH were paired with obligate heterodimeric FokI domains EL and KK. When screened for activity by an SSA, they showed only 40% activity compared to WT FokI domain (**fig.29**). These TALENs were

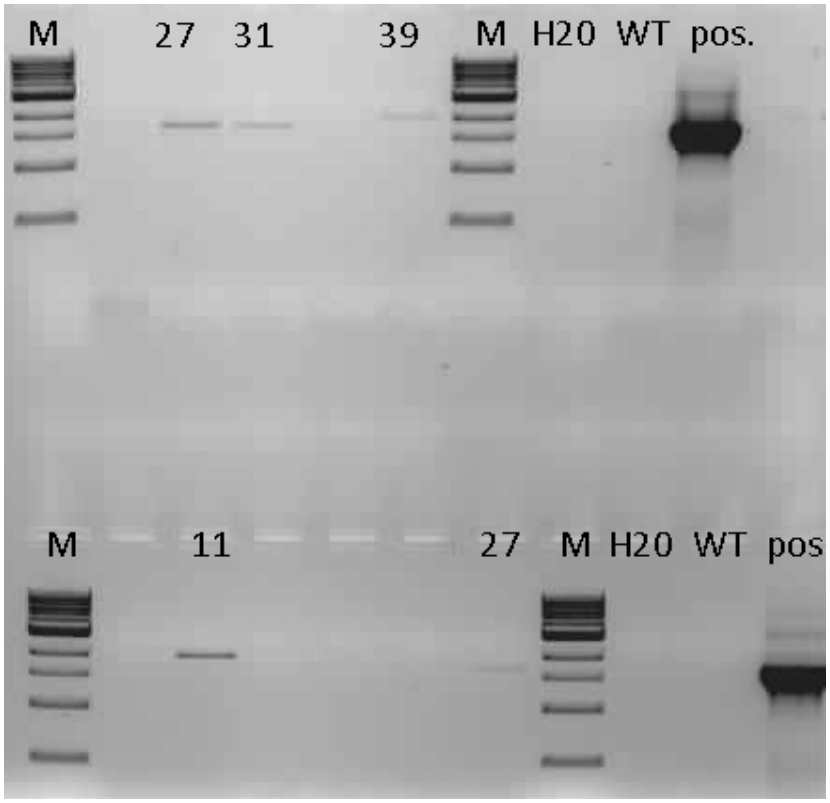

Figure 26: Targeted introduction of a resistance cassette mediated by *JAK3* TAL TZ 5+6. TALEN mediated HDR resulted in introduction of a resistance cassette into the *JAK3* locus, detectable by the presence of a 2.0/1.7 kb PCR fragment. Pos.: positive control; M: 1 kb ladder. 1.0 % TBE agarose gels.

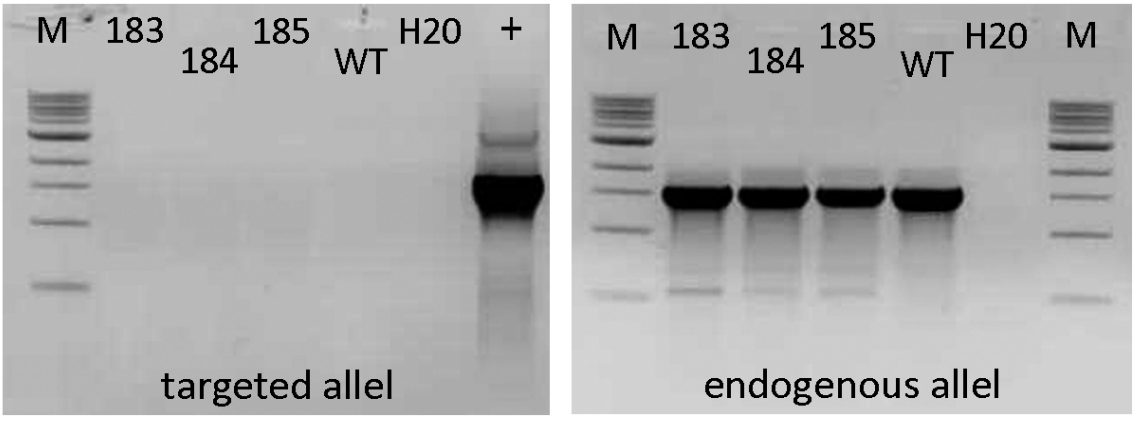

Figure 27: Screening of putative *JAK3* targeted piglets. gDNA isolated from earclips was analysed with PCRs for the targeted and the endogenous allele. + : positive control; M: 1 kb ladder. 1.0 % TBE agarose gels.

also used for transfection of pADMSCs (080812, 350 ng via nucleofection plus 1.3 µg targeting vector), but no positive clones could be detected.

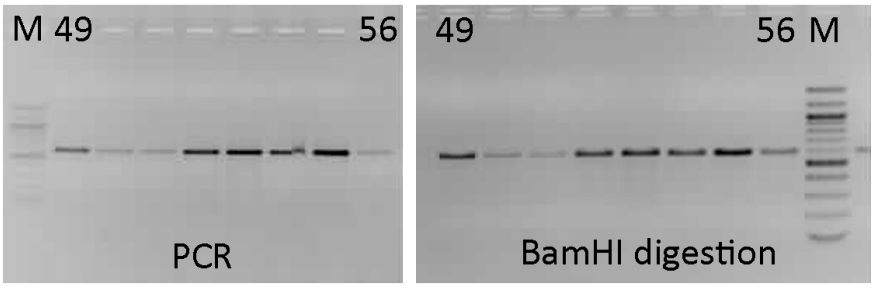

Figure 28: Targeting of *JAK3* with TAL HH and an ssODN. Introduction of the ssODN should provide an novel BamHI site which can be detected by restriction digest of the PCR product. Expected bands: 388/230 bp. No positive clones could be identified. Target sites of TALENs are underlined, additional BamHI site is marked in grey. M: 1 kb ladder. 1.0 % TBE agarose gels.

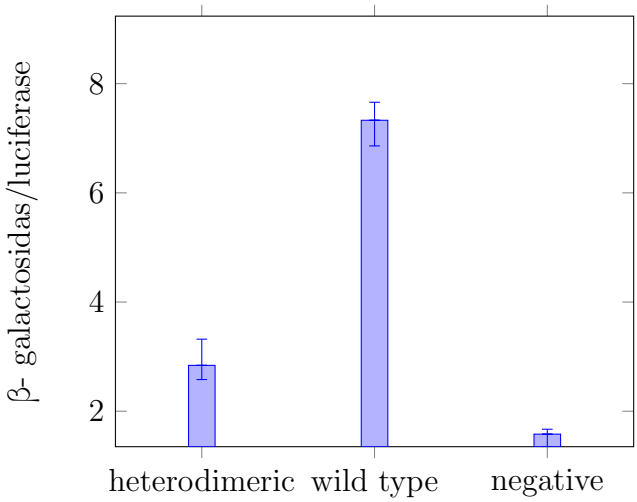

Figure 29: Activity level of obligate heterodimeric TALENs. *JAK3* TAL HH were used in combination with obligate heterodimeric FokI domains EL:KK and showed diminished activity. TALEN activity reconstituted beta-galactosidase activity. Assay was conducted in HEK293 cells.

3.2 CRISPR/Cas9

3.2.1 crRNA production and activity screening

Next, RGEN induced genome editing was tested at the porcine *JAK3* locus. For this, a sgRNA was designed with its target site in exon 1, overlapping the target sites of *JAK3* TAL TZ5+6 (see **fig.20**). It has recently been suggested that reduction of

the size of crRNAs from 20 to 18 nt might increase specificity (Fu et al., 2014). So two crRNAs recognizing the same sequence were generated, one with a 20 nt and one with a 18 nt target site (crRNA 1-20/18). A third crRNA (20 nt) was also designed, targeting exon 1 at a different site (crRNA 2). All components including Cas9 were transcribed *in vitro* into mRNA; for Cas9, additional polyadenylation was performed (**fig.30**, transcription of crRNA 1-18 not shown).

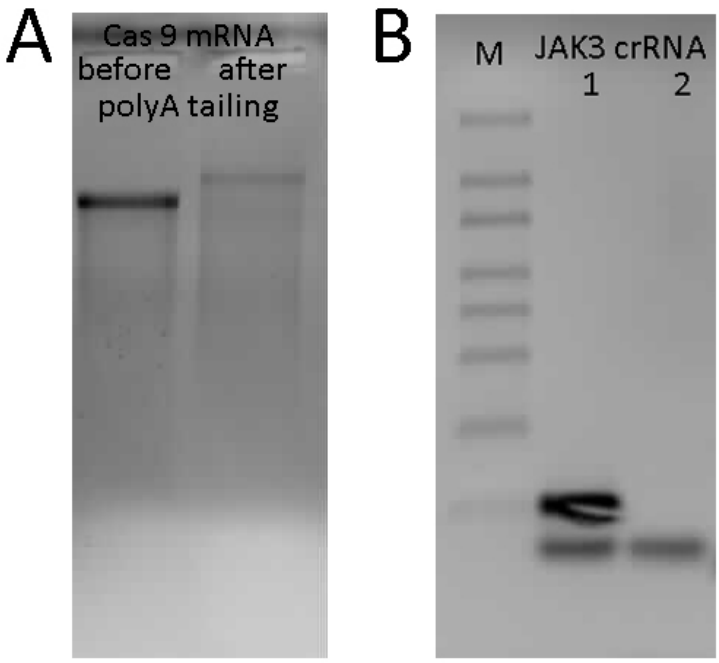

Figure 30: *In vitro* transcription of *JAK3* crRNA 1+2 and Cas9. Cas9 (A) (around 4.2 kb) and *JAK3* crRNAs 1+2 (B) (around 100 bp) were *in vitro* transcribed; the latter was additionally polyadenylated. *JAK3* crRNA 1 showed an additional band for undigested PCR producat at 200 bp. Cas9 mRNA produced by Marlene Edlinger. M: RiboRuler Low Range RNA Ladder. 0.8 and 1.5 % TBE denaturing agarose gels.

These crRNAs were then tested by the same activity assay used to quantify TALEN activity. HEK293 cells were transfected via lipofection with 200 ng of the respective crRNA, 400 ng Cas9 and 600 ng of the respective reporter plasmid. Both crRNAs were functional, but crRNA 2 showed much higher activity than crRNA 1-20 (**fig.31**).

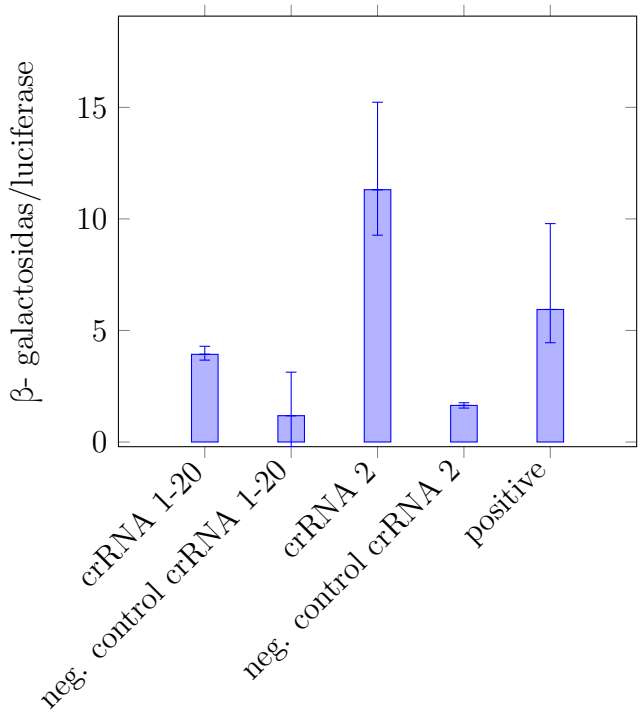

Figure 31: Activity level of crRNAs targeting *JAK3*. *JAK3* crRNAs 1-20 and 2 were tested for their activity, with crRNA 2 showing a higher activity. Assay was conducted in HEK293 cells.

3.2.2 Transfections

poFFs (251113 #4) were then transfected with the Stemfect Kit (Stemgent) and 200 ng of crRNA plus 200 ng Cas9. Transfected clones were screened by amplification of a 700–900 bp region spanning the target site and sequence analysis. Mutation efficiency with crRNA 1-20 (100 ng co-transfected with 200 ng Cas9) was high, yielding 12 positive clones out of 40 (30 %). Some of these showed deletions large enough to be visualised by agarose gel electrophoresis (**fig.32**). Sequencing identified 14 different mutations, with many clones (58%) showing mutations on both alleles. Mutations included mostly deletions (Δ3-107 bp) and a few indels (**fig.33**) Lowering the concentration to 50 ng crRNA and 100 ng Cas9 decreased efficiency below detectable levels.

Unlike recent reports (Fu et al., 2014), a marked decrease in activity ($>$ 90 %) was observed when using 18 nt truncated sgRNAs (crRNA 1-18), with only 1 of 55 clones mutated. However, crRNA 1-18 had only been purified by phenol chloroform precipitation instead of purification with a commercial kit, thus it is possible that residual enzyme or buffer hindered the transfection. Using crRNA 2, no mutated clones could be identified, despite the high activity as shown in the activity assay. Either the genomic locus was less permissive than the one for crRNA 1 or the high

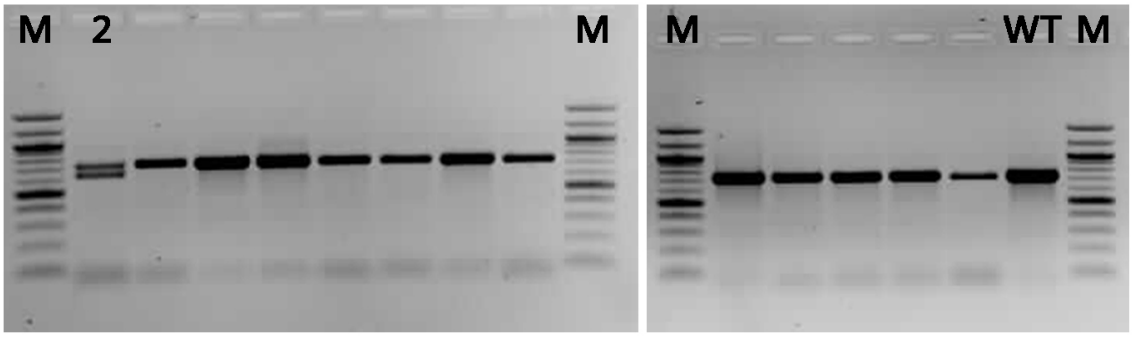

Figure 32: RGEN induced deletions in *JAK3*. After transfection with crRNA 1-20 and Cas9, amplification of a 742 bp amplicon spanning the target site showed deletions of up to 107 bp. M: 100 bp ladder. 1.0 % TBE agarose gels.

```
WT CTGCAGCCTCTCCTCTTCAGAGGCTGGTGCCCTGCAT

   CTGCAGCCTCTCCTCT---G-G----------TGCAT   Δ14
   CTGCA----------------GCTGGTGCCCTGCAT   Δ17 2x
   CTGCAGCCTCTCCTC--------------------     Δ107 2x
  CTGCAGCCTCTCCTCCTCCAGAGGCTGGTGCCCTGCAT   +1
   CTGCAGCCTC--------AGAGGCTGGT-CCCTGCAT   Δ9
   CTGCAGCCTCTCC----CAGAGGCTGGTGCCCTGCAT   Δ4
   CTGCA------------------------CCTGCAT   Δ25
   CTGCAGCCT-T--TCAT--G---CA--TGCCCTGCAT   Δ11 2x
   CTGCAGCC--------TCAGAGGCTGGTGCCCTTCAT   Δ8 2x
   CTGCAGCCTCTCCTCT----------TGCCCTGCAT   Δ11
   CTGCAGCCTCTCC---TCAGAGGCTGGTGCCCTGCAT   Δ3
   CTGCAGCCT--------------CTGGTGCCCTGCAT   Δ14
   CTGCAGCCTCTCACCAGCAGAGGCTGGTGCCCTGCAT
  CTGCAGCCTCTCCTCTTCGAAAGGCTGGTGCCCTGCAT   +1
```

Figure 33: RGEN induced mutations in *JAK3*. Sequencing showed many deletions (Δ3-107) and a few indels. Aa exchanges are marked in **bold**, insertions in grey, crRNA target site is underlined, PAM is denoted with a dotted line.

activiy of crRNA 2 lead to an increased number of off-target cleavage events triggering apoptosis in targeted cells.

3.2.3 Detection of off-target cleavage

Off-target cleavage is a known problem with all CENs. Therefore, the three most prominent off-target sites for *JAK3* crRNA 1-20 were identified via the CRISPR Design

tool (Hsu et al., 2013) and primers designed to amplify a region of 500–1000 bp around each site. PCR products were then sequenced; no mutations were detected for any of the sites in any clone.

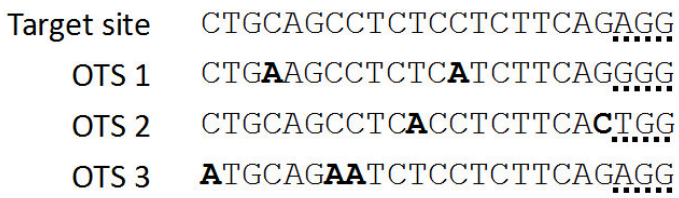

Figure 34: Screening for off-target cleavage of *JAK3* crRNA 1-20. The three most prominent OTS carrying 2-3 mismatches (indicated in **bold**) were identified and screened for off-target cleavage.

3.2.4 Multiplexing of sgRNAs

Multiplexing of crRNAs was also tested by co-transfecting with *RAG1* and *JAK3* crRNA plus Cas9. Whereas screened clones did show mutations in *JAK3*, albeit at a lower frequency (3 % with additional cold shock treatment for 72 hrs at 30°C, 15 % without cold shock treatment) (**fig.35**), no mutations could be detected in *RAG1* (see **2.1**).

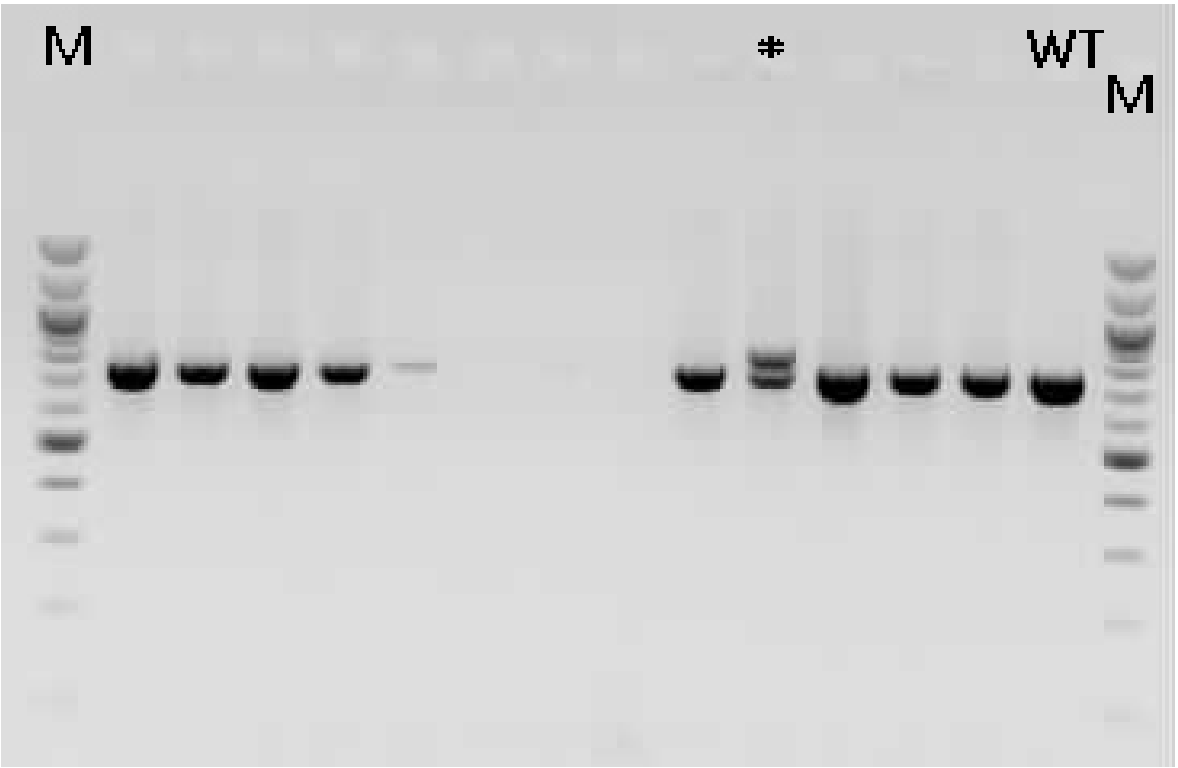

Figure 35: RGEN induced deletions in *JAK3*, but not *RAG1*. After transfection with *JAK3* crRNA 1-20, *RAG1* crRNA and Cas9, amplification of a 742 bp amplicon spanning the *JAK3* target site showed deletions of up to 107 bp. M: 100 bp ladder. 1.0 % TBE agarose gels.

PART V

DISCUSSION

Animal models are important resources for the development of new diagnostic and therapeutic procedures for human medicine. Immunodeficient models are of particular interest because they allow engraftment of human cells and tissue and could be used for the validation of stem cell therapies and in cancer research. Immunodeficiency (SCID) can be traced back to naturally occurring mutations in genes involved in cytokine signalling (*JAK3, IL2Rg*) and antigen receptor diversification (*RAG1/2*)(Macchi et al., 1995; Noguchi et al., 1993; Schwarz et al., 1996). These are all suitable candidates for genetic inactivation to generate SCID animal models and each has been modified in mice (Cao et al., 1995; Mombaerts et al., 1992; Thomis et al., 1995). However, decades of research with such murine models have shown that mice are often not always representative of the human situation (rev. by Flisikowska et al. (2014); Sausville and Burger (2006); Seok et al. (2013)). Efforts have therefore recently been directed towards the generation of alternative animal models, especially the pig because of its similarities with human in terms of genetics, metabolism and size. The advent of customizable endonucleases has greatly facilitated genetic modification of porcine cells (rev. by Tan et al. (2012)); both TALENs and RGENs can easily be generated to target almost any DNA sequence of interest (Cermak et al., 2011; Jinek et al., 2012). Delivery methods and screening processes still have to be optimised in order to ensure high mutational rates.

In this study, novel CENs were investigated as means of the generating an immunodeficient pig model; the main focus was on the *in vitro* modification and subsequent characterization of cell material suitable for SCNT.

1 Genetic modification of porcine cells with CENs

Since they have been first described (Boch, 2011; Christian et al., 2010; Jinek et al., 2012; Mali et al., 2013b; Miller et al., 2011; Mussolino et al., 2011), TALENs and CRISPR/Cas9 enzymes have been widely employed to generate knock-out and genetically modified animals (Bassett et al., 2013; Huang et al., 2011; Jao et al., 2013; Ma et al., 2012; Tesson et al., 2011; Wang et al., 2013). In pigs, Fahrenkrug's group have reported efficient gene editing with TALENs for both gene inactivation and targeted insertion of ssODNs in somatic cells (Carlson et al., 2012; Tan et al., 2013). Microinjection into swine embryos with subsequent generation of live animals has also been reported (Lillico et al., 2013). Here, I used the same techniques to generate both indel knock-outs and targeted insertions in *JAK3* and *RAG1/2*. Because CENs are rather new tools for genome editing, many factors such as choice of target sites, enrichment and screening as well as detection of possible off-target sites had to be established during this study.

1.1 Choice of target sites

As there are few strict requirements known, target sites for both TALENs and RGENs can be chosen relatively freely with the help of various websites (compare **chapter II.10.**). Current understanding is that attention should be paid to potential off-target sites. The ideal sites should have a difference of at least 5–8 mismatches to the next best hit and those hits should not be within any known exons (Kim et al., 2013). Epigenetic modifications can influence the efficiency of cleavage at a given locus; thus, modifications such as histone proteins or cytosine methylations can also have a negative impact on CEN activity, although the causes for this phenomenon have not yet been fully elucidated (Chen et al., 2013b; Guilinger et al., 2014; van Rensburg et al., 2013; Wirt and Porteus, 2012).

Each TALEN half-site should consist of at least 14 bp (Jankele and Svoboda,

2014); increasing the length of the target site may increase specificity (Guilinger et al., 2014) and TALENs targeting up to 23 bp have been reported to cleave DNA effectively (Li et al., 2011b). Long stretches of the same RVD will cause binding instability (Streubel et al., 2012). A preceding T at the target site is desirable, but can be circumvented by using scaffolds with different specificities (Lamb et al., 2013). The length of the spacer between the two half-sites is dependent on the length of the C-terminal domain: a full length C-terminal domain requires 16–31 bp, while 12–21 bp suffice for the commonly used 42–63 C-terminal domain (Li et al., 2011b; Miller et al., 2011).

crRNA target sites are usually 20 bp long and must be followed by a PAM in the form of NGG when using the established *S. pyogenes* derivated system. Some limitation are imposed by the requirements of the respective promoter used; the U6 promoter needs G at the 5'-end of the sequence to transcribe, while the T7 promoter requires GG. These Gs can be either part of the target site, which restricts the number of potential target sites, or can be appended to the full crRNA, which will cause a mismatch between the target site and the crRNA. Both approaches have been applied successfully (Sander and Joung, 2014).

Since the aim of this work was the complete knock-out of the targeted genes, target sites were chosen at the beginning of either exon 1 or 2. For TALENs, target sites were 14–18 bp with spacers of 13–17 bp; all sites started with a T. As crRNA target sites, 20 nt sites and one 18 nt site for comparison were selected; all of these were followed by a canonical PAM, but did not include an obligatory G at the 5'-end. In most cases, several target sites were tested in order to account for differing targeting efficiency at various loci.

1.2 Production of CENs

TALEN repeat arrays can be produced by hierarchical ligation of the individual repeat units. Several different procedures have been developed, some relying on PCR amplification of the repeat units (Sanjana et al., 2012), others on the digestion of previously purified plasmids (Cermak et al., 2011). Both approaches require approximately the same amount of work and time and can be performed using techniques and devices that are regularly used in a standard molecular biology laboratory. In this study, only the original Golden Gate approach (first described by the Voytas group (Cermak et al., 2011)) was tested, i.e. preparation of a plasmid library and subsequent digestion and ligation of the plasmids carrying the desired repeat units. As long as the stock plasmids contained the correct repeat unit, assembly of TALENs worked very efficiently, espe-

cially when paired with further selection procedures. Higher frequency of incompletely assembled TALENs was mostly due to low concentration of one of the RVD plasmids.

For the production of sgRNAs, oligonucleotides carrying the respective target site have to be inserted into a vector containing the rest of the sgRNA sequence. Two rounds of (nested) PCR were necessary to ensure complete absence of plasmid DNA, which considerably hinders RNA transcription when transcribing short fragments (Carolin Wander, personal communication). While individual expression and delivery of sgRNA and Cas9 mRNA is more convenient, particularly in the context of multiplexing, the use of a vector combining sgRNA and Cas9 (such as pSpCas9) is reported to yield better cleavage efficiency (Ran et al., 2013b).

1.3 Delivery of CENs

During this study, TALENs were delivered either as circular pDNA or *in vitro* transcribed mRNA. Components of the CRISPR/Cas9 system were delivered mRNA. For the generation of animal models, the latter approach is preferable because it avoids the problem of plasmid integration, which was observed in all of the cell clones transfected with TALEN pDNA in this study. Prolonged expression of ZFN and Cas9 has been reported to have deleterious effects (Baker, 2014; Gaj et al., 2012). Any integrated TALEN constucts could be bred out at a later stage after animals have been generated via SCNT, but this is time consuming and cannot be controlled (Tan et al., 2012). Furthermore, it has been reported that introduction of TALEN mRNA leads to higher mutation and HDR rates, albeit by an unknown mechanism (Tan et al., 2012).

In principle, any transfection method can be used for delivery of CENs; in the course of this study, no significant difference between various methods for plasmid delivery was observed. CENs can also be introduced as proteins into cells. This was first demonstrated for ZFNs and was shown to decrease off-target effects(Gaj et al., 2012; Yun et al., 2008). TALENs, however, lacking the ZF motif, do not possess cell-penetrating properties *ab initio* and therefore have to be modified or conjugated to enable efficient transduction. This can be achieved by conjugation, for example with poly-Arg peptides (Liu et al., 2014) or transferrin (Chen et al., 2013c); or by fusion with cell penetrating peptides (Mino et al., 2013; Ru et al., 2013). While removal of superfluous conjugating peptides and determination of a suitable ratio of TALEN protein:peptide pose challenges to the first approach, the inefficient production of fusion proteins may hamper application of the latter approach. Cas9 protein seems to be sufficiently cell-penetrating without modification, as shown by experiments with pre-assembled Cas9 protein:sgRNA complexes in *C.elegans* (Cho et al., 2013). An-

other possibility is viral transduction. Various viral vectors such as integrase-deficient lentiviral vectors, adeno-associated virus-derived vectors and lentiviral particles have been used for delivery of CRISPR/Cas9 (Koike-Yusa et al., 2014; Platt et al., 2014), TALENs (Cai et al., 2014; Holkers et al., 2012) and ZFNs (Ellis et al., 2013; Lombardo et al., 2007).

It has been suggested that mild hypothermic treatment directly after transfection increases CEN mutation frequency (Doyon et al., 2010; Gaj et al., 2012; Hauschild et al., 2011); this is most likely due to extended mRNA and protein stability at lower temperatures (Roobol et al., 2009). Extension of the cold shock to up to 7d at 30 °C is reported to lead to enhanced maintenance of mutated alleles when using TALENs(Tan et al., 2012). During this work, a cold shock treatment was routinely administered for all TALEN transfections; for transfection with CRISPR/Cas9, incubation at 30 and at 37°C were compared. Mutation efficiency was higher when no cold shock was applied (3% vs. 15%), but sample sizes were too small to draw statistically relevant conclusions.

1.4 Selection, enrichment and screening of mutants

While many studies have shown that CEN induced mutations occur with frequencies so high that no further selection or enrichment are necessary (Meyer et al., 2010; Santiago et al., 2008; Tong et al., 2012), there are also reports that frequency of NHEJ-mediated mutations decreases over time without appropriate selection (Carlson et al., 2012). Therefore, selection and enrichment techniques can be helpful when working with CENs. For this study, I used a simple enrichment strategy based on co-transfection with the H2-K^kII gene, which encodes a cell surface molecule that enables selection with magnetic beads. This systems effectively enriched transfected cells, but not all cell types survived the selection process.

Kim et al. have described a series of surrogate marker plasmids with similar structure which contain two selection markers separated by the target site of the respective CEN (Kim et al., 2011a, 2013). The second selection marker is out of frame and expressed only after CEN induced mutagenesis of the marker plasmid. Selecting for the second marker favours cells with high CEN concentration and activity; these cells are more likely to also show modifications of the genomic locus. This makes this approach more efficient at the detection of targeted cells than simple co-transfection. Many combinations of resistance cassettes, magnetic and fluorescent markers can be used for the selection process. Because the system leaves no traces in the genome and can be easily applied to various cell types, it could substantially facilitate selection and

enrichment of CEN targeted cells.

Easy enrichment of CEN transfected cells can also be achieved by fusing the respective nuclease to a fluorescent protein, thus allowing FACS selection of cells with high nuclease concentration, which is directly correlated with the number of targeting events (Duda et al., 2014).

For the detection of induced mutations, one can either rely on sequencing or detection of mismatch-induced heteroduplexes. As there has to be a sufficient number of heteroduplexes relative to homoduplexes, these methods are only partially suitable for the screening of cell pools.

Digestion with mismatch specific nucleases such as Cel-I, T7E1 and Surveyor nuclease is the fastest way of detecting CEN induced genetic alterations (Kim et al., 2009). They are also very reliable, with mutants as rare as 1 in 32 being identified by Surveyor nuclease (Qiu et al., 2004). In the course of this study, TALEN induced mutations were successfully detected using the Surveyor nuclease, while digestion of control samples with T7E1 did not produce a defined band pattern. This might have been due to the low quality of template DNA and PCR product, which is crucial for the resolution power of mismatch specific nucleases.

Heteroduplexes of mismatched DNA can also be identified using a heteroduplex mobility assay (HMA). This relies on the slower migration of heteroduplexes through a polyacrylamide gel than their homoduplex counterparts, resulting in distinct bands. Ota et al. used this approach to detect artificially induced deletions of 2–10 bp as well as TALEN induced mutations of 4–11 bp (Ota et al., 2013). Unlike digestion with mismatch-specific nucleases, this approach allows immediate identification of different species of mutants.

SSCP also uses PAGE to separate different DNA species, but here the DNA is single stranded. Therefore, even small sequence variations lead to conformational differences that affect migration behaviour. In the course of this study, SSCP was also briefly tested and different band patterns in wild type and mutated DNA were detected, although no defined band pattern was obtained. Further optimisation would be needed to reliably identify modified cells.

Lastly, high resolution melting analysis (HRMA) requires more technical equipment, but is a very sensitive means of detecting alterations. This method is based on the formation of unstable heteroduplexes between mismatched DNA, which will melt faster than homoduplexes, and can also be used for the characterization of heteregenous mutant population, such as can be expected from microinjection of CENs into embryos (Dahlem et al., 2012).

1.5 Induction of HDR and targeted insertion

A simple knock-out is often all it takes to generate a suitable model for many investigations in forward genetics, but more sophisticated models may require the insertion of precise mutations, as is the case with certain disease-associated mutations. Therefore, CEN mediated HDR was also a point of interest in this study. Conventional HR requires long homologous sequences on the targeting vector (usually several kb), but when working with CENs, homologous regions can be substantially shortened. In this study, targeting vectors with around 1 kb were successfully used for the induction of HDR, with efficiencies ranging between 5 to 40% of analysed cell clones. Unexpectedly a deletion of 300 bp in the 3'-homologous region in *JAK3* was observed in about half of the cases; as this site was 750 bp downstream of the TALEN recognition site, it is unlikely that the deletion resulted from TALEN activity. Probably, the deletion stemmed from incomplete incorporation of the targeting vector and is a locus-specific phenomenon.

More recently, ssODNs have emerged as a tool for targeted introduction of precise mutations (Orlando et al., 2010). Not only do they not require the complex construction of targeting vectors, but they also leave no footprints in the genome, making them ideal tools for modifications in the context of gene therapy (Aarts and te Riele, 2011). ssODNs used for CEN- mediated HDR usually contain around 50 bp of homology on both sides of the target site; silent mutations can be engineered within the target sites to hinder re-cleavage after insertion at the site of interest. Leaving out the initial T from the ssODN sequence is also supposed to reduce TALEN activity, but seems to have little effect *in vivo* (Tan et al., 2013). To facilitate detection of clones carrying the desired modification, additional restriction sites can be built into the ssODN. ssODNs in combination with CENs have been used for modifications in cultured cells (Chen et al., 2011; Rivera-Torres et al., 2014; Strouse et al., 2014; Tan et al., 2012; Wang et al., 2014) as well as in zygotes (Bedell et al., 2012; Wefers et al., 2013a). It was my experience that these results could not be repeated; no targeted insertion of ssODNs co-transfected with TALENs was observed. This might be due to a reduced proliferation phenotype (RPP) of successfully targeted cells, which has been reported following high ssODN concentrations (Borjigin et al., 2012; Ferrara et al., 2007). It is also known that linear donors such as ssODNs have lower intrinsic efficiency than plasmid donors (Orlando et al., 2010) and therefore lead to a great variability in targeting efficiency (Ran et al., 2013b). Further work in this direction would require optimisation of transfection parameters such as delivery method and concentration.

Another interesting idea is to utilise the more efficient NHEJ pathway for

targeted insertion of transgenic cassettes. By furnishing linear donors with overhangs similar to those produced by ZFN activity, Orlando et al. promoted the precise integration of short linear constructs. Maresca et al. further developed this concept by including the target site on the donor plasmid itself, proving that ZFN activity will produce compatible ends *in situ* (Maresca et al., 2012). They avoided re-cleavage by using obligate heterodimeric TALENs and changing the orientation of the target half site on the donor plasmid. With this technique, integration of a 15 kb cassette in various human cell lines was achieved. In the course of this work, I adopted a similar strategy, using the *RAG1* TALENs and a 4.8 kb donor plasmid carrying the respective target site and a neomycin resistance cassette. However, neither pools nor single cell clones of drug selected cells showed evidence of integration events when screened by PCR. Unlike in the study by Maresca et al., a normal TALEN scaffold and not the obligate heterodimeric one was used because of the known lower activity of obligate heterodimeric TALENs. Screening over the intersection of genomic DNA and donor donor DNA rather than over the whole length of the donor plasmid was also tested (data not shown), but did not yield any defined bands. It must therefore be concluded that this approach works best at specific loci with high efficiency for transgene integration. In pigs, such a locus has been found in the ROSA26 locus (Li et al., 2014); in the future, this locus could therefore be used to test whether NHEJ-mediated insertion offers another means of transgene introduction in porcine cells.

1.6 Detection of off-target activity

Utilizing any kind of CEN incurs a risk of undesired off-target activity, i.e. induction of unspecific DSBs throughout the whole genome. While cells with abundant genetic lesions will undergo apoptosis, surviving cells still have to be screened for integrity of the genome. Deep sequencing or whole exome sequencing can provide a faithful image of the genomic landscape, but at present these require considerable investment of resources (Li et al., 2011b). A practical compromise is the sequencing of selected hot spots with known similarity to the target site. For TALENs, studies have shown cleavage activity of up to 2% at sites with 8 or more mismatches (Guilinger et al., 2014). For RGENs, not more than 3 mismatches are tolerated (Ran et al., 2013b), with the 5' bases being more tolerant of mismatches than those within the 3' seed region. While two bp mismatches in the seed region abolishes cleavage activity (Mali et al., 2013a), 5'-truncations of up to 3 bp are tolerated. (Jinek et al., 2012).

Most bioinformatic tools used to identify target sites can also scan the genome of interest for possible off-target sites. These can be analysed by PCR am-

plification and subsequent digestion with a mismatch specific nuclease or sequencing. In this study, the three most prominent off-target sites for *JAK3* crRNA 1-20 were examined for the presence of indel mutations, but none were detected. For TALENs off-target activity was not tested, either because on-target activity was already low or because clones were not used for SCNT.

1.7 Increasing CEN specificity and efficacy

1.7.1 TALENs

Modifications of the FokI domain. Modifying the catalytic domain of TALENs can lower cytotoxicity by reducing off-target activity and can also shift the ratio between NHEJ and HDR events. Mutations can be introduced by either site-directed mutagenesis or splicing by overlaping extensions (SOE) (Heckman and Pease, 2007; Ho et al., 1989). The FokI catalytic domain can itself be easily mutated to generate variants with improved properties. One of the most common obligate heterodimeric scaffolds is the Q486E:I499L and E490K:I538K (short:EL:KK) variant, first described for the use with ZFNs (Miller et al., 2007). During the course of this study, this variant was tested, but activity was lower than the wild type FokI domain in the activity screening and not detectable when used for induction of HDR. This is consistent with earlier reports of decreased activity compared with the wild type FokI domain (Miller et al., 2007; Söllü et al., 2010). Additional mutations (ELD:KKR) or combination with hyperactive FokI domain variants such as Sharkey (S418P:K4441E) (Guo et al., 2010) may restore wild type activity (Doyon et al., 2011). Nickase variants of FokI have been used with both ZFs and TALE DNA-binding domains (Kim et al., 2012; Ramirez et al., 2012; Wu et al., 2014). Conversion of D450 to either alanine or asparagine (Bitinaite et al., 1998; Wang et al., 2012), changes the activity from double strand to single strand cleavage, thus rending off-target cleavage easier to repair and therefore less toxic. Additionally, nickases induce HDR with a greater frequency than the respective nucleases, (Certo et al., 2011; Kim et al., 2012; McConnell Smith et al., 2009; Ramirez et al., 2012; Wang et al., 2012; Wu et al., 2014), possibly by blocking the more efficient NHEJ pathway (Maresca et al., 2012; Perez-Pinera et al., 2012b). However, their overall efficiency is lower than the wild type FokI domain (Ramirez et al., 2012), which also explains why no HDR events mediated by TALENickases were observed in my study. As for obligate heterodimeric FokI domains, further mutations may be needed to increase overall activity.

Other nuclease domains. While FokI is by far the most common nuclease domain used for programmable nucleases, TALE DNA-binding domains can also be combined with cleavage domains that convey their own specifity. The first trials were conducted with so called MegaTALs, which consist of TALE DNA-binding domains and meganucleases (Boissel et al., 2014); others have followed using the catalytic domain of PvuII (Yanik et al., 2013) and I-TevI (Beurdeley et al., 2013). Adding a nuclease domain with intrinsic specificity can result in hyperspecific nucleases that are especially suited for therapeutic use. It is also possible to replace the FokI domain with a monomeric nuclease such as the staphylococcal nuclease (Mineta et al., 2008; Mino et al., 2013), thus widening the criteria for possible target sites.

1.7.2 CRISPR/Cas9 system

crRNA structure. While the *in vivo* system uses a dual-gRNA system with separate crRNA and tracrRNA molecules, using a single-gRNA not only simplifies the assembly and delivery process, but also yields higher activity (Sander and Joung, 2014). sgRNAs can harbor tracrRNA of different length and, generally, longer stretches of tracrRNA convey higher activity. The most commonly used sgRNA scaffold is around 100 nt. In this study, a 101 nt sgRNA scaffold was applied. This showed high editing rates for one locus (3–30% of screened cell clones) (*JAK3* crRNA 1) and no detectable targeting events for two other (*RAG1*, *JAK3* crRNA 2). Complete failure of individual sgRNAs might be due to local chromatin structure or disruptive secondary structure (Shan et al., 2014). These findings serve to illustrate that successful inactivation of a particular gene requires a parallel approach, using various sgRNAs to target different sites.

Recent studies have shown that, unlike with TALENs, increasing the target site of crRNAs (up to 30 bp) does not increase specificity, but rather the opposite (Ran et al., 2013a). This prompted the idea that shorter, truncated sgRNA (tru-sgRNA) with only 17 or 18 nt complementarity could be more specific than the 20 nt type (Böttcher et al., 2014; Fu et al., 2014). However, in this study, a marked decrease in efficiency from 30 to under 2 % was observed when using a 18 bp target site instead of 20 bp. As that specific crRNA was not purified in the same way as the others had been, the efficiency of tru-sgRNA has to be tested in further experiments.

Modifications of Cas9. Mutation of either cleavage domain of Cas9 yields nickases that can be used alone or in pairs. Whereas single Cas9 nickases favour HDR — as expected from similar experiments with ZFNs and TALENs — paired nickases with a suitable spacer between them (4–100 bp) can equally induce NHEJ or HDR when transfected with an ssODN (Cho et al., 2014; Mali et al., 2013a; Ran et al., 2013a; Shen

et al., 2014). While Cas9 nickases have been reported to reduce off-target cleavage compared to the respective nucleases (Ran et al., 2013a), introducing a pair still risks off-target cleavage at two separate sets of possible sites. This could be solved by replacing the momomeric catalytic domains with co-dependent, dimeric versions such as FokI (Sander and Joung, 2014; Tsai et al., 2014).

Other RGEN systems. The requirement of *S. pyogenes* Cas9 for an NGG PAM motif at the 3'-end of the target site might limit the number of possible target sites; in this case, other, recently described Cas9 variants from *Streptococcus thermophilus*, *Neisseria meningitidis* or *Treponema denticola* could be used (Esvelt et al., 2013; Hou et al., 2013), with many more yet to be explored (Chylinski et al., 2013; Fonfara et al., 2014).

1.8 Concluding remarks on CENs

Leaving aside homing endonucleases since they are not readily customizable, the toolbox for genome editing offers three distinct systems for targeted modifications, ZFNs, TALENs and RGENs. While TALENs share a basic structure with ZFNs, the CRISPR/Cas system has different molecular roots; its beauty lies in utilizing the gold standard of DNA recognition — Watson-Crick base pairing. Each of the three systems empowers researches to carry out precise mutagenesis; comprehensive long-term studies comparing both efficacy and adverse effects are required before a definite recommendation for any of the three can be given. It is quite likely that all will continue to be used, each offering its own benefits for particular applications.

Research on ZFNs has supplied a range of tools to modify and enhance specificity, enrich and screen for targeting events and detect off-target cleavage. Most can be put to use with TALENs and RGENs, although their reduced toxicity profiles might render some of the measures unnecessary.

CENs are applicable for both directed mutagenesis and HDR. The assembly of huge libraries of cells carrying simple knock-outs provides scientists with an unprecedented wealth of readily available models for genetic studies in species other than the established mouse. The facile construction of HDR based models, on the other hand, enables study of known disease-associated mutations. To this end, ssODNS have recently evolved as a promising alternative to conventional homology donors that are easy to produce and leave minimal genomic footprints. CENs are already revolutionizing biological and medical research, but the scientific community is only beginning to grasp their remarkable possibilities. With Cas9 transgenic animals (Gratz et al., 2014; Platt

et al., 2014), a powerful tool for the easy generation of multiplex knock-out animals has been created. In combination with the well studied Cre-system, these animals allows temporal and spatial induction of gene inactivation, providing exciting possibilities to study the interplay of genes. Beyond the realm of genome editing, DNA recognition domains of CENs can be combined with activating or repressive elements to alter gene transcription profiles. Fusing with fluorescent domains yields highly specific marker proteins.

Taken together, the outstanding potential of CENs for a myriad of therapeutical applications is bound to have a lasting impact on biomedical research.

2 Porcine models for immunodeficiency

2.1 Recently developed models

2.1.1 *IL2Rg* knock-out pigs

Two porcine models carrying a knock-out in *IL2Rg* have been recently described. Suzuki et al. used conventional gene targeting to remove exon 6 from *IL2Rg*, thereby inactivating the gene. Male offspring of animals generated via SCNT lacked a thymus and showed low counts of T and NK cells. Allogenic bone marrow transplantation rescued the immunodeficient phenotype; female offspring was healthy (Suzuki et al., 2012). Watanabe et al. generated the same phenotype using zinc finger nucleases; their animals were also athymic and showed severe deficiency in T and NK cells (Watanabe et al., 2013).

2.1.2 *RAG1*/2 knock-out pigs

RAG1 and *RAG2* have also been targeted to generate immunodeficient pigs. Huang et al. genetically engineered porcine foetal fibroblasts with the help of TALENs and used both *RAG1* and *RAG2* deficient cell clones for SCNT. Generated animals with biallelic mutations in either *RAG1* or *RAG2* had atrophic thymi and spleens and lacked mature T and B cells (Huang et al., 2014). Under standard housing conditions, biallelic knock-out animals died within 29 days, while heterozygous animals developed normally.

Lee et al. focused on *RAG2*, also using TALENs to generate knock-out animals (Lee et al., 2014). Biallelic knock-out animals failed to thrive in a conventional housing environment, possibly due to increased inflammation, apoptosis and infections. As expected, these animals also lacked functional thymi and showed absence of T and B cells. Kept in a cleaner environment, piglets lived to be transplanted with human

iPSCs, which gave rise to mature human teratomas. This demonstrates that RAG deficient pig models permit engraftment of human cells and tissue.

2.2 Generation of immunodeficient pig models

2.2.1 Modifications of somatic cells with CENs

This study focused on the same molecular pathways as the models described above, with the exception that *JAK3* was favoured over *IL2Rg*. Both *JAK3* and *IL2Rg* are involved in γc mediated cytokine signalling, but as *JAK3*, unlike *IL2Rg*, is inherited in an autosomal recessive manner, only *JAK3* $^{-/-}$ offspring will be immunodeficient, while heterozygous male offspring can be bred normally.

Random indel mutations were introduced into the first exon of *RAG1* with the help of TALENs. This was very efficient and, depending on the amount of TALEN pDNA used, between 4 and 50% of the cells analysed carried a mutation. However, this experiment used the bone marrow MSC preparation 071210, which was later shown not to support development of healthy foetuses in SCNT (unpublished data). Thus, these clones were not used for cloning and not analysed further. The experiment was repeated in other primary cells, but these did not survive selective enrichment with magnetic microbeads.

JAK3 was first targeted with TALENs and a homologous donor plasmid; as cells, pADSMSC 110111 and 080812 were used which are known to yield healthy piglets. Efficiency was around 6% of analysed cell clones following selection with an antibiotic. Later, sgRNAs targeting *JAK3* were applied in poFF 251113, which resulted in 30% mutated clones, of which half were mutated on both alleles. TALEN and RGEN mutated clones were used for SCNT, but did not give rise to foetuses.

Given the similarities between the porcine and humane immune system, a double knock-out of RAG genes and *JAK3* might be necessary to completely abolish both innate and adaptive immune response in pigs. Multiplexing with the CRISPR/Cas9 system provides a suitable platform for this approach; however, during this study, no double knock-outs were obtained. This was most likely due to inactivity of the *RAG1* crRNA. Despite using a verified pair of TALENs for the modification of *RAG2*, no mutated clones could be isolated, indicating that the delivery method might offer room for improvement.

Viability of cell clones modified with CENs

During all experiments, it was noted that cells transfected with CENs did not grow as fast as cells subjected to conventional gene targeting. Other than prolonged proliferation time, a higher percentage of cells within a colony showed signs of replicative senescence and characteristic elongated morphology. Cell clones that exhibited normal growth were mostly negative for the desired mutation, indicating that CEN activity somehow reduced proliferation. This reduction was progressed with time in culture and more pronounced when selection with an antibiotic was applied.

Primary cells are defined by a finite life span in cell culture, thus a reduction of proliferation rate is expected over time. But high doses of CENs have also been reported to be toxic (Peng et al., 2014). Reducing the overall amount of CEN pDNA or mRNA introduced into the cell can lower cytotoxicity (Mussolino et al., 2011), but is not always feasible since it reduces the overall frequency of cleavage. Further optimisation is therefore needed to decrease the harmful effects of CENs on transfected cells. One possibility is to regulate CEN protein stability, for example by adding an uncleavable ubiquitin moiety leading to fusion degradation (Dantuma et al., 2000; Pruett-Miller et al., 2009). Another interesting approach is the incorporation of a sterically demanding, light sensitive artificial amino acids at the catalytic centre. Only upon UV irradiation the CEN protein can cleave DNA at the target site, thus making introduction of DSBs highly controllable (Chou and Deiters, 2011).

2.2.2 SCNT

Targeted cell clones generated in the course of this study were used several times for SCNT. Firstly, a mixed cell population containing, among others, *JAK3* TALEN targeted pADMSCs 110111 was used for SCNT; these fat-derived MSCs has been shown to generate healthy live piglets (Li et al., 2014). A pregnancy was established and three piglets born, but all of them had arisen from other cell clones carrying other mutations not related to this study. On two other occasions, a mix of *JAK3* monoallelic targeted cell clones gained from transfection with CRISPR/Cas9 was used for SCNT; these cells were poFF 251113 and were also mixed with cell clones containing different mutations. No pregnancies were established from these NTs. poFF 251113 had not been used successfully for SCNT before. In order to identify the exact nature of the problem, it would be helpful to use not a mixture of cells, but only CEN targeted cells as donors for one or more SCNT experiments. Thus, a bias from the experimenters during the SCNT experiment towards healthier looking cells or overgrowth of CEN targeted cells by other cell clones could be excluded. If the problem persists, it could by caused

by three factors: general problems with SCNT; problems with the cell types used; or problems caused directly by CEN treatment.

General considerations concerning SCNT failure. Long-term studies have identified a number of factors that influence the success of SCNT experiments (Kurome et al., 2013). Thus, the season during which SCNT is performed is crucial; SCNT performed in winter gives rise to more healthy live piglets than in any other season. On the other hand, winter sees a low maturation rate of oocytes. SCNT for the cell clones generated in this study was performed twice in March and once April, which could have affected the outcome. A better distribution through the year would be desirable to exclude any seasonal effects.

Choice of donor cell type. Various cell types were tested for the generation of immunodeficient pigs, MSCs isolated from bone marrow or adipose tissue, foetal fibroblasts, postnatal fibroblasts and kidney cells. All have been shown to be able to support SCNT and development of live piglets (Jin et al., 2007; Lee et al., 2010; Richter et al., 2012). However, not only the donor cell type can influence cloning efficiency, but also the individual cell preparation. Different cell preparation can vary widely in their ability to support normal development after SCNT (Kurome et al., 2013). With pADMSC 110111, a cell preparation known for its high cloning efficiency and ability to undergo correct reprogramming (as judged by the lack of developmental defects associated with NT) was chosen (unpublished data). poFF 251113, on the other, have not been tested yet for their ability to give rise to healthy piglets, but poFFs can undergo 30-50 cell cycles in culture (Polejaeva and Campbell, 2000) and have also been used in combination with TALENs for the generation of genetically modified pigs (Lee et al., 2014). In order to exclude negative influence of cell type and line, targeting experiments, especially with CRISPR/Cas9, should be repeated in other cell preparations and obtained cell clones used for comparative SCNT studies. Of particular interest would be transfection of kidney cells, since they possibly contribute to healthier offspring (Kurome et al., 2013).

Influence of CEN treatment. It is also conceivable that CEN targeted cells *per se* do not easily give rise to healthy foetuses. Long-term studies have shown that prolonged *in vivo* culture reduces cloning efficiency (Kurome et al., 2013). Cells targeted with CENs were kept in culture for three weeks and more because single cell dilution was required in order to gain pure cell clones in the absence of a selectable marker. Given the high mutation rate of, for example, CRISPR/Cas9, it would be feasible to generate mini pools from 2-5 single cell clones instead of isolating single cell clones.

These pools could be analysed sooner and, albeit a heterozygous mixture of different mutations, would still give rise to pigs with defined mutations. Other than extended time in culture, undesired off-target activity might induce DSBs in genes important for embryonic development. Karyotyping could ensure that cells have not undergone any gross chromosomal arrangement. At any rate, live piglets have been generated via SCNT from CEN modified cell clones (Hauschild et al., 2011; Huang et al., 2014; Lee et al., 2014; Li et al., 2013c; Xin et al., 2013; Yang et al., 2011), showing that the combination of CEN modification of somatic cells and SCNT is feasible.

2.2.3 Genome editing in early embryos

Microinjection of CENs into zygotes or embryos has recently been developed as an alternative to SCNT of somatic cells modified *in vitro*. The advantage lies in the fact that this avoids SCNT and its associated problems such as faulty reprogramming (Whitworth et al., 2014).

This approach was first established with ZFNs in zebrafish and later rats and rabbits (Doyon et al., 2008b; Flisikowska et al., 2011; Geurts et al., 2009). For TALENs, the system was applied in rats (Tesson et al., 2011) and mice (Wefers et al., 2013a) and first studies with the CRISPR/Cas9 system applied in mouse and rat zygotes have now been published (Li et al., 2013a). In pigs, several models have been generated using this approach. Lillico et al. showed a 16 % editing rate for TALEN and ZFN induced modifications of the RELA locus, which is associated with severity of African Swine Fever infection (Lillico et al., 2013). Hai et al. targeted the *vwF* gene with CRISPR/Cas9 to create a model for von Willebrand disease and reported an efficiency of 11% of analysed piglets, with half of the piglets showing biallelic knock-outs (Hai et al., 2014). Models for biomedicine and agriculture, with targeted CD163 and CD1D, were generated via microinjection of components of the CRISPR/Cas9 system by Whitworth et al. Whitworth et al. (2014).

Microinjection of CENs, however, brings its own range problems; mosaicism is often observed after zygote injection and prolonged activity of CENs will increase this problem. It has not been fully elucidated yet whether CENs are active after the first cleavage or not; some groups claim that that this is not the case (Sung et al., 2013), while others observed a variety of mutations in founder animals that can be only explained by CEN activity in two-cell stage or beyond (Qiu et al., 2013).

In any case, injection of mRNA is preferable over DNA injection, since an early onset of CEN activity should result in more mutant cells contributing to the

embryo (Qiu et al., 2013).

Co-transfection with an HDR donor such as ssODNs could also help to overcome the problem of re-cleavage and would ensure that modified alleles show the same mutation. And as long as there is germline transmission, as shown in most studies (Bedell et al., 2012; Gupta et al., 2013; Hwang et al., 2013; Lei et al., 2012), subsequent rounds of breeding will result in animals with a defined mutation (Song et al., 2013).

3 Conclusion

This study has shown that TALENs and RGENs can be successfully applied for the generation of genetically modified porcine cells. Both types of CENs proved to be easy to design and produce and highly efficient in the induction of targeted mutations and knock-ins. Mutational rates (up to 50% and 30% of cell clones analysed) greatly surpassed those observed with conventional gene targeting.

Screening of indel mutations caused by CENs can be carried out either by detecting loss of a restriction site, digestion with mismatch-specific nucleases or SSCP. In this study, the first approach gave the best results; in future, target sites can be designed to cover a suitable restriction site with the help of freely available bioinformatic tools. Mismatch specific nucleases were also able to identify small mutations; with SSCP, differences between mutated and wild type cell were also visible, but the method would require further adjustments.

TALENs were used both for the introduction of random indel mutations at the target site and targeted insertion of a transgenic cassette. While the latter approach offers the possibility of selecting positive targeting events, the first approach leaves no traces in the genome other than the induced mutation, making it especially suitable for gene therapy. In order to minimise potentially deleterious effects, obligate heterodimeric scaffolds as well as nickases for TALENs were constructed. As they exhibited decreased on-target efficiency, further modifications are necessary to restore wild type activity. The CRISPR/Cas9 system proved to be highly efficient for targeted mutagenesis. A single transfection resulted in a large variety of mutations, with many clones showing independent mutations on both alleles. While biallelic targeted clones might not be desirable for the establishment of an immunodeficient pig strain, they could be used for SCNT and preliminary studies to verify the immunodeficient phenotype. Off-target activity was not observed.

In this study, three genes belonging to the immune system were targeted — *JAK3*, *RAG1* and *RAG2*. By modifying target sites within the first exons of *RAG1* and *JAK3*, cellular material suitable for the cloning of immunodeficient pig models was produced. While SCNT offers a well described path for the generation of genetically

modified animals, microinjection of CEN mRNA, possibly in combination with ssODNs, could provide a short cut for the establishment of transgenic strains.

4 Future directions

The advent of CENs has unlocked a new era for the generation of animal models in species where no ES cells are available. Thanks to ZFNs, TALENs and RGENs, it is now possible to induce modifications at almost any desired genomic site with high efficiency in porcine cells, which will unquestionably lead to the establishment of a variety of animal models in pigs.

JAK3 and *RAG1* targeted cell clones generated during this study can be used for further SCNT experiments and will, in all likelihood, eventually lead to the establishment of an immunodeficient pig line. With all of the induced mutations exhibiting a recessive phenotype, heterozygous animals can be maintained for breeding, while homozygous or biallelic targeted animals will exhibit an immunocompromised phenotype. Under standard housing conditions, these animals will quickly develop chronic inflammation and are unlikely to survive for more than a few weeks; therefore, the installation of specific pathogen free facilities is indispensable for long-term studies.

To verify the immunophenotype of animals with a biallelic knock-out, a series of examinations and analyses can be performed. Macroscopically and histologically, size and composition of lymphoid organs such as thymus and spleen can be determined; at the cellular level, samples from peripheral blood, bone marrow, thymus and spleen have to be tested for the presence of T, B and NK cells. State of the art is the analysis via flow cytometry, with many antibodies for markers of porcine lymphocytes already established. For *RAG1*/2 deficient animals, PCR analysis can additionally detect the lack of rearrangement of V(D)J genes. And as a proof for xenograft tolerance, teratoma formation assays with human stem cells or induced pluripotent stem cells (iPSCs) can be carried out.

SCID mice are now used routinely in cancer research, xenotransplant studies and immunology and many other areas. The generation of a similar porcine line will be a significant advance in establishing pigs as model species for biomedical research. Not only will it provide the base for primary tumourgraft models, but it can also function as a model for the human immune system, similar to humanised mouse models. Because of the extensive similarities between humane and porcine immune system (Butler and

Šinkora, 2007), porcine cytokines might be able to stimulate human lymphocytes, thus precluding the requirement for additional gene knock-ins as necessary for humanised mouse models (Rongvaux et al., 2014). Such a model will be a valuable tool for the study of virus infections, e.g. with HIV or hepatitis C virus, and could also be used in regenerative medicine to measure both safety and efficacy of stem cell therapies. Mice are unlikely to be supplanted by pigs for most basic mammalian research, but their limitations in applications beyond fundamental studies have long been evident. Large animal models may complement biomedical research and with the toolbox of genome editing at hand, these models can now be generated in a fraction of the time previously needed. Bridging the gap between bench and bedside has thus become a matter of taking out the molecular scissors.

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List of Abbreviations

aa	Amino acid
AD	Activation domain
bp	Basepair
cas	CRISPR associated
CEN	Customizable engineered nuclease
CRISPR	Clustered, regularly interspaced, short palindromic repeats
crRNA	CRISPR-derived RNA
CTS	C-terminal segment
dCas9	Dead Cas9
DNA-Pkc	DNA-protein kinase complex
DSB	Double strand break
gDNA	genomic DNA
gRNA	Guide RNA
HDR	Homology directed repair
HMA	Heteroduplex mobility assay
HR	Homologous recombination
HRMA	High resolution melting analysis
HSCT	Hematopoetic stem cell transplantation
Indels	Insertions and mutations

iPSCs	Induced pluripotent stem cells
Lyst	Lysosomal trafficking regulator
MHC	Majoy histocompatibility complex
NHEJ	Non-homologous end joining
NLS	Nuclear localization sequence
NTS	N-terminal segment
OTS	Off-target sites
PAGE	Polyacrylamid gel electrophoresis
PAM	Protospacer adjacent motif
pDNA	plasmid DNA
PGK	Phosphoglycerate kinase
precrRNA	Precursor crRNA
Prkdc	Protein kinase, DNA activated, catalytic polypeptide
RGEN	RNA guided endonucleases
RSS	Recombination signal sequence
RVD	Repeat-variable diresidues
SCID	Severe combined immunodeficiency
SCNT	Somatic cell nuclear transfer
sgRNA	Single guide RNA
SSA	Single strand annealing
SSA	Single strand annealing
SSB	Single strand break
SSCP	Single strand conformation polymorphism
ssODN	Single stranded DNA oligonucleotide

TALE	Transcription activator like effector
TALEN	Transcription activator like effector nuclease
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
tracrRNA	Trans-activating crRNA
tru-sgRNA	Truncated sgRNA
WT	Wild type
ZF	Zinc finger
ZFN	Zinc finger nuclease

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