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Meganucleases Revolutionize the Production of Genetically Engineered Pigs for the Study of Human Diseases

Bethany K. Redel[1] and **Randall S. Prather**[1,2]

[1]Division of Animal Science, University of Missouri, Columbia, Missouri

[2]National Swine Resource and Research Center, University of Missouri, Columbia, Missouri

Abstract

Animal models of human disease are critically necessary for developing an in-depth knowledge of disease development and progression. In addition, animal models are vital to the development of potential treatments or even cures for human disease. Pigs are exceptional models as their size, physiology and genetics are closer to that of humans than rodents. In this review, we discuss the use of pigs in human translational research and the evolving technology that has increased the efficiency of genetically engineering pigs. With the emergence of the clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas system technology, the cost and time it takes to genetically engineer pigs has markedly decreased. We will also discuss the use of another meganuclease, the transcription activator-like effector nucleases (TALENs), to produce pigs with severe combined immunodeficiency by developing targeted modifications of the recombination activating gene 2 (*RAG2*). *RAG2* mutant pigs may become excellent animals to facilitate the development of xenotransplantation, regenerative medicine and tumor biology. The use of pig biomedical models is vital for furthering knowledge of, and for treating human disease.

Keywords

Meganucleases; CRISPR; Pig Models; Zinc Finger Nucleases; Transcription Activator-Like Effector Nucleases; Zygote Injections

INTRODUCTION

Medical technology is advancing the knowledge and treatment of human disease. New medical treatments are developed every day but before clinical trials can be approved for treatment on humans, pre-clinical animal experimentation must be completed. Animal models are undeniably essential to understand the mechanisms of disease, develop treatments, identify preventive measures and even cure human diseases. The Nuremberg Code states that human experimental treatment should only be completed based on the results of preclinical studies using animals as models (Shuster, 1997). Therefore, it is crucial that the most accurate physiological animal model of the specific human disease is created.

Corresponding Author: Randall S. Prather, 573-882-6414, PratherR@Missouri.Edu 920 East Campus Drive, Division of Animal Sciences, Animal Science Research Center, Columbia, MO 65211, U.S.A.

There is an urgency to identify animal models that are best suited to reflect or mimic human disease, although there are limitations to each model.

The most common animal species used for pre-clinical human trials is the mouse. The mouse is advantageous because it is small, inexpensive, has a short gestation period and reaches maturity relatively quickly. Researchers are also capable of obtaining embryonic stem cells (ESCs) from mice which can improve the efficiency of genome modification procedures. However, there are a few limitations that make the mouse model not as efficient to replicate and study the human disease. One of the main limitations is the obvious size difference between mice and humans. The size of the mouse is dramatically smaller which makes surgical and clinical monitoring almost impractical. Another limitation for the use of mice to model human disease is the life span of a mouse is significantly shorter than that of a human. This makes mice an inefficient model for age-dependent diseases such as Huntington's disease. Huntington's disease (HD) is neurodegenerative disease that is caused by an expansion of a CAG repeat greater than 36 repeats in exon 1 of huntingtin (*HTT*) (Pouladi *et al.*, 2013). This age dependent neurodegeneration is identified by the formation of aggregates caused by mis-folding of proteins due to the mutated *HTT* gene (Li and Li, 2012). There are many rodent models of HD but because of their short lifespan, they have not become useful models to study the age-dependent neurodegenerative effects seen in humans. Also, their small brain size is an issue for studies involving medical device-delivered drugs and imaging. However, an HD transgenic pig model has been created that exhibits the neurodegeneration similar to what is seen humans (Yang *et al.*, 2010). Although the mouse model is useful in studying the pathogenesis of HD, this pig model is beneficial to study the age related aspects of HD, imaging of the development of HD, as well drug dosage, delivery, and efficacy in a human sized model.

The ease of creating transgenic mouse models is another advantage to using mice; however, some transgenic mouse models fail to present the same symptoms of human disease. One example of this is mice with mutations in the chloride ion channel, *CFTR* (CF transmembrane conductance regulator), to model cystic fibrosis (CF). These transgenic mice possess a defective chloride ion channel but do not present the characteristic features of human CF such as abnormalities of the pancreas, lung, intestine, liver and other organs (Wilke *et al.*, 2011, Rogers *et al.*, 2008). Transgenic pigs with a mutated *CFTR* gene were created and exhibit the characteristic meconium ileus, exocrine pancreatic destruction and focal biliary cirrhosis which are also seen in newborn children with CF (Rogers *et al.*, 2008).

Although the species divergence between pig and human occurred around the same time as the species divergence between the mouse and human, the pig sequence is more closely related to human sequence (Wernersson *et al.*, 2005). Pigs have been valued as an excellent animal model for human disease, since they have similar physiology to humans and have been found useful in surgical, anatomical, nutritional and toxicological testing (Swindle *et al.*, 2012, Swindle *et al.*, 1994). Minipigs have also shown value and utility as a model for humans and biomedical research (Bode *et al.*, 2010, Vodicka *et al.*, 2005). Pigs possess similar sized organs to humans, have relatively short gestation periods and give birth to large liters. For each of the aforementioned reasons, pigs may lend themselves to mimic the human disease more closely than rodent models. The National Swine Resource and

Research Center at the University of Missouri (see http://nsrrc.missouri.edu/) has been established to assist with the growing need of pig models.

METHODS TO PRODUCE GENETICALLY ENGINEERED PIGS

One of the first genetic engineering approaches used in pigs was pronuclear injection of DNA into pig zygotes (Hammer *et al.*, 1985). This technique is very inefficient with only about 1% of injected zygotes producing transgenic piglets (Prather *et al.*, 2008). Additionally, while large constructs can be injected there is no control over the site of integration(s). Generally, large numbers of founders are created to determine where the transgene has integrated, tissue specificity and level of expression.

Genetic modification of somatic cells followed by nuclear transfer (SCNT) (Whyte and Prather, 2011) offers the advantages of knowing the site of integration, the number of copies integrated, and expression (at least in vitro) prior to creating the animal. The SCNT procedure involves removing the metaphase plate and polar body from oocytes arrested at the metaphase II stage of meiosis. Next, a donor cell that has been genetically modified is transferred to the oocyte and fusion of the donor cell with the oocyte cytoplasm is induced. This transfers the nucleus into the cytoplasm of the oocyte. Finally, the oocyte is activated. Many reconstructed embryos do not progress past the early stages of development; therefore, many embryos are transferred to a recipient at one time. The SCNT process is inefficient and piglets may be born with abnormal phenotypes that may be due to epigenetic reprogramming errors (Prather *et al.*, 2013).

Other methods of genetically engineering pigs include oocyte transduction and sperm mediated gene transfer. Oocyte transduction uses an inactive virus that does not replicate to deliver the transgene. The inactivated virus is injected under the zona pellucida into the perivitilline space of metaphase II stage oocytes (Cabot *et al.*, 2001). Since these retroviruses integrate into chromosomes that are in metaphase and the oocyte is arrested in metaphase this makes for a very efficient system. The oocyte can then be fertilized and transferred to a surrogate for gestation. Again, while retroviruses have preferred sites of integration, this method cannot be used to target where the DNA is integrated or control the number of copies that are integrated (Prather *et al.*, 2013). Another approach used to produce transgenic pigs is the sperm mediated transfection approach (Lavitrano *et al.*, 2003). Here, the transgene is introduced with the sperm at the time of fertilization. The sperm facilitate bringing the transgene into the oocyte where integration can occur. Again, there is no control over the site of integration.

More sophisticated genetic modifications require a system whereby precise genome edits can be made. Such edits may disrupt a gene's function (knockout), delete a domain (interfere with the intron exon boundary such that an exon is skipped), or cut the DNA for integration of donor DNA by homologous recombination. These editing tools (meganucleases) are proving to be very valuable for the creation of animal models.

GENOME EDITING WITH MEGANUCLEASES

Recently, meganucleases have advanced gene targeting and transgenic approaches to improve the efficiency of transgenic animal production. This review will briefly describe this technology but for an exhaustive review of each meganuclease and their application in genetically engineering livestock see Petersen and Niemann, 2015. These targeted meganucleases create site specific DNA double strand breaks and cell repair mechanisms can cause random mutations at that location via non-homologous end joining (NHEJ), or if donor DNA is provided then homologous recombination (HR) can occur. When a cell repairs DNA by NHEJ, insertion/deletion mutations can be introduced which can then disrupt the reading frame of a coding sequence. Although there are many meganucleases that have been used for gene editing; including designed endonucleases and engineered meganucleases, here we will discuss only the three most commonly used meganucleases (Menoret *et al.*, 2013, Ashworth *et al.*, 2006). The first, zinc-finger nucleases (ZFNs), are composed of a DNA binding zinc finger protein domain and the nuclease domain derived from the *Fok1* restriction enzyme (Kim and Kim, 2014). The Cys_2-His_2 zinc finger domain is one of the most abundant types of DNA binding motifs found in eukaryotes (Gaj *et al.*, 2013). Each zinc finger is approximately 30 amino acids that generally bind to three DNA base pairs. Typically, there are 3–6 zinc fingers joined together to create a DNA binding domain with a specificity of 9–18 base pairs per ZFN monomer (Carlson *et al.*, 2012a). To create a double stranded DNA break, dimerization of two *FokI* nuclease domains is needed. This requires a pair of ZFNs to bind to opposing DNA strands, thus allowing *FokI* to dimerize and cut the DNA. Since two sets are required, the genome wide DNA-specificity increases to 18–36 bp. The cleaved DNA can then be repaired by either HR, if donor DNA is provided, or error-prone NHEJ.

ZFNs have been used successfully to create modification of pig genomes by either electroporation of fetal fibroblast cells followed by SCNT with the mutated cells or by microinjecting ZFNs directly into zygotes. The first example of ZFN use in pigs was to knock out an enhanced green florescent protein (eGFP) transgene in pigs (Whyte *et al.*, 2011). Later that same year an α1,3-galactosyl-transferase (*GGTA1*) biallelic knockout model was created by transfecting porcine fibroblasts with ZFNs and SCNT (Hauschild *et al.*, 2011). While, another group used ZFNs to disrupt the myostatin gene by both injection into parthenogenetic embryos and by modification of fibroblast cells followed by SCNT (Huang *et al.*, 2014b). While efficient at inducing DNA edits, ZFNs are cumbersome and time consuming to assemble.

The second meganuclease system to be discussed is the transcription activator-like effector nucleases (TALENs). The TALENs have been introduced as an alternative to genome editing with ZFNs. TALENs are similar to ZFNs as they use a *FokI* restriction enzyme domain that is fused to a tailored DNA binding domain. However, this DNA binding domain is comprised of highly conserved repeats from transcription activator-like effectors, which are proteins that are secreted by *Xanthomonas* spp. bacteria to alter transcription in plant cells (Joung and Sander, 2013). TALENs have been directly injected into porcine zygotes targeting the porcine *RELA* gene, for which a tolerance allele for African Swine Fever has been suggested (Carlson *et al.*, 2012b). About 25% of the modified embryos exhibited

biallelic modifications (Carlson *et al.*, 2012b). Similarly, Lee and coworkers (Lee *et al.*, 2014) used TALENs to efficiently create biallelic modifications in the *RAG2* gene producing pigs with severe combined immunodeficiency (this will be discussed in more detail in this review).

The use of TALENs allows greater flexibility in selecting target sequences than that of ZFNs (Cermak *et al.*, 2011) and the constructs are easier to build. However, one limitation to ZFNs and TALENs is that they are not efficient at making biallelic modifications and additional cloning steps or animal breeding are required to produce animals with the wanted biallelic mutations. This process of waiting for the founders to reach puberty in domestic animals is both time consuming and expensive. In pigs the generation interval is up to 1 year. Recently, a relatively new endonuclease (our third meganuclease system), the clustered regularly interspaced, short palindromic repeat (CRISPR)/CRISPR associated (Cas) protein 9 system has revolutionized genome editing (Cong *et al.*, 2013, Hwang *et al.*, 2013, Mali *et al.*, 2013, Cho *et al.*, 2013). This CRISPR technology is based on a bacterial Cas9 nuclease from *Streptococcus pyogenes* and is part of the bacteria's adaptive immune response. This immune defense mechanism utilizes short RNA to direct the degradation of foreign DNA. The CRISPR locus consists of four genes, including the Cas9 nuclease, as well as two noncoding CRISPR RNAs (crRNAs) (Cong *et al.*, 2013). Each crRNA hybridizes with tracrRNAs (called guide RNAs) and this pair of RNAs complex with the Cas9 nuclease (Sander and Joung, 2014). The protospacer encoded portion of the crRNA directs Cas9 to cleave complementary target DNA sequences if they lie near a protospacer adjacent motif (PAM) sequence (Sander and Joung, 2014).

The CRISPR/Cas9 system was used successfully to produce transgenic *CD163* and *CD1D* pigs both by modifying somatic cells followed by SCNT and by injection of in vitro derived zygotes (Whitworth *et al.*, 2014). Whitworth and coworkers had 100% efficiency creating genetic modifications in injected zygotes and more importantly, they found embryos with homozygous or bialleleic modifications illustrating the high efficiency of this technology in pigs. We have injected zygotes and produced piglets attempting to edit 4 different genes (*CD1D* and *CD163* are included). Twenty three piglets have been born and 22 had biallelic mutations (Table 1 and Fig. 1). Not only is the technique very efficient at producing gene edited pigs, but we have seen none of the problems associated with cloning, i.e. large tongues, contracted tendons, etc. (Carter *et al.*, 2002).

Since the CRISPR/Cas9 system was introduced a number of gene edited pigs have been produced. Hai and coworkers (Hai *et al.*, 2014) found that by injecting CRISPR/Cas9 against the *vWF* gene into zygotes they were able to create piglets with mutations in the *vWF* gene at about 63% efficiency. Similarly, Chinese Bama miniature pig zygotes were injected with *Npc1l1* single guide RNA/Cas9 and again 100% genetic modification was detected (Wang *et al.*, 2015). Another group used the CRISPR/Cas9 system to modify porcine fetal fibroblasts followed by SCNT for *TYR* or *PARK2* and *PINK1* and the knockout pigs were successfully produced (Zhou *et al.*, 2015). While reports show that CRISPRs have the same specificity as ZFNS and TALENs, it is very easy to quickly assemble them at a minimal cost (Petersen and Niemann, 2015). Prior to the introduction of meganucleases the timeline for creating the knockout somatic cells was months to years prior to even

performing SCNT to generate the pigs. In contrast the timeline for generation of a pig with a gene knocked out by using the CRISPR/Cas9 technology is now on the order of 5 months.

USEFUL MODELS OF HUMAN DISEASE

Severe Combined Immunodeficiency (SCID)

Animals possessing a compromised immune system can be used as a recipient for human induced pluripotent stem cells (iPSCs) for the potential to regenerate human organs/tissues or for xenotransplantation (Roberts *et al.*, 2015). SCID mice have been developed and are routinely used for tumor growth and treatment (Schmidt-Wolf *et al.*, 1991, Bastide *et al.*, 2002, Lunardi *et al.*, 2014, Clohessy and Pandolfi, 2015). Recently, two reports of pigs with SCID have been generated by mutating recombination activating gene 1(*RAG1*) or recombination activating gene 2 (*RAG2*) (Lee *et al.*, 2014, Huang *et al.*, 2014a). TALENs were used for transfection of porcine Minnesota minipig fetal fibroblast cells and colonies with modifications were identified. The cells with genetic *RAG2* modifications were used for SCNT and mutant Minnesota minipigs were generated (Fig. 2). The pigs with a biallelic modification were predicted to have a non functioning *RAG2* gene as a stop codon was generated in one allele. These *RAG2* mutants exhibit a SCID phenotype and lack T and B cells. Three out of the four biallelic modified pigs did not have a thymus and the thymus of the fourth was very small compared to age matched control pigs. To determine if these pigs are able to accept human grafts, iPSCs were injected. Not only did the cells survive but by day 12 post injection tumors were palpable, and by day 28 the tumors were solid. These teratomas contained all three germ cell layers, and human mitochondria DNA. This is a great step in identifying a model for testing safety and efficacy of stem cell therapies. In addition, such pigs would be very useful for whole organ transplants and others studying immune-deficiencies.

Cancer

Cancer is one of the leading causes of death worldwide (National Cancer Institute, 2015) which makes the need for cancer detection, diagnosis and treatment crucial. Having an animal model that recapitulates human tumorigenesis and tumor progression is vital to develop new imaging approaches for non-invasive cancer detection and monitoring, understand drug metabolism studies, implementation of radiation oncology regimes and for performing surgical training procedures (Sieren *et al.*, 2014, Adam *et al.*, 2007, Schook *et al.*, 2015). *TP53* is a tumor suppressor that is important for regulation of cell cycle, apoptosis and senescence and is very frequently mutated in human cancers (Levine and Oren, 2009). The majority of mutations in *TP53* cause impairment in its binding to DNA, including the R175H mutations (Sieren *et al.*, 2014). This same mutation is also found in Li-Fraumeni patients. Recently, a porcine *TP53* model was created with the R175H mutation which corresponds to R167H in pigs (Sieren *et al.*, 2014). The R167H mutation was introduced by gene targeting in the endogenous *TP53* gene and delivered by using recombinant adeno-associated virus to Yucatan minipigs. The R167H cells were used for SCNT and pigs were generated. All $TP53^{R167H/R167H}$ pigs that reached maturity developed some type of cancer. They primarily developed lymphomas and osteogenic tumors which is also seen in humans with the R175H mutation.

Another “oncopig” model was generated by creating Cre recombinase inducible porcine transgenes encoding *KRAS*^{G12D} and *TP53*^{R167H}. *KRAS* is a commonly mutated oncogene in human cancers (Jancik *et al.*, 2010). Fibroblasts collected from the transgenic oncopigs treated with AdCre activated the transgene and lead to formation of an in vitro transformed cell with increased proliferation and migration. These oncopig cells were also highly tumorigenic when explanted into immune-compromised mice. When the oncopigs were injected with AdCre, development of a mesenchymal tumor arose. This was highly reproducible within different injection sites on the same pig and also in different littermate oncopigs. Together, both of these models provide a platform to better understand and treat cancer in humans.

CONCLUSIONS

Pigs have profoundly improved the understanding of human disease. They make a useful biomedical model to continue to study the pathogenesis, treatment of symptoms and even identify a cure for the disease. These pigs share many similarities with humans that, along with the advancement of new genetic engineering technology, will result in treatments, therapies and cures for human diseases.

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Abbreviations

ESCs	embryonic stem cells
CRISPR	clustered, regularly interspaced, short palindromic repeat
Cas	CRISPR associated
RAG2	recombination activating gene 2
HD	Huntington’s disease
HTT	huntingtin
CFTR	CF transmembrane conductance regulator
CF	cystic fibrosis
SCNT	somatic cell nuclear transfer
NHEJ	non-homologous end joining
HR	homologous recombination
ZFNs	zinc-finger nucleases
eGFP	enhanced green florescent protein
GGTA1	α1,3-galactosyl-transferase
TALENs	transcription activator-like effector nucleases

crRNAs	CRISPR RNAs
tracrRNAs	trans-activating RNAs
PAM	protospacer adjacent motif
PINK1	PTEN-induced putative kinase 1
PARK2	parkin RBR E3 ubiquitin protein ligase
TYR	tyrosinase
Npc1l1	NPC1-like 1
SCID	severe combined immunodeficiency
iPSCs	induced pluripotent stem cells
TP53	tumor protein 53
KRAS	Kirsten rat sarcoma viral oncogene homolog

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Figure 1.
CD163 gene edited piglets produced by using the CRISPR/Cas9 technology.

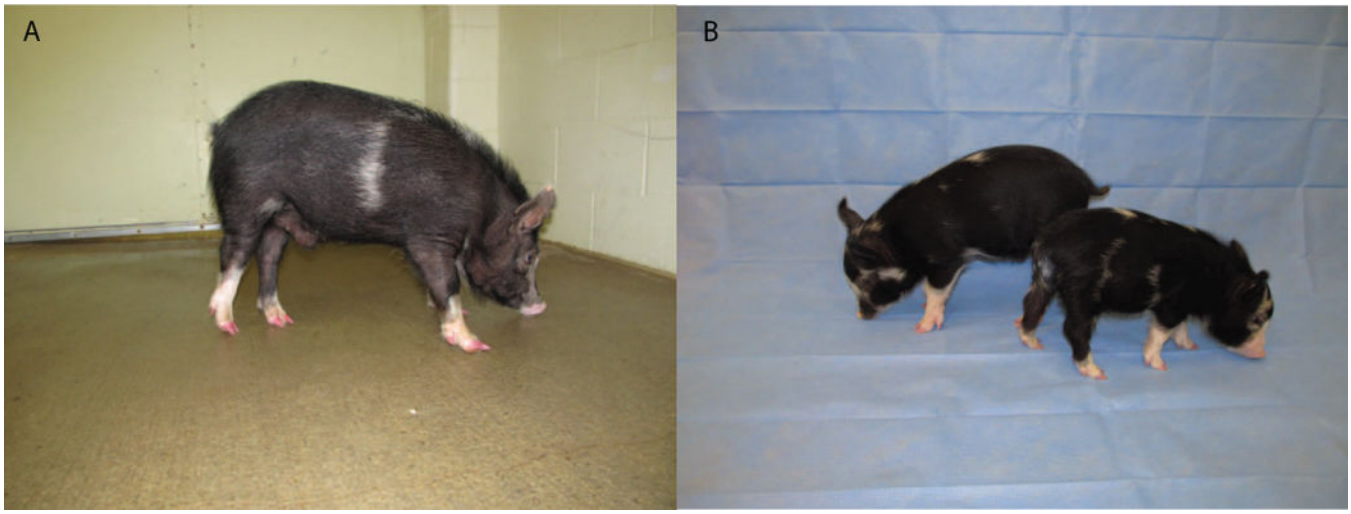

Figure 2.
RAG2 monoallelic gene edited (A) and *RAG2* mono- and bi-allelic gene edited pigs at 4 weeks of age (B).

Table 1

Generation of biallelic edited pigs by using the CRISPR/Cas9 system into in vitro produced zygotes (unpublished).

Gene	# ETs	# Pregnant	# Piglets	# Edited	%
CD1D	2	1	4	4	100%
CD163	2	1	4	4	100%
Unnamed A	2	1	4	3	75%
Unnamed B	2	2	12	12	100%