ABSTRACT: Engineered zinc finger nucleases (ZFN) are rapidly gaining popularity as a means to enhance the rate and specificity of DNA modifications in plant and animal cells. Repair-mediated gene modification by ZFN is driven by introducing DNA double-strand breaks via a nonspecific nuclease domain linked to a sequence-specific zinc finger nucleotide recognition domain. This review examines the use of ZFN to produce genetically modified swine and the potential of this technology for the future. By combining conventional gene targeting methods with somatic cell nuclear transfer, several genetically modified pig models have been produced. These conventional techniques are inefficient in mammalian somatic cells and provide little control over the site specificity and rate of exogenous DNA integration. The use of engineered ZFN that bind and cleave genomic DNA at specific loci can enhance targeting efficiencies by orders of magnitude. Recent publication of the first genetic modification in pigs by combining ZFN technology with somatic cell nuclear transfer has opened the door to genome targeting with a precision that was not previously possible in a large animal model. This review will examine the use of ZFN to generate these pig models and the potential of ZFN to accelerate the production of genetically modified pigs of agricultural and biomedical importance. Current methods of ZFN design, important considerations for their safe and effective use in modification of the swine genome, and future innovative applications of this technology in pigs will be discussed.

Key words: genetic modification, swine, zinc finger nuclease

INTRODUCTION

For decades, pigs have been recognized by the medical community as an excellent surgical model based on their similarity to the human heart, coronary vasculature, liver, kidney, lungs, and uterine histology (Lind et al., 2007; Swindle, 2007). There is a good correspondence between the porcine and human genomes in terms of size and shared synteny (Bendixen et al., 2010), providing many prospects in the area of gene modification. Alterations of key swine genes in disease pathways will improve our understanding of the causes of many human genetic disorders and their potential treatments. For certain disease models, it has been demonstrated that genetically modified pigs may better represent the human condition than analogous rodent models [e.g., cystic fibrosis symptoms in cystic fibrosis transmembrane conductance regulator (CFTR) knockouts (Bragonzi, 2010)]. The completed sequencing of the swine genome coupled with advances in techniques to insert, delete, or replace genetic material in pigs will significantly enhance the specificity of genetic modifications and allow more accurate representations of human disease.

GENETICALLY MODIFIED SWINE AS BIOMEDICAL MODELS

The first transgenic pigs resulted from the stable integration of exogenous DNA into the swine genome via pronuclear microinjection (Hammer et al., 1985). Methods such as sperm-mediated gene transfer (Sperandio et al., 1996) and oocyte transduction (Cabot et al.,
2001) have also been used to produce transgenic pigs. Each of these methods is limited, however, due to the inability to prescreen embryos for transgene integration before embryo transfer, the random integration of foreign DNA, and the fact that only transgene addition is permitted, not deletion. It was the advent of cloning through porcine somatic cell nuclear transfer (SCNT; Onishi et al., 2000) that led to the production of the first gene knockout models in pigs. Endogenous swine genes can be targeted for deletion in SCNT donor cells by the method of homologous recombination (HR), as originally described in mice (Smithies et al., 1985; Thomas and Capecchi, 1987). This technique was used to generate cloned swine lacking a copy of the gene for α-1,3-galactosyltransferase (GGTA1) involved in the hyperacute rejection of pig organs transplanted into humans (Lai et al., 2002). Production of the GGTA1 knockout pigs was a major step forward in the field of xenotransplantation.

The established techniques for swine gene modification have resulted in the production of more than 60 swine models of agricultural and biomedical importance, and this number is steadily increasing (Whyte and Prather, 2011). The National Swine Resource and Research Center (http://www.nsrrc.missouri.edu/) was established in 2003 to ensure investigator access to these swine models for research. Although manipulation of the pig genome has advanced, genetically modified mice continue to be the organism of choice when developing models for use in translational medicine (Peters et al., 2007; Trancikova et al., 2011). Beyond the practical benefits of using mice, such as less space required for housing and a shorter gestational period, the production of genetically altered swine by SCNT presents additional challenges due to the finite lifespan of porcine fibroblast donor cells and the unavailability of stably cultured pig embryonic stem cells. Without the proliferative potential of embryonic stem cells, rapid and efficient modification in somatic SCNT donor cells is critical. In this review, engineered zinc finger nucleases (ZFN) are presented as a means to increase the rate and specificity of gene modification in pigs by orders of magnitude over conventional gene targeting. This novel approach has the potential to dramatically influence the development of swine models for human disease.

**ZINC FINGER NUCLEASES**

Zinc finger nucleases are specifically designed proteins composed of an engineered zinc finger DNA binding domain fused to a separate endonuclease cleavage domain (Durai et al., 2005; Porteus and Carroll, 2005; Urnov et al., 2010). The zinc finger (ZF) is a protein motif capable of binding specific DNA sequences dictated by hydrogen bonding between nucleotide bases and key AA in the ZF α-helix (Figure 1; Klug, 2010). Biochemical analysis established the structure and role ZF as transcription factors more than a quarter century ago (Miller et al., 1985). Each tandemly linked ZF recognizes 3 successive DNA bases, enabling binding to nucleotide sequences of different lengths. This versatile modular design explains why ZF are extremely common in mammalian transcription factors (Papworth et al., 2006). The fusion of specific ZF modules with the FokI endonuclease domain to produce ZFN was first described by the Chandrasegaran laboratory (Kim et al., 1996). Engineered pairs of ZFN with recognition sequences on opposing DNA strands permit 2 FokI endonuclease domains to dimerize and become active, cleaving the DNA at the target site (Figure 1).

Genetic modification by ZFN is driven by the repair of the resulting DNA double-strand breaks (DSB; Urnov et al., 2010). Repair of DSB in eukaryotic cells is efficiently carried out by the highly conserved non-homologous end joining (NHEJ) or homology-directed...
repair pathways (Hartlerode and Scully, 2009). Briefly, NHEJ provides a mechanism for the repair of DSB mainly during G0, G1, and early S phase of mitotic cells. The rapid ligation of the 2 broken ends can introduce short errors (10 to 20 bp) resulting in the gain or loss of genetic information (Figure 1). This “error-prone” repair can be exploited by ZFN to introduce small insertions or deletions or both at the site of the break to disrupt a target gene (Porteus and Carroll, 2005). The alternative HR-dependent repair pathway generally occurs during the G2 and S phase of mitotic cells. The sister chromatid sequence serves as a repair template rather than the sister chromatid, leading to incorporation of the exogenous donor DNA at the target locus (Beumer et al., 2006). It should be noted that unlike traditional HR-dependent gene knockout where the targeting efficiency is near 1 event in $1 \times 10^6$, the DSB introduced by ZFN dramatically increase HR-mediated gene modification, creating gene knockouts in 1 to 50% of all cells (Urnov et al., 2010).

**DESIGN AND PRODUCTION OF ZFN**

At first glance, the design of ZFN that target a desired nucleotide sequence appears to be a straightforward task. Two ZFN for example, each composed of 3 ZF modules that bind to opposing DNA strands would recognize a sequence totaling 18 bp in length (i.e., 2 × 9 bp). This recognition sequence would be sufficiently long to constitute a highly specific genomic target (Klug, 2010). Methods to assemble ZFN through modular addition of individual ZF are established (Bibikova et al., 2002; Wright et al., 2006), including standardized reagents and a free web-based predictive tool (ZiFIT; Sander et al., 2007, 2010) to identify potential ZFN target sites in a gene of interest. It was soon recognized, however, that not all ZF combinations result in optimal DNA binding specificity (Ramirez et al., 2008). More recent selection-based methods to assemble ZFN (e.g., oligomerized pooled engineering context-dependent assembly) have had greater success by avoiding positional combinations of ZF that interfere with optimal DNA binding (Maeder et al., 2009; Sander et al., 2011). This specificity comes at an upfront investment cost of acquiring specific reagents and method optimization. An in silico ZFN design and rapid assembly method that does not require specialized reagents has recently been reported (Osborn et al., 2011) and has the potential to significantly reduce the time required to engineer target-specific ZFN. For researchers who wish to forgo the ZFN design and testing phase, Sigma-Aldrich (St. Louis, MO) offers the CompoZr service that produces custom-made ZFN for specified gene targets in the organism of interest. This proprietary method delivers ZFN plasmids or mRNA ready to be used with an estimated cost of approximately US $12,500. Such an investment for ZFN-based modification may seem prohibitive, until one considers that the production of genetically modified livestock by conventional methods can cost as much as $300,000 per animal (Whitelaw, 2004).

**ZFN FOR GENOME EDITING IN SWINE**

Early work with ZFN to produce genetically modified organisms started with injection into oocyte nuclei of the African clawed frog, *Xenopus laevis* (Bibikova et al., 2001). Although live frogs were not produced, this study demonstrated the in vivo activity of ZFN and HR repair-mediated genome alteration. The first genetic modifications in organisms produced through ZFN-based mutation were in the fruit fly, *Drosophila melanogaster* (Bibikova et al., 2002). The mechanism of NHEJ led to mutation of the yellow (y) gene in fruit flies exposed to ZFN specific for this gene target. Since these initial studies, engineered ZFN have been used for genome editing to produce heritable traits in zebrafish (Doyon et al., 2008; Meng et al., 2008), mice (Carbery et al., 2010), and rats (Geurts et al., 2009; Moreno et al., 2011). However, it was not until recently that this technology was used in large animals, with the production of genetically modified swine.

The first report on ZFN gene knockout in swine somatic cells came from Watanabe et al. (2010). It was demonstrated that electroporation of swine cells with ZFN mRNA targeting an exogenous enhanced green fluorescent protein transgene (eGFP) resulted in ZFN-induced cleavage of the target sequence. Confirmation of cleavage with the Surveyor nuclease assay (Transgenomic Inc., Omaha, NE) was substantiated by the disappearance of eGFP fluorescence expression. Although the study was not designed to generate live pigs, it demonstrated the potential for ZFN to modify cells that could eventually be used for SCNT. Two months after that report, Whyte, Zhao, and coworkers (Whyte et al., 2011) reported on the generation of ZFN-mediated eGFP knockout pigs from donor cells with a single copy of the transgene in their genome. This study built on the rat ZFN modification reported by Geurts et al. (2009), combining ZFN DNA modification with SCNT to generate knockout pigs. Both rat and swine ZFN were designed and provided by Sigma-Aldrich (i.e., CompoZr). Unlike the eGFP gene knockout in the rat, where 1-cell embryos were injected with ZFN mRNA, knockout pigs were produced by electroporation of the swine donor cells (i.e., fibroblasts) with separate plasmids encoding each of the ZFN and a transient selectable fluorophore (i.e., CAG-tomato plasmid). Cells were sorted by fluorescence automated cell sorting for the selectable fluorophore to enrich for swine fibroblast cells that received the plasmids. Cells confirmed to have NHEJ-induced deletions by Sanger sequencing were used as donor cells for SCNT followed...
by embryo transfer to 3 surrogate females. Six of the 7 initial cloned piglets carried ZFN-mediated mutations in eGFP that resulted in disruption of eGFP expression. A third litter of 6 cloned piglets was delivered 1 wk later, all of which were eGFP knockouts. The nature of the mutations, determined by Sanger sequencing, was typically a 2- to 9-bp deletion for the first 2 piglet litters. The majority of the piglets in the third litter had identical 21-bp deletions, although 1 piglet had an unusual 700-bp deletion that removed almost the entire eGFP coding sequence. The variability of mutation size from NHEJ-repair may not be a concern if gene disruption is the goal, but it is an important factor to be aware of for other types of modifications. The relatively large number of cloned offspring produced in this study and the high proportion of eGFP knockouts (i.e., in 12 of 13 piglets) demonstrates the feasibility of ZFN-based gene modification with no perceptible impact on offspring health.

The proof of concept established by the eGFP swine knockout was soon followed with the first report of an endogenous gene knockout in cloned pigs using ZFN. Yang et al. (2011) combined ZFN technology with SCNT to produce heterozygous KO pigs with a defined mutation in PPAR-γ. Three pairs of ZFN (i.e., CompoZr; Sigma-Aldrich) were individually tested for mutational activity by mRNA microinjection into parthenogenetically activated porcine embryos. It was determined that only the second pair of ZFN introduced targeted mutations. Experiments to generate PPAR-γ knockout pigs used ZFN plasmids and not mRNA, presumably due to poor development of embryos associated with ZFN mRNA microinjection in pigs. An important advantage of ZFN genome editing is that the greater rate of HR events eliminates the need for drug selection strategies. However, investigators may still choose to include a selection cassette, as was done in this swine study (G418 selection). Modified swine donor cells were identified by PCR sequencing of the target PPAR-γ region. These donor cells were used for SCNT to produce 1,340 cloned embryos. Embryos were transferred to 8 surrogate females. Four of these surrogates produced a total of 10 live piglets; the other 4 surrogates did not produce offspring. Sequence analysis identified 2 piglets carrying the predicted ZFN-induced mutation in their genome. The large loss of embryos is a characteristic of SCNT and would be expected with or without the presence of ZFN. The authors determined that the use of ZFN increased the efficiency of monoallelic gene-disruption in primary pig cells from less than one in 1 × 10^6 to greater than 4% (Yang et al., 2011).

The greater rate of ZFN-induced alterations at a specific locus has an added benefit: the potential for biallelic knockout of an endogenous gene. This property was effectively demonstrated by Hauschild et al. (2011) through the production of cloned swine with both copies of the GGTA1 gene knocked out in approximately 1% of donor cells after a single ZFN treatment. To produce the pigs, primary fibroblasts were transfected with ZFN plasmids designed to disrupt the catalytic region of GGTA1. Targeted cells were identified by PCR- and Surveyor nuclease-based methods, and the ZFN-modified cells were enriched for with magnetic bead selection. Fetuses were collected from the first round of SCNT at d 25 to verify genetic modification. Those fetuses characterized as GGTA1 biallelic knockout pigs were then used for serial cloning. In that study, embryo transfer was performed in 9 surrogate females. Five of these surrogate females produced a total of 9 live GGTA1 knockout piglets. The remaining 4 surrogate females did not produce piglets. Cells with modified GGTA1 were resistant to lysis by human antibody complement exposure, indicating a protective phenotype against hyperacute rejection.

Biallelic knockout via ZFN provides a significant time advantage compared with traditional knockout techniques (Figure 2). Disruption of both alleles by conventional HR generally involves production of monoallelic knockout clones followed by breeding with other heterozygous knockouts to obtain homozygous knockout in 25% of the offspring. Because cloned pigs are generally not bred until 7 to 8 mo of age, this can introduce a substantial time lag in the generation of double knockouts. The authors of the ZFN-GGTA1 knockout study report a timeline of 5 mo for ZFN transfection, identification of modified donor cells for SCNT, and birth of cloned pigs (Hauschild et al., 2011). The ability to generate biallelic knockouts in less than one-half the time as previous techniques would significantly streamline the production of relevant swine biomedical models. The actual benefit of ZFN-produced double knockouts will be determined on a gene-by-gene basis because certain biallelic modifications may result in an embryonic lethal phenotype.

**POTENTIAL LIMITATIONS OF ZFN FOR PRODUCTION OF GENETICALLY MODIFIED SWINE**

Initial studies have demonstrated the effectiveness of ZFN in producing genome modifications in swine, but to ensure the desired study outcome, 2 important caveats should be considered before conducting such studies in pigs. These are the possibility of ZFN plasmid integration into the swine genome and potential “off-site” or nonspecific cleavage of genomic DNA by ZFN. Plasmids for ZFN are transfected in their supercoiled form so that expression is only transient. Supercoiled plasmids do not undergo integration into the host genome at the same rate as linearized plasmids do (Clark et al., 2007). For a functional ZFN cleaving pair to be expressed permanently, both ZFN plasmids for the left and right binding regions would have to be integrated, an unlikely event. Even the integration of a single ZFN from the pair would mean incorporation of unwanted foreign DNA, so any integration of plasmid DNA is not desired. Primers for PCR specific to the ZFN plasmids
can be used to verify that no integration has occurred in either swine SCNT donor cells or offspring (Hauschild et al., 2011). An alternative to using ZFN plasmids is to microinject porcine embryos or transfect SCNT donor cells with ZFN mRNA. This method will ensure no integration of ZFN DNA, but the success rate of mRNA microinjection in swine has been reported to be less than if ZFN plasmids are used (Yang et al., 2011). The second caveat, off-site cleavage by ZFN, can induce toxicity and decrease the viability of treated cells (Ramalingam et al., 2011). Although the length of the targeting site can be designed to span highly specific ranges from 18 to 24 bp, it is still important to validate the uniqueness of the target site sequence. For organisms with a completed genome sequence, bioinformatic tools can be used to scan the genome assembly to identify putative off-site sequences matching the predicted binding target (Geurts et al., 2009). The most recent assembly of the pig genome (Sscrofa10; currently being annotated) represents about 98% of the porcine genome (Groenen et al., 2011), so such predictive bioinformatic tools could rule out most off-site cleavage activity in the pig. Not all off-site cleavage can be predicted by sequence alone, however. By design, the FokI nuclease domain of ZFN must dimerize to cleave DNA. Although the left and right ZF binding domains are designed to bring the nonspecific FokI nuclease monomeric domains into close proximity, there is the potential for FokI homodimers to form and induce off-target cleavage (Miller et al., 2007). Methods to address FokI homodimer toxicity include redesign of FokI cleavage domains to create obligate heterodimer variants or the creation of ZFN with shortened half-lives, or both (described in Ramalingam et al., 2011). The use of these improved ZFN design techniques when targeting swine genes will increase the specificity of ZFN while reducing toxicity caused by nonspecific binding or nuclease homodimer formation. The increased use and reduced cost of high-throughput sequencing techniques may also serve as a means of assessing off-site cleavage events (Perez et al., 2008). If an off-site cutting event occurs, it may not have consequences that influence the alteration of the intended gene target. In this case, genetically modified cloned pigs could be bred to unmodified pigs with the original genotype to eliminate any mutations that could interfere with modification. With proper planning and design, however, such occurrences should not be a common problem.

SUMMARY AND CONCLUSIONS

Genetic modification of swine with ZFN is a rapidly emerging area of research. This technology has the potential to accelerate the production of pigs to be used as a source of organs for xenotransplantation or therapeutically for humans and pig models as realistic representations of human disease. There is a wealth of existing data on ZFN design and application in other species that can be directly transferred to swine studies. Based on results from the first examples of their use in swine, ZFN offer many advantages over conventional methods to produce knockout pig models. One area that has yet to be explored in pigs is the use of ZFN to drive the insertion of donor fragments of DNA (i.e., knock-in pigs). Targeted integration of exogenous expression cassettes or the insertion of fragments of DNA with point mutations to “correct” the existing genomic sequence in pigs could lead to new models for gene therapy in humans. The nature of DSB repair at the ZFN cut site triggers a high rate of integration for exogenous DNA constructs with only 400- to 800-bp arms of homology and a genetic payload consisting of a few base pairs to more than 8,000 bp (Moehle et al., 2007). By enabling precise control over the location of gene knock-ins, ZFN offer many exciting opportunities in the pig such as the addition of ubiquitous promoters to genes not normally active in specific tissues or at different stages of development.

To date, many pig models have been produced that are critical to improving human health and nutrition. Zinc finger nucleases provide an opportunity to increase the rate of successful production for these models by...
reducing the time in culture for SCNT donor cells and enhancing the specificity of genomic alterations. However, several aspects of ZFN still require further study in order for them to be used with confidence. As more is learned about the nature of ZFN off-site cutting and potential toxicity, better design techniques will become available. Nevertheless, initial successes employing ZFN to modify the swine genome gives many reasons to be optimistic about their future use.

LITERATURE CITED


