Published in final edited form as:

Reprod Fertil Dev. 2019 January ; 32(2): 40–49. doi:10.1071/RD19273.

Application of genome editing systems to enhance available pig resources for agriculture and biomedicine

Kiho Lee, Kayla Farrell, Kyungjun Uh

Department of Animal and Poultry Sciences, Virginia Tech

Abstract

Traditionally, genetic engineering in the pig was a challenging task. Genetic engineering of somatic cells followed by somatic cell nuclear transfer (SCNT) could produce genetically engineered (GE) pigs carrying site-specific modifications. However, due to difficulties in engineering the genome of somatic cells and developmental defects associated with SCNT, a limited number of GE pig models were reported. Recent developments in genome editing tools, such as ZFNs, TALENs, CRISPR/Cas9 system, have dramatically changed the effort and time required to produce GE pig models. The frequency of genetic engineering in somatic cells is now practical. In addition, SCNT is no longer essential in producing GE pigs carrying site-specific modifications, as direct injection of genome editing systems into developing embryos introduces targeted modifications. To date, CRISPR/Cas9 system is the most convenient, cost effective, timely, and commonly used genome editing technology. A number of applicable biomedicine and agriculture pig models have been generated using CRISPR/Cas9 system. Although efficiency of genetic engineering has been remarkably enhanced with the use of genome editing systems, improvements are still needed to optimally utilize the emerging technology. The current and future advancements in genome editing strategies will have a monumental impact on pig models used in agriculture and biomedicine.

Introduction

The pig is a suitable model in biomedicine and an important animal species in the meat industry. Similarities in physiology and genetics constitutes the pig as an ideal model to represent human physiology and phenotype (Prather *et al.* 2013). For instance, symptoms of X-linked primary immunodeficiency are better recapitulated in the pig when compared to mice (Suzuki *et al.* 2012); disruption of *IL2RG* leads to T-B+NK- phenotype in the human and pig, whereas *Il2rg* knockout mice exhibit T-B-NK- phenotype. The pig is also an important source of protein (Grillenberger *et al.* 2003; Murphy and Allen 2003). In fact, according to the US Department of Agriculture livestock report, the swine industry supplied over 9 Billion kilograms of meat in 2017 (<https://www.ers.usda.gov/publications>, accessed 25 October 2019)¹. Meat consumption is projected to rise dramatically due to the desire for a

Correspondence to: Kiho Lee, kiholee@vt.edu, 540-231-1906.

Conflicts of Interest

Authors declare no conflict of interest.

modern diet as incomes improve and urbanization increases. However, the current level of meat productivity is unlikely to secure the rising demand.

Genetic enhancement through genetic engineering approaches could generate humanized pig models for biomedical applications and improve the productivity of pigs for meat production. Unfortunately, the efficiency of genetic engineering has been poor in the pig, thus limiting the use of genetically engineered (GE) pigs in various fields. For example, the first report of GE pigs carrying a targeted modification (Lai *et al.* 2002) was announced more than a decade later than the first knockout mouse (Zijlstra *et al.* 1989), and GE pigs were only applied in limited fields. Recent developments in genome editing technology have transformed the production and use of GE pigs. The efficiency of genetic engineering has been dramatically improved and a new route to produce GE pigs has been introduced. As a result, the number of available pig models for biomedicine and agriculture has increased dramatically. Here, we summarize how genome editing systems were developed and applied in the pig and discuss potential implications.

Conventional genetic engineering in the pig

Unlike the mouse, there is no established porcine pluripotent stem cell line, which prohibits the production of GE pigs carrying targeted modifications and hinders the use of pigs for gene-based research. Persistent efforts were made to establish pluripotent stem cells that can be used for GE pig production, unfortunately, the attempts have not been successful (Koh and Piedrahita 2014). The birth of the first cloned mammal, Dolly, through somatic cell nuclear transfer (SCNT) (Wilmut *et al.* 1997) enlightened another route for GE pig production without having to apply pluripotent stem cells. Specifically, targeted modifications were introduced into the genome of somatic cells by utilizing the endogenous homologous recombination mechanism; those genetically modified somatic cells were used as a donor for SCNT to produce GE animals. The feasibility of this approach was first reported in the sheep (McCreath *et al.* 2000; Denning *et al.* 2001) and quickly expanded to non-rodent species, including the pig. Since the first report of cloned pigs (Onishi *et al.* 2000; Polejaeva *et al.* 2000), this approach was used to produce the first knockout pigs (Dai *et al.* 2002; Lai *et al.* 2002). The pigs lacked a gene (*GGTA1*) responsible for α -(1,3)-galactosyl transferase, which synthesizes galactose- α -(1,3)-galactose (α -Gal) and causes hyper acute rejection after xenotransplantation. These *GGTA1* modified pigs laid a foundation for the progression towards xenotransplantation using pig models; most GE pigs designed for xenotransplantation carry a modified *GGTA1* gene.

Reports of the first knockout pigs led to the development of more pig models in the biomedicine area. One pig model lacking a functional cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Rogers *et al.* 2008a), responsible for cystic fibrosis disease, demonstrated the suitability of pig models in biomedicine. The genetic element of the disease has been clearly identified, but existing *CFTR* rodent models do not exhibit the typical symptoms observed in *CFTR* patients (Semaniakou *et al.* 2018). Similar to *GGTA1* knockout pigs, targeted disruption of *CFTR* was introduced into the genome of pig somatic cells, then SCNT was completed to produce *CFTR* knockout pigs. When homozygous pig models were produced through breeding, the pigs (*CFTR*^{-/-}) represented all known

symptoms of a CFTR patient (Rogers *et al.* 2008b), demonstrating the practicality of this model. Because the efficiency of genetic engineering in somatic cells is extremely low, all of these modifications were introduced in a heterozygous fashion and breeding steps were required to generate GE pigs with homozygous modifications. Considering the gestation period of pigs (114 days) and days to reach puberty (around 6 months), a single round of breeding can easily take over one year in the pig. If only a limited number of founder pigs are generated through the conventional approach, multiple breeding steps through backcrossing are inevitable, which will require years of breeding schemes to produce a sufficient number of GE pigs.

Another obstacle of utilizing the conventional approach is the inefficiency of SCNT. The average term development from SCNT is 1–2% (Zhao *et al.* 2010). However, the efficiencies vary depending on donor cell lines. SCNT is also highly laborious and technically demanding; more importantly, pigs born through SCNT often present sudden death or health complications associated with the process of SCNT (Carter *et al.* 2002; Park *et al.* 2005). The SCNT associated developmental defects are known to be corrected during germ cell reprogramming. However, the health complications associated with SCNT hinders successful breeding to expand the number of GE pig models.

Due to these challenges of the conventional SCNT approach, only a small number of GE pig models have been produced using SCNT. Therefore, there has been an immense interest within the pig community to improve the efficiency of GE pig production. The development and application of genome editing technology in the past ten years has significantly changed the design and process of producing GE pigs.

Development of the genome editing system

The application of genome editing systems has transformed the methods used to produce GE pigs. Although different variations do exist, the foundation of genome editing technology is in its ability to recognize specific sequences on the genome and introduce a site-specific double strand break (DSB) (Fernandez *et al.* 2017). Because DSBs are toxic and may lead to cell death if unresolved, DNA repair pathways are rigorously activated to restore the DSBs. Two main DNA repair pathways are involved in this process: non-homologous end joining (NHEJ) or homology-directed repair (HDR). After a DSB, small insertions or deletions (indels) can be introduced through NHEJ, which often cause a frame-shift of the amino acid codons and leads to a premature stop codon. Specific modifications at the nucleotide level can be introduced through HDR pathway in the presence of donor DNA. The repair pathways are well-organized processes and specific proteins are involved in each repair pathway; therefore, efforts have been made to modulate the frequency of NHEJ/HDR using chemical inhibitors (Maruyama *et al.* 2015; Song *et al.* 2016). Currently, there are three different types of genome editing systems: Zinc-Finger Nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), and Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) system.

ZFNs were the first genome editing system developed to modify the genome in mammals. Zinc-fingers are known to recognize specific sequences and by fusing the non-specific

endonuclease, FokI, the system can introduce DSBs on a target locus. The application of ZFNs could increase the efficiency of gene targeting in mammalian somatic cells up to 1000 folds (Urnov *et al.* 2005). In the pig, the first application of ZFNs was to disrupt an exogenous *GFP* gene. Specifically, by introducing ZFNs into GFP positive cells, the *GFP* gene was inactivated and *GFP* knockout pigs were produced using SCNT (Whyte *et al.* 2011). In the same year, ZFNs were used to produce GE pigs carrying a modified *PPAR- γ* gene (Yang *et al.* 2011), which was the first report of endogenous gene disruption using a genome editing system. The efficiency of targeted disruption was significantly improved compared to the conventional approach, but only a heterozygous modification was introduced into the pig genome. Later that year, it was successfully demonstrated that ZFNs could effectively disrupt both alleles in somatic cells (Hauschild *et al.* 2011); *GGTA1* knockout pigs were produced by introducing targeted modifications to the *GGTA1* gene in somatic cells and using the cells as a donor for SCNT. The successful generation of null or homozygous knockout pigs without a breeding step suggested that genome editing systems could dramatically reduce the time required to establish and expand GE pig models.

The development of TALENs further expanded the use of genome editing systems in GE pig production. TALENs originate from a plant pathogenic bacteria, *Xanthomona* (Gohre and Robatzek 2008). Similar to the action of ZFNs, the engineering of TALENs allows them to bind and introduce site-specific DSBs, thus introducing targeted modifications to the genome. The use of TALENs provided more flexibility when choosing target locations on the genome because TALENs can effectively recognize AT-rich regions (Gaj *et al.* 2013), whereas the activity of ZFNs is favorable in GC-rich target regions. TALENs were first applied for GE pig production in 2012 (Carlson *et al.* 2012). The study demonstrated that TALENs could effectively introduce targeted modifications, including biallelic or homozygous mutations, on various loci of the pig genome. Another important outcome of applying TALENs in GE pig production was the introduction of targeted modifications during embryogenesis (Lillico *et al.* 2013). In this study, injection of mRNA coding for TALENs or ZFNs into developing pig embryos could successfully introduce targeted modifications during embryogenesis and produce GE piglets. As mentioned above, GE pig production required the employment of SCNT due to the lack of pig pluripotent stem cells. The study demonstrated for the first time that SCNT was no longer the only way to produce GE pigs with site-specific modifications.

The application of ZFNs and TALENs significantly lowered the technical difficulties encountered when producing GE pigs. However, the assembly of ZFNs and TALENs could be troublesome and these genome editing systems did not always exhibit credible site-specific nuclease activity. For instance, multiple ligation reactions are required to complete ZFNs and TALENs ready for genetic engineering experiments (Wright *et al.* 2006; Cermak *et al.* 2011). It was the development of CRISPR/Cas9 system that remarkably lowered the efforts of utilizing genome editing systems in the pig. The first CRISPR/Cas9 system was developed based on the adaptive immune system of prokaryotes, specifically *Streptococcus pyogenes* (Gaj *et al.* 2013). The engineered complementary RNA (crRNA) combined with tracrRNA, called single guide RNA (sgRNA), can recognize a target sequence on the genome and recruit the Cas9 protein to the target site. Then, a DSB can be introduced by the Cas9 protein if a protospacer adjacent motif sequence (PAM), 5'-NGG-3', is present at the

locus (Cong *et al.* 2013b). Unlike ZFNs and TALENs, the CRISPR/Cas9 system is easy to assemble. For instance, vectors systems which require only an insertion of crRNA to the vectors are available (Cong *et al.* 2013b) and sgRNA can be commercially synthesized. In addition, the design of sgRNAs can be easily conducted through web-based software such as CHOPCHOP (<https://chopchop.cbu.uib.no/>), GPP sgRNA designer (<https://portals.broadinstitute.org/gpp/public/>), or CRISPOR (<http://crispor.tefor.net/>).

The first use of CRISPR/Cas9 system for pig genetic engineering was to introduce targeted mutations in somatic cells (Tan *et al.* 2013). The following year, two reports were published demonstrating that CRISPR/Cas9 system could be used to produce GE pigs (Hai *et al.* 2014; Whitworth *et al.* 2014). Both studies introduced the RNA form of CRISPR/Cas9 system into developing embryos to induce targeted disruptions through NHEJ pathway. Unlike the lower efficiencies reported with ZFNs and TALENs, the direct injection of CRISPR/Cas9 system into pig embryos could reach efficiencies as high as 100%. One of the studies also demonstrated that targeted modifications could be introduced into the genome of somatic cells, then GE pigs were produced via SCNT (Whitworth *et al.* 2014), confirming that somatic cells modified by CRISPR/Cas9 system are developmentally competent *in vivo*.

The previous reports demonstrated that CRISPR/Cas9 system could be utilized to effectively generate GE pigs and SCNT was not the only approach to develop GE pigs carrying targeted modifications. With the development of CRISPR/Cas9 system, currently, two different routes are used to produce GE pigs carrying targeted modifications (Figure 1). Because the exact genotype of GE somatic cells can be identified prior to SCNT, more complicated modifications, such as targeted insertions, are introduced through somatic cell based genetic engineering (Figure 1A). The direct injection of CRISPR/Cas9 system can remove developmental defects associated with SCNT; therefore, the strategy is also successfully applied in GE pig production (Figure 1B). The application of genome editing systems, especially the CRISPR/Cas9 system, allowed for the production of GE pigs that have an impact on both biomedicine and agriculture.

Pig models introduced with the incorporation of genome editing systems

As introduced earlier, the first knockout pigs were designed for xenotransplantation application. The use of genome editing systems now allows for the introduction of edits to multiple alleles and genes, referred to as multiplexing, which allows for the rapid generation of GE pigs carrying multiple genetic modifications to improve pig models for xenotransplantation. For instance, using a genome editing system (ZFNs), GE pigs lacking *GGTA1* and *CMAH* were produced (Lutz *et al.* 2013) to reduce pig carbohydrates that can be recognized by human antibodies after xenotransplantation. Furthermore, CRISPR/Cas9 system allowed for the production of a triple knockout pig carrying modified *GGTA1/CMAH/β4GalNT2* without having to incorporate any breeding steps; serum from the *GGTA1/CMAH/β4GalNT2* knockout pig reduced human antibody binding (Estrada *et al.* 2015). These double or triple knockout pigs would have required years of preparation through the conventional genetic engineering approach combined with breeding steps. Another important accomplishment when using genome editing systems in the field of xenotransplantation was the disruption of a pig virus sequence that could be transmitted to a

patient after xenotransplantation. Specifically, genome wide disruption of porcine endogenous retrovirus (PERVs) sequence was reported using CRISPR/Cas9 system (Yang *et al.* 2015); the CRISPR/Cas9 system inserted modifications to all 62 copies of the PERV sequence in the genome of pig somatic cells. When the somatic cells were used for SCNT, PERV inactivated GE pigs were produced and no health issues were observed from the pigs (Niu *et al.* 2017). A recent publication demonstrated that a heart from a GE pig could support the cardiac function of non-human primates (Langin *et al.* 2018), indicating that clinical application of orthotopic transplantation of organs from GE pigs may occur in the near future. Advancements in genome editing systems will facilitate the design and production of customized GE pigs for xenotransplantation.

The development of genome editing systems assisted in the production of GE pigs presenting immunodeficient phenotype. Animal models lacking an immune system can be used for studying the mechanism of pathogenesis and immune response upon infection, vaccine development, and cell transplantation (Zhang *et al.* 2009; Ito *et al.* 2018). Although the first report of severe combined immunodeficiency (SCID) pigs was made in 2012 (Suzuki *et al.* 2012), the efficiency was poor because the pigs were produced using the conventional genetic engineering approach and no xenografting was possible using the pigs. Using TALENs, we produced GE pigs lacking functional RAG2 and demonstrated that the pigs could support proliferation and differentiation of transplanted induced pluripotent stem (iPS) cells (Lee *et al.* 2014). This was the first report of a successful human stem cell transplantation into a pig model, suggesting that SCID pigs could be used to predict the outcomes of stem cell transplantation or progression of cancer cells.

The NOD-scid gamma (NSG) mouse model has been the leading animal model used to perform xenograft experiments using human stem cells (Shultz *et al.* 2005; Shultz *et al.* 2007) because the mice lack all major lymphocytes (T-B-NK-). Pigs lacking functional *RAG* and *IL2RG* gene would resemble the phenotype of NSG mice; however, pigs deficient in functional *RAG* or *IL2RG* do not reach puberty under the conventional housing due to their immunodeficiency status (Suzuki *et al.* 2012; Lee *et al.* 2014). Unlike SCID rodents, SCID pigs are extremely difficult to manage and distribute because of their limited lifespan under the conventional housing system. Their limited life expectancy is similar to what has been reported in X-linked SCID patients (Suzuki *et al.* 2012; Allenspach and Torgerson 2016), which provides more supporting evidence of how pig models can recapitulate human phenotypes. The limited lifespan of SCID pigs impedes the incorporation of these mutations into a single pig through breeding; breeding heterozygous knockout pigs (*RAG2*^{+/-} and *IL2RG*^{+/-}) as founder pigs would require the production of 256 pigs before obtaining one *RAG*^{-/-}/*IL2RG*^{+/-} pig. Recently, we reported the production of *RAG2/IL2RG* double knockout pigs by injecting the RNA form of CRISPR/Cas9 system into developing embryos (Lei *et al.* 2016). All 17 piglets derived in the study carried mutations on both *RAG2* and *IL2RG* and no wild-type sequence was found from genotyping analysis. As expected, the NSG-like SCID pigs displayed T-B-NK- phenotype, confirmed by FACS analysis. The study demonstrated that the multiplexing ability of CRISPR/Cas9 system was robust enough to disrupt all target genes during embryogenesis, thus eliminating any need of breeding steps during the GE pig production. Because of the high efficiency in genome editing (100%), the pigs were introduced into a human norovirus challenge study prior to genotyping and

demonstrated that lymphocytes are important for clearance of human norovirus (Lei *et al.* 2016). Since the study, we were able to obtain a consistent efficacy (100%) from the direct injection of CRISPR/Cas9 system (Kang *et al.* 2016a; Yugo *et al.* 2018; Carey *et al.* 2019). Currently, NSG-like SCID pigs are used for human stem cell transplantation, and in general, housed in the gnotobiotic housing system during cell transplantation experiments due to their SCID phenotype (Figure 2).

The examples above highlight GE pig models in the biomedicine area; however, the genome editing system also assists in the production of GE pigs relevant to agriculture.

Outbreaks of porcine reproductive and respiratory syndrome virus (PRRSV) cause a considerable loss to the pig industry and vaccines against the virus are often suboptimal (Lunney *et al.* 2016). The application of genome editing systems, specifically CRISPR/Cas9 system, facilitated the targeted disruption of *CD163*, a potential key receptor of PRRSV pathogenesis (Whitworth *et al.* 2014). When *CD163* knockout pigs were used for a PRRSV challenge study, the pigs were completely resistant to the virus (Whitworth *et al.* 2016), indicating that introducing the pigs into the production system will significantly improve profit made by producers. A similar strategy was used to generate pigs resistant to transmissible gastroenteritis virus by disrupting a putative receptor, amino peptidase N (ANPEP) (Whitworth *et al.* 2019). These studies prove that GE pigs produced by utilizing genome editing systems can be resistant to viruses, which greatly impact pig production. In other words, the use of the GE pigs in production will dramatically increase productivity of the pig industry and assist in securing the food supply.

Another example of GE pigs for agricultural improvements is the generation of pigs harboring a modified myostatin (*MSTN*) gene, which plays a negative regulatory role in muscle development. Naturally occurring mutations in *MSTN* have been identified in multiple species such as cattle (Grobet *et al.* 1997), famously known in the Belgian Blue breed, and sheep (Clop *et al.* 2006). Targeted disruption of myostatin in the pig using ZFNs or TALENs resulted in better feed efficiency, growth rate, and higher muscle mass (Cyranoski 2015; Qian *et al.* 2015). Similarly, CRISPR/Cas9 system was used to alter physiology to improve the productivity of pigs. The uncoupling protein 1 (*UCPI*) gene, known to be a key element for brown adipose tissue-mediated thermogenesis, has lost its function through evolution. Replacing the endogenous *UCPI* gene with the functional murine *UCPI* gene via CRISPR/Cas9 mediated HDR pathway, could protect piglets resistant against the cold and assist in the regulation of energy homeostasis (Zheng *et al.* 2017a). These GE pigs exhibited improved ability to maintain body temperature, decreased fat deposition, and increased carcass lean percentage.

Before the era of genome editing systems, less than ten knockout pig models were reported. As demonstrated above, genome editing systems have greatly increased the number of available pig models for biomedicine and agriculture as rapid production of GE pigs is now achievable.

Limitations and forthcoming improvements of genome editing systems in pigs

Although genome editing systems, especially CRISPR/Cas9 system, have significantly transformed the production of GE pigs, there are some shortcomings and limitations of genome editing systems. For instance, genetic modifications, introduced through genome editing systems, commonly rely on the error prone nature of the NHEJ pathway. However, because NHEJ-mediated mutations are random at the nucleotide level, a frame shift, introducing a premature stop codon, is not always generated. In other words, NHEJ-mediated indels in triplets may not lead to the full disruption of target genes. This shortcoming of genome editing systems was observed in our previous study (Lei *et al.* 2016). Specifically, a subpopulation of *RAG2/IL2RG* double knockout pigs, generated with CRISPR/Cas9 system, presented a normal level of NK cells, indicating the *IL2RG* gene was still functional. Genotyping analysis confirmed that all the knockout pigs carried indels in triplets; deletion of 45bp in the coding region was identified in one pig, which still maintained functional *IL2RG*. Interestingly, any type of modifications on *RAG2* led to inactivation of the gene, indicating not all mutations in triplets allow target genes to maintain their function. Similarly, another report suggested that the function of a target gene remained when indels in triplets were present (Park *et al.* 2017a), indicating that targeted knockout events may not be effectively introduced via NHEJ-mediated genome editing events.

Unlike NHEJ-mediated mutations, genome editing through HDR pathway can be error-free. As mentioned earlier, the presence of donor DNA during genome editing induces the HDR pathway, which allows the donor DNA sequence to be inserted into the target locus. Therefore, genome editing can be accurately performed, even at the nucleotide level. Previously, we reported that donor DNA with 800bp homology to a target gene could successfully moderate the HDR pathway during ZFNs-mediated genome editing in somatic cells (Kwon *et al.* 2013); with the use of a drug selectable marker, the efficiency was over 40%. Other reports also suggest that the HDR mechanism is active in both cells (Tan *et al.* 2013) and embryos (Wang *et al.* 2015a). Single strand DNA (ssDNA) has also been successfully used as a donor DNA to introduce targeted modifications through HDR (Wang *et al.* 2015a; Park *et al.* 2017b). Utilization of HDR is possible, but inducing HDR is a challenge because of the nature of the NHEJ and HDR pathways. The frequency of NHEJ is, in general, known to be higher compared to that of HDR in most cell types because the HDR mechanism is only active during the late S and G2 phases when sister chromatids are available to serve as a repair template (Heyer *et al.* 2010). Because of the high frequency of NHEJ, modifications introduced via HDR in homozygous fashion are rarely observed. Improvements in HDR could assist in the utilization of HDR during genome editing.

The use of genome editing systems now allows us to induce targeted modifications during embryogenesis, thus offering a SCNT-free way of producing GE pigs. Previous publications from our group indicate that the frequency of targeted disruption via direct injection of CRISPR/Cas9 system can consistently reach 100% (Lei *et al.* 2016; Yugo *et al.* 2018; Carey *et al.* 2019). High genome editing efficiency from the direct injection of CRISPR/Cas9 system was utilized to induce targeted modifications in SCNT embryos after the cloning

process (Sheets *et al.* 2016). Specifically, somatic cells from a specific genetic background (Ossabaw pigs) were used for SCNT as a donor cell, then SCNT embryos were injected with CRISPR/Cas9 system. Direct injection of CRISPR/Cas9 system has shown to be practical in introducing targeted modifications through NHEJ and reported efficiencies are often 100%. Although encouraging, limitations exist when applying the direct injection of genome editing strategy. Exact genotype of CRISPR/Cas9 injected embryos is difficult to determine prior to embryo transfer to generate GE pigs; therefore, examples of the aforementioned NHEJ-mediated indels in triplets are commonly observed in GE pigs produced through the direct injection approach. In addition, abattoir-derived oocytes are typically used for this approach due to convenience and the number of available embryos needed for embryo transfer in the pig. It is difficult to employ this approach to generate GE pigs with a specific genetic background as it requires collecting *in vivo* embryos from the selected pigs or introduction of genome editing reagents into SCNT embryos. Another shortcoming of the direct injection approach is the occurrence of mosaic genotype; more than two allele sequences for a target gene are detected in these GE pigs. If intended modifications are not introduced in one-cell stage embryos, mosaic genotype could occur, which causes an issue when phenotyping the founder animals and breeding to propagate the intended genotype. Under our GE pig production, the range of GE pigs carrying the mosaic genotype is 10–20% (Lei *et al.* 2016; Yugo *et al.* 2018).

Because CRISPR/Cas9 introduces targeted DSBs at the “PAM” site (NGG), effective CRISPR sequences are typically found in GC rich regions. Therefore, it can be troublesome to design CRISPR/Cas9 systems that recognize sequences in AT rich regions, such as introns. To address this issue, CRISPR/Cas9 systems from other bacteria organisms possessing different “PAM” sequences have been developed (Hou *et al.* 2013; Karvelis *et al.* 2015; Nishimasu *et al.* 2015; Ran *et al.* 2015). Unfortunately, their efficacy is not as high as the original Cas9 protein. The development of Cpf1 (Cas2) suggests that the new system could solve the issue of CRISPR/Cas9 system as the “PAM” sequence of the system is 5’-TTTN-3’. The Cpf1 system originates from another bacterial system and provides higher diversity to target sequences (Zetsche *et al.* 2015). The technology was successfully applied to produce GE pigs (Wu *et al.* 2018). Unfortunately, the efficiency of targeted disruptions using the system is not as consistent as that of the CRISPR/Cas9 system in the pig (through personal communications). Therefore, the approach is not widely used in GE pig production. Another genome editing system that could solve some shortcomings of the current genome editing systems is base pair editor (Komor *et al.* 2018). Because only missense mutations are introduced through the system, instead of indels introduced by the other genome editing systems, concerns from the previous genome editing systems can be significantly alleviated. In fact, a recent study demonstrated that the system could be used to produce GE pigs (Li *et al.* 2018b). Although promising, recent studies suggest that base pair editor could potentially introduce unintended modifications to the genome (Kim *et al.* 2017; Kim *et al.* 2019; Zuo *et al.* 2019). Therefore, safety of the approach needs to be further verified.

The foundation of genome editing systems is their ability to induce DSBs. However, DSBs on unintended locations could cause undesirable mutations, i.e. off-targeting. Previous studies using CRISPR/Cas9 system indicate that off-targeting events can be introduced (Zhang *et al.* 2015; Doench *et al.* 2016; Li *et al.* 2018a). Frequency of off-targeting in GE

pigs have shown to be absent in some publications (Kang *et al.* 2016b; Han *et al.* 2017; Li *et al.* 2017); there was no polymorphic sequences on potential off-target genes selected through a bioinformatic analysis. However, some sgRNAs caused unintended modifications to the genome of GE pigs when modifications were introduced during embryogenesis (Wang *et al.* 2015b; Carey *et al.* 2019). In our previous study, one out of four sgRNAs introduced off-targeting events (Carey *et al.* 2019). Interestingly, previous studies indicate that mismatches on the 5' side of sgRNAs, specifically up to 5–10 base pairs downstream from the 5' start, permit off-targeting events to occur (Cong *et al.* 2013a; Zhang *et al.* 2015; Zheng *et al.* 2017b). However, our results demonstrate that the logic does not consistently translate into all occasions because off-targeting was detected on a gene carrying a mismatch on the 13th base pair downstream from the 5' start sequence (Carey *et al.* 2019). The use of Cas9-nikase, a variant of CRISPR/Cas9 system which induces only a single strand break instead of DSBs, can be used to reduce off-targeting events and enrich HDR events; however, use of the approach is rarely reported in the pig (Ruan *et al.* 2015; Park *et al.* 2017b). Thorough evaluations of designed CRISPR/Cas9 system and verification of the prevalence of off-targeting from the sgRNA is important to obtain optimal genome editing outcomes free of off-targeting events.

Concluding remarks

Recent developments in genome editing technology has expanded the availability of GE pig models for agriculture and biomedicine. Rapid GE pig production is now possible when utilizing genome editing systems because the disadvantages of employing pig models (prolonged gestation period and days to reach puberty) are overcome. Genome editing tools are now often commercially available; therefore, no specialized trainings are necessary to perform genome editing in mammals, including the pig. Genome editing events during embryogenesis offer an exciting opportunity considering the approach can excise complications associated with SCNT during GE pig production. Shortcomings of introducing site-specific modifications during embryogenesis persist (Figure 3); however, continuous advancements in genome editing technology may resolve the obstacles. *In vitro* embryo production in the pig has been significantly improved, which facilitates the use of direct injection approach. GE pigs have been produced solely using oocytes and fertilized embryos derived *in vitro* (Whitworth *et al.* 2014; Lei *et al.* 2016; Yugo *et al.* 2018). A recent report on embryonic stem cells in the pig (Gao *et al.* 2019) suggests that there is now a hope to introduce complicated modifications at the cell level and produce GE pigs through embryo complementation, as demonstrated in the mouse. These technical advancements in genome editing technologies have set forth a foundation for complex modifications that can enhance the production of GE pigs for agriculture and biomedicine.

Acknowledgement

This project was supported in part by the Virginia Agricultural Experiment Station through the NIFA, U.S. Department of Agriculture and NIH grant 1R21OD027062.

References

- Allenspach E, and Torgerson TR (2016) Autoimmunity and Primary Immunodeficiency Disorders. *J Clin Immunol* 36 Suppl 1, 57–67 [PubMed: 27210535]
- Carey K, Ryu J, Uh K, Lengi AJ, Clark-Deener S, Corl BA, and Lee K (2019) Frequency of off-targeting in genome edited pigs produced via direct injection of the CRISPR/Cas9 system into developing embryos. *BMC Biotechnol* 19(1), 25 [PubMed: 31060546]
- Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, Voytas DF, Long CR, Whitelaw CB, and Fahrenkrug SC (2012) Efficient TALEN-mediated gene knockout in livestock. *Proc Natl Acad Sci U S A* 109(43), 17382–7 [PubMed: 23027955]
- Carter DB, Lai L, Park KW, Samuel M, Lattimer JC, Jordan KR, Estes DM, Besch-Williford C, and Prather RS (2002) Phenotyping of transgenic cloned piglets. *Cloning Stem Cells* 4(2), 131–45 [PubMed: 12171705]
- Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, and Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* 39(12), e82 [PubMed: 21493687]
- Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibe B, Bouix J, Caiment F, Elsen JM, Eychenne F, Larzul C, Laville E, Meish F, Milenkovic D, Tobin J, Charlier C, and Georges M (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet* 38(7), 813–8 [PubMed: 16751773]
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, and Zhang F (2013a) Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* (New York, N.Y.) 339(6121), 819–823
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, and Zhang F (2013b) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121), 819–23 [PubMed: 23287718]
- Cyranoski D (2015) Super-muscle pigs created by small genetic tweak. *Nature* 523(7558), 13–4 [PubMed: 26135425]
- Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, and Ayares DL (2002) Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. *Nat Biotechnol* 20(3), 251–5 [PubMed: 11875425]
- Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie WA, Rollo M, de Sousa P, Travers A, Wilmut I, and Clark AJ (2001) Deletion of the alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. *Nat Biotechnol* 19(6), 559–62 [PubMed: 11385461]
- Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R, Virgin HW, Listgarten J, and Root DE (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* 34(2), 184–191 [PubMed: 26780180]
- Estrada JL, Martens G, Li P, Adams A, Newell KA, Ford ML, Butler JR, Sidner R, Tector M, and Tector J (2015) Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GalNT2 genes. *Xenotransplantation* 22(3), 194–202 [PubMed: 25728481]
- Fernandez A, Josa S, and Montoliu L (2017) A history of genome editing in mammals. *Mamm Genome* 28(7–8), 237–246 [PubMed: 28589393]
- Gaj T, Gersbach CA, and Barbas CF 3rd (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 31(7), 397–405 [PubMed: 23664777]
- Gao X, Nowak-Imialek M, Chen X, Chen D, Herrmann D, Ruan D, Chen ACH, Eckersley-Maslin MA, Ahmad S, Lee YL, Kobayashi T, Ryan D, Zhong J, Zhu J, Wu J, Lan G, Petkov S, Yang J, Antunes L, Campos LS, Fu B, Wang S, Yong Y, Wang X, Xue SG, Ge L, Liu Z, Huang Y, Nie T, Li P, Wu D, Pei D, Zhang Y, Lu L, Yang F, Kimber SJ, Reik W, Zou X, Shang Z, Lai L, Surani A, Tam PPL, Ahmed A, Yeung WSB, Teichmann SA, Niemann H, and Liu P (2019) Establishment of porcine and human expanded potential stem cells. *Nat Cell Biol* 21(6), 687–699 [PubMed: 31160711]

- Gohre V, and Robatzek S (2008) Breaking the barriers: microbial effector molecules subvert plant immunity. *Annu Rev Phytopathol* 46, 189–215 [PubMed: 18422429]
- Grillenberger M, Neumann CG, Murphy SP, Bwibo NO, van't Veer P, Hautvast JG, and West CE (2003) Food supplements have a positive impact on weight gain and the addition of animal source foods increases lean body mass of Kenyan schoolchildren. *J Nutr* 133(11 Suppl 2), 3957s–3964s [PubMed: 14672296]
- Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Menissier F, Massabanda J, Fries R, Hanset R, and Georges M (1997) A deletion in the bovine myostatin gene causes the double-muscling phenotype in cattle. *Nat Genet* 17(1), 71–74 [PubMed: 9288100]
- Hai T, Teng F, Guo R, Li W, and Zhou Q (2014) One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell Res*
- Han K, Liang L, Li L, Ouyang Z, Zhao B, Wang Q, Liu Z, Zhao Y, Ren X, Jiang F, Lai C, Wang K, Yan S, Huang L, Guo L, Zeng K, Lai L, and Fan N (2017) Generation of Hoxc13 knockout pigs recapitulates human ectodermal dysplasia-9. *Hum Mol Genet* 26(1), 184–191 [PubMed: 28011715]
- Hauschild J, Petersen B, Santiago Y, Queisser AL, Carnwath JW, Lucas-Hahn A, Zhang L, Meng X, Gregory PD, Schwinzer R, Cost GJ, and Niemann H (2011) Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proc Natl Acad Sci U S A* 108(29), 12013–7 [PubMed: 21730124]
- Heyer W-D, Ehmsen KT, and Liu J (2010) Regulation of Homologous Recombination in Eukaryotes. *Annual Review of Genetics* 44(1), 113–139
- Hou Z, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ, and Thomson JA (2013) Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proc Natl Acad Sci U S A* 110(39), 15644–9 [PubMed: 23940360]
- Ito R, Takahashi T, and Ito M (2018) Humanized mouse models: Application to human diseases. *J Cell Physiol* 233(5), 3723–3728 [PubMed: 28598567]
- Kang JT, Cho B, Ryu J, Ray C, Lee EJ, Yun YJ, Ahn S, Lee J, Ji DY, Jue N, Clark-Deener S, Lee K, and Park KW (2016a) Biallelic modification of IL2RG leads to severe combined immunodeficiency in pigs. *Reprod Biol Endocrinol* 14(1), 74 [PubMed: 27809915]
- Kang JT, Ryu J, Cho B, Lee EJ, Yun YJ, Ahn S, Lee J, Ji DY, Lee K, and Park KW (2016b) Generation of RUNX3 knockout pigs using CRISPR/Cas9-mediated gene targeting. *Reprod Domest Anim* 51(6), 970–978 [PubMed: 27696566]
- Karvelis T, Gasiunas G, Young J, Bigelyte G, Silanskas A, Cigan M, and Siksnys V (2015) Rapid characterization of CRISPR-Cas9 protospacer adjacent motif sequence elements. *Genome Biol* 16, 253 [PubMed: 26585795]
- Kim D, Kim DE, Lee G, Cho SI, and Kim JS (2019) Genome-wide target specificity of CRISPR RNA-guided adenine base editors. *Nat Biotechnol* 37(4), 430–435 [PubMed: 30833658]
- Kim D, Lim K, Kim ST, Yoon SH, Kim K, Ryu SM, and Kim JS (2017) Genome-wide target specificities of CRISPR RNA-guided programmable deaminases. *Nat Biotechnol* 35(5), 475–480 [PubMed: 28398345]
- Koh S, and Piedrahita JA (2014) From “ES-like” cells to induced pluripotent stem cells: a historical perspective in domestic animals. *Theriogenology* 81(1), 103–11 [PubMed: 24274415]
- Komor AC, Badran AH, and Liu DR (2018) Editing the Genome Without Double-Stranded DNA Breaks. *ACS Chem Biol* 13(2), 383–388 [PubMed: 28957631]
- Kwon DN, Lee K, Kang MJ, Choi YJ, Park C, Whyte JJ, Brown AN, Kim JH, Samuel M, Mao J, Park KW, Murphy CN, Prather RS, and Kim JH (2013) Production of biallelic CMP-Neu5Ac hydroxylase knock-out pigs. *Sci Rep* 3, 1981 [PubMed: 23760311]
- Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, and Prather RS (2002) Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* 295(5557), 1089–92 [PubMed: 11778012]
- Langin M, Mayr T, Reichart B, Michel S, Buchholz S, Guethoff S, Dashkevich A, Baehr A, Egerer S, Bauer A, Mihalj M, Panelli A, Issl L, Ying J, Fresch AK, Buttgerit I, Mokolke M, Radan J,

Werner F, Lutzmann I, Steen S, Sjoberg T, Paskevicius A, Qiuming L, Sfriso R, Rieben R, Dahlhoff M, Kessler B, Kemter E, Kurome M, Zakhartchenko V, Klett K, Hinkel R, Kupatt C, Falkenau A, Reu S, Ellgass R, Herzog R, Binder U, Wich G, Skerra A, Ayares D, Kind A, Schonmann U, Kaup FJ, Hagl C, Wolf E, Klymiuk N, Brenner P, and Abicht JM (2018) Consistent success in life-supporting porcine cardiac xenotransplantation. *Nature* 564(7736), 430–433 [PubMed: 30518863]

Lee K, Kwon DN, Ezashi T, Choi YJ, Park C, Ericsson AC, Brown AN, Samuel MS, Park KW, Walters EM, Kim DY, Kim JH, Franklin CL, Murphy CN, Roberts RM, Prather RS, and Kim JH (2014) Engraftment of human iPS cells and allogeneic porcine cells into pigs with inactivated RAG2 and accompanying severe combined immunodeficiency. *Proc Natl Acad Sci U S A* 111(20), 7260–5 [PubMed: 24799706]

Lei S, Ryu J, Wen K, Twitchell E, Bui T, Ramesh A, Weiss M, Li G, Samuel H, Clark-Deener S, Jiang X, Lee K, and Yuan L (2016) Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency. *Sci Rep* 6, 25222 [PubMed: 27118081]

Li C, Zhou S, Li Y, Li G, Ding Y, Li L, Liu J, Qu L, Sonstegard T, Huang X, Jiang Y, Chen Y, Petersen B, and Wang X (2018a) Trio-Based Deep Sequencing Reveals a Low Incidence of Off-Target Mutations in the Offspring of Genetically Edited Goats. *Front Genet* 9, 449 [PubMed: 30356875]

Li Z, Duan X, An X, Feng T, Li P, Li L, Liu J, Wu P, Pan D, Du X, and Wu S (2018b) Efficient RNA-guided base editing for disease modeling in pigs. *Cell Discov* 4, 64 [PubMed: 30588328]

Li Z, Yang HY, Wang Y, Zhang ML, Liu XR, Xiong Q, Zhang LN, Jin Y, Mou LS, Liu Y, Li RF, Rao Y, and Dai YF (2017) Generation of tryptophan hydroxylase 2 gene knockout pigs by CRISPR/Cas9-mediated gene targeting. *J Biomed Res* 31(5), 445–452 [PubMed: 28866660]

Lillico SG, Proudfoot C, Carlson DF, Stverakova D, Neil C, Blain C, King TJ, Ritchie WA, Tan W, Mileham AJ, McLaren DG, Fahrenkrug SC, and Whitelaw CB (2013) Live pigs produced from genome edited zygotes. *Sci Rep* 3, 2847 [PubMed: 24108318]

Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B, and Renukaradhya GJ (2016) Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. *Annu Rev Anim Biosci* 4, 129–54 [PubMed: 26646630]

Lutz AJ, Li P, Estrada JL, Sidner RA, Chihara RK, Downey SM, Burlak C, Wang ZY, Reyes LM, Ivary B, Yin F, Blankenship RL, Paris LL, and Tector AJ (2013) Double knockout pigs deficient in N-glycolylneuraminic acid and galactose alpha-1,3-galactose reduce the humoral barrier to xenotransplantation. *Xenotransplantation* 20(1), 27–35 [PubMed: 23384142]

Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, and Ploegh HL (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol* 33(5), 538–42 [PubMed: 25798939]

McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, and Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* 405(6790), 1066–9 [PubMed: 10890449]

Murphy SP, and Allen LH (2003) Nutritional importance of animal source foods. *J Nutr* 133(11 Suppl 2), 3932s–3935s [PubMed: 14672292]

Nishimasu H, Cong L, Yan WX, Ran FA, Zetsche B, Li Y, Kurabayashi A, Ishitani R, Zhang F, and Nureki O (2015) Crystal Structure of *Staphylococcus aureus* Cas9. *Cell* 162(5), 1113–26 [PubMed: 26317473]

Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, Zhao HY, Wang Y, Kan Y, Shrock E, Lesha E, Wang G, Luo Y, Qing Y, Jiao D, Zhao H, Zhou X, Wang S, Wei H, Guell M, Church GM, and Yang L (2017) Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science* 357(6357), 1303–1307 [PubMed: 28798043]

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, and Perry AC (2000) Pig cloning by microinjection of fetal fibroblast nuclei. *Science* 289(5482), 1188–90 [PubMed: 10947985]

Park KE, Kaucher AV, Powell A, Waqas MS, Sandmaier SE, Oatley MJ, Park CH, Tibary A, Donovan DM, Blomberg LA, Lillico SG, Whitelaw CB, Mileham A, Telugu BP, and Oatley JM (2017a) Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. *Sci Rep* 7, 40176 [PubMed: 28071690]

- Park KE, Powell A, Sandmaier SE, Kim CM, Mileham A, Donovan DM, and Telugu BP (2017b) Targeted gene knock-in by CRISPR/Cas ribonucleoproteins in porcine zygotes. *Sci Rep* 7, 42458 [PubMed: 28195163]
- Park MR, Cho SK, Lee SY, Choi YJ, Park JY, Kwon DN, Son WJ, Paik SS, Kim T, Han YM, and Kim JH (2005) A rare and often unrecognized cerebromeningitis and hemodynamic disorder: a major cause of sudden death in somatic cell cloned piglets. *Proteomics* 5(7), 1928–39 [PubMed: 15832370]
- Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, and Campbell KH (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 407(6800), 86–90 [PubMed: 10993078]
- Prather RS, Lorson M, Ross JW, Whyte JJ, and Walters E (2013) Genetically engineered pig models for human diseases. *Annu Rev Anim Biosci* 1, 203–19 [PubMed: 25387017]
- Qian L, Tang M, Yang J, Wang Q, Cai C, Jiang S, Li H, Jiang K, Gao P, Ma D, Chen Y, An X, Li K, and Cui W (2015) Targeted mutations in myostatin by zinc-finger nucleases result in double-muscling phenotype in Meishan pigs. *Sci Rep* 5, 14435 [PubMed: 26400270]
- Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, and Zhang F (2015) In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature* 520(7546), 186–91 [PubMed: 25830891]
- Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, and Prather RS (2008a) Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J Clin Invest* 118(4), 1571–7 [PubMed: 18324337]
- Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr., Zabner J, Prather RS, and Welsh MJ (2008b) Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science* 321(5897), 1837–41 [PubMed: 18818360]
- Ruan J, Li H, Xu K, Wu T, Wei J, Zhou R, Liu Z, Mu Y, Yang S, Ouyang H, Chen-Tsai RY, and Li K (2015) Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. *Sci Rep* 5, 14253 [PubMed: 26381350]
- Semaniakou A, Croll RP, and Chappe V (2018) Animal Models in the Pathophysiology of Cystic Fibrosis. *Front Pharmacol* 9, 1475 [PubMed: 30662403]
- Sheets TP, Park CH, Park KE, Powell A, Donovan DM, and Telugu BP (2016) Somatic Cell Nuclear Transfer Followed by CRISPR/Cas9 Microinjection Results in Highly Efficient Genome Editing in Cloned Pigs. *Int J Mol Sci* 17(12)
- Shultz LD, Ishikawa F, and Greiner DL (2007) Humanized mice in translational biomedical research. *Nat Rev Immunol* 7(2), 118–30 [PubMed: 17259968]
- Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL, and Handgretinger R (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* 174(10), 6477–89 [PubMed: 15879151]
- Song J, Yang D, Xu J, Zhu T, Chen YE, and Zhang J (2016) RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. *Nat Commun* 7, 10548 [PubMed: 26817820]
- Suzuki S, Iwamoto M, Saito Y, Fuchimoto D, Sembon S, Suzuki M, Mikawa S, Hashimoto M, Aoki Y, Najima Y, Takagi S, Suzuki N, Suzuki E, Kubo M, Mimuro J, Kashiwakura Y, Madoiwa S, Sakata Y, Perry AC, Ishikawa F, and Onishi A (2012) Il2rg gene-targeted severe combined immunodeficiency pigs. *Cell Stem Cell* 10(6), 753–8 [PubMed: 22704516]
- Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB, and Fahrenkrug SC (2013) Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proc Natl Acad Sci U S A* 110(41), 16526–31 [PubMed: 24014591]

- Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, and Holmes MC (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435(7042), 646–51 [PubMed: 15806097]
- Wang X, Zhou J, Cao C, Huang J, Hai T, Wang Y, Zheng Q, Zhang H, Qin G, Miao X, Wang H, Cao S, Zhou Q, and Zhao J (2015a) Efficient CRISPR/Cas9-mediated biallelic gene disruption and site-specific knockin after rapid selection of highly active sgRNAs in pigs. *Sci Rep* 5, 13348 [PubMed: 26293209]
- Wang Y, Du Y, Shen B, Zhou X, Li J, Liu Y, Wang J, Zhou J, Hu B, Kang N, Gao J, Yu L, Huang X, and Wei H (2015b) Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. *Sci Rep* 5, 8256 [PubMed: 25653176]
- Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, Samuel MS, Mao J, O’Gorman C, Walters EM, Murphy CN, Driver JP, Mileham A, McLaren D, Wells KD, and Prather RS (2014) Use of the CRISPR/Cas9 System to Produce Genetically Engineered Pigs from In Vitro-Derived Oocytes and Embryos. *Biol Reprod* 91(3), 78 [PubMed: 25100712]
- Whitworth KM, Rowland RR, Ewen CL, Tribble BR, Kerrigan MA, Cino-Ozuna AG, Samuel MS, Lightner JE, McLaren DG, Mileham AJ, Wells KD, and Prather RS (2016) Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. *Nat Biotechnol* 34(1), 20–2 [PubMed: 26641533]
- Whitworth KM, Rowland RRR, Petrovan V, Sheahan M, Cino-Ozuna AG, Fang Y, Hesse R, Mileham A, Samuel MS, Wells KD, and Prather RS (2019) Resistance to coronavirus infection in amino peptidase N-deficient pigs. *Transgenic Res* 28(1), 21–32 [PubMed: 30315482]
- Whyte JJ, Zhao J, Wells KD, Samuel MS, Whitworth KM, Walters EM, Laughlin MH, and Prather RS (2011) Gene targeting with zinc finger nucleases to produce cloned eGFP knockout pigs. *Mol Reprod Dev* 78(1), 2 [PubMed: 21268178]
- Wilmot I, Schnieke AE, McWhir J, Kind AJ, and Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385(6619), 810–3 [PubMed: 9039911]
- Wright DA, Thibodeau-Beganny S, Sander JD, Winfrey RJ, Hirsh AS, Eichinger M, Fu F, Porteus MH, Dobbs D, Voytas DF, and Joung JK (2006) Standardized reagents and protocols for engineering zinc finger nucleases by modular assembly. *Nat Protoc* 1(3), 1637–52 [PubMed: 17406455]
- Wu H, Liu Q, Shi H, Xie J, Zhang Q, Ouyang Z, Li N, Yang Y, Liu Z, Zhao Y, Lai C, Ruan D, Peng J, Ge W, Chen F, Fan N, Jin Q, Liang Y, Lan T, Yang X, Wang X, Lei Z, Doevendans PA, Sluijter JPG, Wang K, Li X, and Lai L (2018) Engineering CRISPR/Cpf1 with tRNA promotes genome editing capability in mammalian systems. *Cell Mol Life Sci* 75(19), 3593–3607 [PubMed: 29637228]
- Yang D, Yang H, Li W, Zhao B, Ouyang Z, Liu Z, Zhao Y, Fan N, Song J, Tian J, Li F, Zhang J, Chang L, Pei D, Chen YE, and Lai L (2011) Generation of PPARgamma mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. *Cell Res* 21(6), 979–82 [PubMed: 21502977]
- Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, Cortazio R, Wilkinson RA, Fishman JA, and Church G (2015) Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science*
- Yugo DM, Heffron CL, Ryu J, Uh K, Subramaniam S, Matzinger SR, Overend C, Cao D, Kenney SP, Sooryanarain H, Cecere T, LeRoith T, Yuan L, Jue N, Clark-Deener S, Lee K, and Meng XJ (2018) Infection dynamics of hepatitis E virus in wild-type and immunoglobulin heavy chain knockout JH (–/–) gnotobiotic piglets. *J Virol*
- Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, and Zhang F (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163(3), 759–71 [PubMed: 26422227]
- Zhang B, Duan Z, and Zhao Y (2009) Mouse models with human immunity and their application in biomedical research. *J Cell Mol Med* 13(6), 1043–58 [PubMed: 18419795]
- Zhang XH, Tee LY, Wang XG, Huang QS, and Yang SH (2015) Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol Ther Nucleic Acids* 4, e264 [PubMed: 26575098]
- Zhao J, Whyte J, and Prather RS (2010) Effect of epigenetic regulation during swine embryogenesis and on cloning by nuclear transfer. *Cell Tissue Res* 341(1), 13–21 [PubMed: 20563602]

- Zheng Q, Lin J, Huang J, Zhang H, Zhang R, Zhang X, Cao C, Hambly C, Qin G, and Yao J (2017a) Reconstitution of UCP1 using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and improves thermogenic capacity. *Proceedings of the National Academy of Sciences* 114(45), E9474–E9482
- Zheng T, Hou Y, Zhang P, Zhang Z, Xu Y, Zhang L, Niu L, Yang Y, Liang D, Yi F, Peng W, Feng W, Yang Y, Chen J, Zhu YY, Zhang LH, and Du Q (2017b) Profiling single-guide RNA specificity reveals a mismatch sensitive core sequence. *Sci Rep* 7, 40638 [PubMed: 28098181]
- Zijlstra M, Li E, Sajjadi F, Subramani S, and Jaenisch R (1989) Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature* 342(6248), 435–8 [PubMed: 2685607]
- Zuo E, Sun Y, Wei W, Yuan T, Ying W, Sun H, Yuan L, Steinmetz LM, Li Y, and Yang H (2019) Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science* 364(6437), 289–292 [PubMed: 30819928]

Figure 1.

Production of GE pigs utilizing genome editing systems. (A) Somatic cell nuclear transfer of genetically modified cells to produce GE pigs. First, primary cultured cell lines are collected from the desired animal. After the cells are cultured, transfection of the genome editing system is conducted. Cells containing the desired genotype are selected for SCNT. An oocyte is first enucleated, and then nuclear transfer is completed with the GE cells. Next, the reconstructed SCNT embryos are artificially activated. Lastly, blastocysts are collected and used for an embryo transfer to generate GE piglets. (B) Direct injection of genome editing system to produce GE pigs. First, *in vitro* fertilization (IVF) is conducted. After fertilization is complete, CRISPR/Cas9 system is injected into the presumable zygotes. Lastly, blastocysts are collected and used for an embryo transfer to generate GE piglets.

Figure 2.
Image of NSG-like SCID pigs in gnotobiotic housing. *RAG2/IL2RG* double knockout pigs are generally housed under the gnotobiotic system due to their immunodeficiency phenotype.

Figure 3.

Shortcomings of gene editing by direct embryo injection. Direct injection often produces mosaicism when the editing event occurs in zygotes after DNA replication. Knock-in efficiency remains low even after the advent of the CRISPR/Cas9 system. Genotypes of piglets are not predictable and unintended off-targeting can occur in the piglet genome.