Application of gene-editing in livestock – updates and prospects

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Introduction

Genetic improvements are essential for sustainable production in livestock. Traditionally, genetic selection has been based on phenotypes related to efficient production or economically valuable traits; livestock has been bred for these traits to enhance profit. For instance, cattle have been selectively bred to produce more meat or more milk, and pigs have been crossed to improve productivity, reproductive traits, and resistance to diseases. These breeding programs have utilized the natural genetic variation present in an animal population. On the other hand, genetic engineering technology can precisely modify a genome to improve genetic value of livestock.[1] The approach can selectively edit genetic traits without segregating other economically valuable traits seen in breeding-based genetic improvements; therefore, the approach can dramatically reduce the genetic value of livestock with a minimum number of breedings. Success of genetic engineering in livestock has been historically low due to technical challenges. However, the recent development of gene-editing technology allows these modifications to be highly efficient.[2]

Historical approach of genetic engineering

The first report of genetic engineering in mammals was in 1976 by introducing exogenous Moloney leukemia virus (M-MuLV) into a mouse genome and transmitting it to its offspring.[3] Engineering of these viral vector systems allowed us to deliver exogenous DNA into livestock genomes as a form of transgenesis including pigs[4] and cattle.[5] Although successful, application of the approach has been limited because of difficulties in constructing effective viral vectors and integration of viral genes into host genomes. Pronuclear injection, on the other hand, is technically more convenient, and unlike virus-mediated transgenesis, there is no limit to the size of exogenous DNA that can be integrated into the genome. In 1980, Gordon et al.[6] successfully introduced exogenous DNA into the mouse genome by pronuclear microinjection. The pronuclear injection approach was also applied to develop numerous transgenic pig lines[7-9] and cattle.[10] However, this technique has limitations because the exogenous DNA integrates to a random location of genome[11] therefore, no targeted disruption is possible and the copy number of integrated exogenous DNA cannot be controlled. In mice, use of embryonic stem (ES) cell based genetic engineering allow us to overcome these issues. Homologous recombination-based gene targeting approach permits us to modify genome in ES cells, then ES cells can be used to generate a chimeric mouse carrying the genetic modifications.[12] Breeding the chimeric mice establishes genetically engineered mouse models carrying desired genetic modifications. This technique has been the main approach to generate knockout mouse models, which are valuable animal models in biological sciences.[13-15] However, the lack of true ES cells in livestock has prohibited this approach in producing genetically engineered livestock. Alternatively, application of somatic cell nuclear transfer (SCNT), i.e. cloning, allows us to bypass the need of ES cells in production of genetically engineered livestock. Early experiments used embryonic cells from cleavage stage embryos or blastocysts as donor nuclei: cattle[16,17] and pigs.[18] Later, it was demonstrated that somatic cells, such as fetal derived fibroblasts[19] or adult derived cells,[20] could have full term development through SCNT when used as donor cells. This was a groundbreaking report in cellular reprogramming, but also indicated that genetic engineering of donor cells followed by SCNT could be an ideal system to produce genetically engineered livestock. The strategy was quickly adopted and resulted in the production of genetically engineered livestock[21-24] for various applications. However, efficiency of gene targeting through conventional HR is extremely poor thus only limited reports of genetically engineered livestock have been made. Recent developments in gene-editing technology allow us to introduce site-specific genetic modifications in somatic cells at high efficiency. Specifically, the use of engineered endonucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) system can introduce homozygous or biallelic modifications in somatic cells in various livestock species.[25-27] In addition, the systems are powerful enough to introduce genetic modifications during embryogenesis, thus bypassing the need of SCNT in generating genetically engineered livestock.[28] These gene-editing systems furnish unprecedented opportunities to enhance the genetic value of livestock species.

Mechanistic action of gene-editing system: engineered endonucleases

The gene-editing systems are labeled as engineered endonucleases because they can bind to a specific region of genome and induce a double-strand break (DSB) in the chromosome, allowing introduction of targeted modification into the locus (reviewed in[2,29]). Endogenous DNA repair pathways, non-homologous end joining (NHEJ) or homologous recombination (HR), are vigorously activated by response to the DSB. The repair through NHEJ does not rely on template DNA, thus it may result in the formation of small insertion or deletion (indels). These small indels often cause frameshifts on the coding region of the target gene, resulting in a targeted disruption. The repair through HR is accurate at the nucleotide level, as it utilizes the DNA template, in nature sister chromatid, to correct damages caused by the DSB. Introducing donor DNA with engineered endonucleases can induce targeted insertion of specific DNA sequences or exogenous genes into the DSB locus through homology-directed repair (HDR) pathway. The modifications through HDR is specific at the nucleotide level.

Genetically engineered livestock by ZFNs

Zinc-finger nucleases (ZFNs) were the first developed engineered endonucleases, consisting of zinc-finger proteins, which recognize specific DNA sequences, and *FokI* enzyme, a non-specific endonuclease. Each zinc-finger protein motif, originally identified from *Xenopus* oocytes,[30] can recognize and bind to three nucleotides. The zinc-finger proteins can be engineered to recognize any three nucleotides in the genome, and these proteins can be connected to recognize a longer DNA sequence. These zinc-finger proteins are fused with a restriction endonuclease *FokI* to induce a site-specific DSB in the genome. The use of ZFNs dramatically improved the efficiency of gene targeting. The first publication using human cells demonstrated that the system can induce targeted modifications at 1,000-fold higher, compared to the conventional gene targeting approach.[31]

The ZFNs were first applied in livestock to generate biomedical pig models.[32,33] For agricultural application, ZFNs were first used to generate genetically engineered cattle with a different milk composition. Genetically engineered cattle carrying modified beta-lactoglobulin (*BLG*) gene, known as one of the major milk allergens, was produced by utilizing ZFNs.[34] In this study, first ZFNs were used to alter *BLG* gene in bovine cell lines through NHEJ repair pathway. Then, two cell lines containing biallelic mutation in the *BLG* locus were used as nucleus donors for SCNT. A total of eight live animals were born through the SCNT, but only one cow survived over a month. The report did not comment on the quality of milk from the cow, probably due to extended time required to breed and obtain milk from the cow.

Another study applied zinc-finger nickases (ZFNickases) to produce dairy cattle which can be resistant to *Staphylococcus aureus* (*S. aureus*)-induced mastitis. Use of ZFNikase instead of ZFNs allowed them to utilize HDR pathway instead of NHEJ because ZFNikases trigger site-specific single-strand break (SSB) without causing DSB. In this study, through the ZFNickases-mediated HDR, the lysostaphin gene was inserted into the endogenous β-casein locus (CSN2) in bovine fetal fibroblasts.[35] Lysostaphin, which is naturally produced in *Staphylococcus simulans* (*S. simulans*), is known to be effective in treating *Staphylococcus aureus* (*S. aureus*)-induced mastitis, the most consequential disease in dairy cattle. The lysostaphin knock-in fibroblast cells were used for SCNT and the gene-targeted calves were born. Analysis of milk from the cows indicated that the lysostaphin was present in the milk and *in vitro* assay demonstrated the milk's ability to kill *S. aureus*.

The ZFNs were also applied to generate genetic engineered pigs for agricultural improvements. Myostatin (*MSTN*) gene plays a negative regulatory role in muscle development and naturally occurring mutation in *MSTN* has been identified in multiple species such as sheep[36] and cattle,[37] famously known in the Belgian Blue breed of cattle. These animals present better feed efficiency, growth rate, and higher muscle mass. This naturally occurring mutation was introduced into pigs by disrupting the *MSTN* in somatic cells using ZFNs followed by SCNT.[38] The *MSTN* null pigs displayed higher growth rate and increased muscle mass compared to wild-type control, indicating these line of pigs can increase productivity, if incorporated into production system.

As shown above, the use of ZFNs significantly increased the efficiency of generating genetically engineered livestock and provided new opportunities to design animals with higher genetic values. However, assembly of ZFNs can be technically challenging, and effectiveness of ZFNs was limited to GC-rich regions of the genome.[39]

Genetically engineered livestock by TALENs

Transcription activator-like effector nucleases (TALENs) are designed by fusing a TAL effector DNA binding domain to *FokI* enzyme, which introduces a DSB. The TAL effector was originally isolated from plant pathogenic bacteria, *Xanthomonas*.[40,41] The binding specificity of TALEN is determined by a central domain of tandem, 33–35 amino acid repeats, and one TALEN motif can bind to a single base pair.[42,43] In contrast to the ZFNs, TALENs have a greater design flexibility with the ability of single base recognition of TAL effector.

The first application of TALENs on genetically engineered livestock production was on the pig and cattle. Use of TALENs effectively introduced targeted modifications in somatic cells, as well as in embryos.27 The paper produced a pig model for human metabolic syndromes as a proof of concept. The same group reported the possibility of introducing the hornless phenotype, exists in breeds such as Angus,[44] into dairy cattle by introgression of a candidate *POLLED* allele into the genome of somatic cells isolated from dairy cattle. In a follow-up study, these cell lines were used for SCNT to produce five hornless calves, which, as expected, presented the hornless phenotype.[45] Physical dehorning of cattle is typically carried out in the dairy industry to protect animals and producers from accidental injury. However, the process is costly and painful for the animals. This TALEN-mediated introgression of a specific allele into a breed demonstrates that gene-editing technology can be used to improve economic value to producers and contribute to animal welfare as well.

The TALENs were also used to generate pigs carrying targeted modifications without having to apply SCNT.[28] Genetically engineered pigs potentially resistant to African swine fever virus were produced by direct injection of TALEN mRNAs targeting RELA gene. In this study, ZFNs were also able to introduce a targeted modification in the RELA region, but only one allele was modified through ZFNs, whereas TALENs were able to disrupt both alleles during embryogenesis. This was the first report of producing genetically engineered livestock with site specific modifications without having to apply SCNT. Similar approaches were used to generate *MSTN*-mutant cattle and sheep.[46] In this study, oocytes were fertilized *in vitro*, and then TALEN mRNA targeting *MSTN* was injected into the zygotes. The cultured blastocysts were transferred to surrogates and live *MSTN*-mutant animals were born. Not all animals showed increased muscle phenotype because the modifications introduced by TALENs did not cause a frame shift of coding sequences in many of the gene-edited animals. These studies demonstrated that zygote injection of TALEN mRNA can be an alternative to the laborious and technically difficult SCNT technique for genetically engineering livestock. Use of TALEN also allowed for generation of *MSTN*-mutant goats by a targeted disruption of *MSTN* in somatic cells followed by SCNT.[47]

Similar to the application of ZFNickase, transcription activator-like effector nickase (TALE nickase) was also applied to introduce a specific gene into a specific location of the bovine genome. In 2015, TALE nickase system was used to produce cattle resistant to tuberculosis, a chronic infectious disease, by introducing the *SP110* gene under the control of a macrophage specific promoter, macrophage scavenger receptor 1 (*MSR1*) promoter.[48] Expression of the *SP110* gene, obtained from tuberculosis-resistant mouse strain, allowed the genetically engineered cows to manage the growth of *Mycobacterium bovis* (*M. bovis*) in their system and efficiently be resistant to the low dose of *M. bovis*.

Application of TALENs expanded the production of gene-edited animals and demonstrated that introducing specific mutations during embryogenesis was possible. However, although simper than ZFN assembly, constructing effective TALEN sets has been the main limitation on the wide use of the technology.

Genetically engineered livestock by CRISPR/Cas9 system

Clustered regularly interspaced palindromic repeats (CRISPR) is an adaptive immune mechanism presented in bacteria cells against exogenous DNA from virus or plasmid.[49,50] The guide sequences within the CRISPR system, which corresponds to the viral DNA, can be easily replaced by a target of interest. This engineered single guide RNA (sgRNA) combined with tracr-RNA can bind to a target DNA sequence, and recruit Cas9 endonuclease protein to induce DSB on the target site.[51,52] Both ZFN and TALEN systems require a series of assembly to generate a construct; however, design of 20 bp gRNA for the target sequence is only needed to establish a CRISPR/Cas9 system for gene-editing. Due to the simplicity, CRISPR/Cas9 system has become the leading gene-editing system to develop gene-edited livestock.

The first application of CRISPR/Cas9 system in livestock was to produce a pig model for von Willebrand disease in humans.[53] For agricultural purposes, the first report was to produce pigs, resistant to porcine reproductive and respiratory syndrome (PRRS) virus.[54,55] Although PRRS virus causes substantial economic losses in North America, Europe, and Asia, there is no effective vaccine against the virus. Piglets lacking functional *SIGLEC1* (CD169), a candidate primary viral receptor for PRRS virus infection, were generated by the conventional approach without the use of gene-editing system.56 Establishing the pigs required two breeding cycles because only one *SIGLEC1* allele could be modified through the conventional gene targeting approach. Unfortunately, these pigs were not resistant to the PRRS virus. When CRISPR/Cas9 was used to disrupt CD163, another putative receptor for PRRS virus, CD163 null pigs were established without incorporating any breeding.[55] Some of these pigs were generated by injecting CRISPR/Cas9 system into fertilized embryos. Challenging these pigs with PRRS virus demonstrated that, indeed, the CD163 null piglets were resistant to PRRS,[54] indicating that incorporation of these pigs into production can dramatically reduce economic losses associated with the PRRS virus. The first CD163 knockout paper also proved that direct injection of CRISPR/Cas9 system could be highly effective in generating knockout pigs; efficiencies were 100% in the study. Using a similar approach, we generated *RAG2/IL2RG* double knockout pigs without breeding or SCNT.[57] All seventeen pigs produced through the approaches carried modification on both *RAG2* and *IL2RG*. They also presented expected phenotype, primary immunodeficiency. To the best of our knowledge, this was the first report of pigs lacking all major lymphocytes: B, T, and NK cells. The direct injection of CRISPR/Cas9 system indicates that SCNT may not always be necessary to generate genetically engineered livestock carrying multiple gene-edits, thus avoiding developmental abnormalities associated with SCNT. The study utilized NHEJ to introduce site-specific modifications; however, we also showed that exogenous DNA fragment could be integrated into a specific locus through HDR during embryogenesis in pigs.[58]

CRISPR/Cas9 system has also been applied to generate genetically engineered cattle to prevent diseases. The bovine *PRNP* gene, responsible for mad cow disease, was successfully modified using CRISPR/Cas9 system,[59] similar to a previous study in which *PRNP* gene was disrupted by using TALEN system.[60] Although *PRNP*-mutant cattle have not been generated, this study suggested the possibility that cattle with a resistance to mad cow disease can be created through CRISPR/Cas9 system. More importantly, the efficiency of targeted disruptions in these studies was much more efficient compared to publications related to the original production of *PRNP* knockout cattle.24, 61 In addition to disrupting endogenous genes to improve disease resistance, CRISPR/Cas9 system targeting an African swine fever virus gene, CP204L, was incorporated into the pig genome to effectively prevent the viral infection. Porcine cell lines carrying the CRISPR/Cas9 system was resistant to the African swine fever virus,[62] indicating the approach can be used to provide an enhanced immunity to pigs.

Above examples focused on generating gene-edited livestock for disease prevention. The CRISPR/Cas9 system was also used to improve genetic value of livestock by introducing or replacing specific DNA sequences. The Japanese Black cattle breed is known for producing high quality meat. However, the breed is susceptible to isoleucyl-tRNA synthetase (IARS) syndrome, a recessive disease in Japanese Black cattle caused by a single nucleotide substitution.[63] The recessive genetic trait was successfully replaced/repaired by utilizing CRISPR/Cas9 system.[64] The study demonstrates that harmful genetic traits can be precisely replaced, while keeping valuable genetic information unmodified. Similarly, gene-edited pigs that have improved thermogenesis capability were produced by replacing endogenous uncoupling protein 1 (*UCP1*) gene, known to be a key element for brown adipose tissue-mediated thermogenesis and regulating energy homeostasis, by utilizing CRISPR/Cas9 system.[65] Pigs have lost functional *UCP1* during evolution, thus showing susceptibility to cold, which leads to high neonatal mortality and decreased production efficiency. In this study, first adiponectin promoter driven mouse *UCP1* gene was inserted into the endogenous porcine *UCP1* locus through CRISPR/Cas9-mediated gene insertion in porcine fetal fibroblast cells. Then, murine *UCP1* overexpressing pigs were produced by SCNT using the cells. The gene-edited pigs showed improved ability to maintain body temperature, decreased fat deposition, and increased carcass lean percentage. This report indicates that gene-editing technology can improve genetic value of pigs, production efficiency, and animal welfare.

Conclusion

The use of gene-editing systems has provided new opportunities to improve the genetic value of livestock. Due to available funding opportunities, most gene-edited livestock is generated for biomedical purposes.[26,57,66-69] However, examples introduced here certainly illustrate the potential impact of gene-edited livestock in the agriculture industry and an upcoming line of animals that may be introduced into the food production system. Many of the gene-edited animals only rely on endogenous NHEJ pathway to introduce targeted modification, thus no foot-print remained in their genome. Because there is no integration of exogenous DNA sequence, the word 'transgenic' does not apply to these animals. The FDA approval of transgenic salmon also suggests that more genetically engineered or gene-edited animal products may be introduced into the food production system.[70] The development of gene-editing technology will certainly lower the existing barriers in producing genetically engineered livestock, thus widening the use of genetically engineered livestock in agriculture.

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