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Genome editing with targetable nucleases makes large animals catch up with mice

Jian Hou

Genetic modifications in mammalian species provide valuable tools for basic research as well as biomedical and agricultural applications. Over the last 35 years, great efforts have been made by academics as well as industry to develop and improve technologies for modifying animal genomes. Transgenesis by microinjection of foreign DNA into pronuclei of fertilized zygotes, so-called pronuclear injection, was first established in mice in early 1980s [1]. This technique provided the first platform for genetic modification in mammals and enabled generation of transgenic farm animals in the early days [2]. Due to its very low efficiency, pronuclear injection has been largely discarded in livestock transgenics since the emergence of somatic cell nuclear transfer technology in late 1990s. However, this method still remains the main choice in production of transgenic rodents.

Different from transgenesis that is generally characterized by random DNA insertion into genomes, gene targeting is the way of precisely modifying the genome at defined loci, such as knocking out a specific endogenous gene or knocking in an exogenous sequence to a specific site. Conventional gene targeting technology depends on homologous recombination (HR) between the introduced foreign DNA sequences and their homologous targets in cells. However, due to the extremely low HR efficiency in mammalian cells, it is not practicable to directly perform gene targeting in an embryo to create genetically modified animals. Instead, HR in murine embryonic stem (ES) cells has provided an effective approach for routinely generating gene-targeted mice since its initial success in late 1980s [3]. However, ES cell-based gene targeting has never been achieved in most other species, including farm animals, due to the lack of established ES cells for these species.

Jian Hou
Affiliations: State Key Laboratory of Agrobiotechnology and College of Biological Science, China Agricultural University, Beijing, China.
Corresponding Author: Jian Hou, No.2 Yuan-Ming-Yuan-WestRoad, HaidianDistrict,Beijing, China,100193; E-mail: houjian@cau.edu.cn

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Gene targeting in farm animals became possible only with the birth of the sheep Dolly, the first mammal cloned by somatic cells nuclear transfer (SCNT) in 1996 [4]. With the help of SCNT, genetic modification (transgenesis or gene targeting) can be performed in cultured somatic cells, and then the modified cells can be used as donors to produce entire animals by nuclear transfer. Therefore, the technology of SCNT provided an alternative way to produce either transgenic or gene-targeted animals. In particular, in the absence of ES cells, SCNT is the only available approach for achieving gene targeting in livestock species [5–8]. However, due the extremely low HR efficiency in somatic cells as well as the technical difficulty and inefficiency of SCNT, this technology had not been adopted by most researchers and only few gene-targeted livestock have been generated in this way during the last 15 years.

The situation of genetic modification in both murine and livestock species has been dramatically changed with the recent advent of targetable nucleases, including zinc-finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and clustered regularly interspaced palindromic repeats (CRISPR)-associated protein (Cas9). The nuclease-based technology is variously termed "gene editing", "genome editing" or "genome engineering" to distinguish it from HR-mediated conventional gene targeting. The core effect of these nucleases is that they can cleave chromosomes and create double-strand breaks (DSBs) at the genomic sites to be modified. In cells, nuclease-induced DSBs can be repaired through error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. Repair by NHEJ frequently induces targeted gene disruption (gene knock-out), while HDR-mediated repair can introduce a sequence into the genome (gene knock-in) that leads to precise genomic modifications, such as point mutations, gene correction and gene insertion.

In accordance with their functions of creating site-specific DSBs, all three types of nucleases consist of two

essential associated components: one responsible for recognizing and binding DNA at defined genomic loci, and the other having an endonuclease activity of cutting DNA at the sites anchored by the former. However, each nuclease has its own unique characteristics in DNA recognition, structure and construction. ZFNs and TALENs are fusion proteins that consist of two domains: a customizable DNA binding domain and the common Fok I nuclease domain. In ZFNs, the DNA-binding domain is zinc finger proteins (ZFP) based on eukaryotic transcription factors, while in TALENs, the domain is transcription activator–like effector (TALE) protein derived from plant *Xanthomonas* bacteria. The specificity and versatility of DNA recognition are conferred by programmability of ZFPs or TALEs. Both ZFNs and TALENs use the same Fok I nuclease domain to cleave the DNA targets. The recently developed CRISPR/Cas9 is a RNA-guided nuclease system. In this system, a short RNA, named guide RNA (gRNA), mediates DNA recognition through Watson-Crick base pairing and directs Cas9 nuclease to cleave the DNA targets. Due to the great simplicity of designing and making a RNA for a new target site, CRISPR/Cas9 has represented a facile and efficient alternative to ZFNs and TALENs for genome editing in various organisms.

In the last five years, all three types of nucleases have been shown to be powerful tools for targeted genomic modification in mammals. However, even with the nucleases, the technology of genome editing has gone through different methodological evolution between model animals and large animals. Starting with ZFNs, it has been firmly established that zygote injection is a feasible strategy for efficient generation of knockout murine animals. The first proof-of-evidence study was reported on rats in 2009 [9]. By direct injection of ZFN mRNAs into pronuclei or cytoplasm of zygotes, an average of 28% (5–75%) of resultant pups carried the targeted mutations. This efficiency is high enough and acceptable for generation of knockout murine models by direct embryo targeting. Using the same technique, subsequent studies on TALENs and CRISPR/Cas9 achieved even higher knockout efficiency than previously on ZFNs [10–13]. The most striking efficiency was with CRISPR/Cas9; more than 90% of newborns carried bi-allelic mutations in the targeted gene and considerable proportions of the animals could be bi-allelic mutants in two or more targeted genes if multiple corresponding gRNAs were injected simultaneously [10]. In addition to gene knockout, gene knockin has also been achieved in both rats and mice by co-injection of nucleases and a DNA repair template into zygotes, albeit at lower efficiencies than NHEJ-mediated mutagenesis [14–17]. With zygote injection, the timeframe of producing targeted animals is dramatically shortened by bypassing ES cells. Thus, zygote injection has been a commonly used method in nuclease-mediated genome editing in rats and mice.

After the success in model animals, each of these nucleases was soon adapted to livestock. However, different from their use in rats and mice, the great utility of nucleases in livestock is that they can induce high efficiency of gene targeting in cultured somatic cells. In this case, SCNT remains the major method of choice for genome editing in livestock. Initial studies with ZFNs showed that commercially derived-custom ZFNs could efficiently induce gene knockout in cultured fibroblast cells of livestock, with the efficiency of around 10% (ranging from 1 to 20% in different studies) [18–20]. Such efficiency is several orders of magnitude higher than HR-mediated conventional gene targeting and allowed efficient recovery of targeted cells, especially bi-allelic mutant cells, thus facilitating production of gene knockout livestock by SCNT [18–20]. Subsequent studies with TALEN- or CRISPR/Cas9 achieved similar or higher targeting efficiency than that reported using ZFNs, and knockout livestock, including pigs and goats, have been generated by SCNT from TALENs or CRISPR/Cas9-targeted cells [21–28]. Moreover, gene knockin has also been achieved in livestock somatic cells with the help of nucleases, and consequently livestock carrying targeted transgenes or subtle mutations have been born [29–33].

In addition to SCNT, zygote injection has also been successfully used to produce genome-edited livestock. The first report described the generation of knockout pigs by zygote injection of ZFN or TALEN mRNAs; 21% and 11% of born piglets harbored the desired mutations from TALEN and ZFN injection, respectively [34]. More recently, by zygote injection of TALEN mRNAs, the same group of authors reported 75% and 11% of targeting efficiency in cattle and sheep, respectively [35]. Recently, several groups have performed gene targeting in livestock by zygote injection of Cas9 mRNAs and gRNAs. The most impressive is the efficiency reported in pigs. The first report showed that 66.8% of newborn piglets carried the desired mutations of the targeted gene, and more than half of them were bi-allelic mutants [36]. More strikingly, the other two reports described nearly 100% of targeting efficiency: almost all piglets born from zygote injection carried bi-allelic mutations of the targeted gene [25, 37]. Knockout sheep have also been produced by zygote injection of CRISPR/Cas9 components [38], albeit with lower efficiency than that reported in pigs. These studies suggest that zygote injection in large animals could be as effective as in murine animals. Thus, this method provides an appealing and straightforward alternative to SCNT to produce targeted livestock.

Zygote injection has several advantages over SCNT. First, it is relatively simpler in technique than SCNT. Second, zygote injection completely avoids the laborious work that is needed for cell-mediated genetic manipulation, such as cell preparation, transfection and screening. Third, zygote injection-derived embryos or animals have less developmental problems than those generated from SCNT. Therefore, zygote injection would offer a convenient, efficient and cost-effective means of producing targeted large animals.

Zygote injection method also makes it possible for genome editing in the species that traditional cell-based gene targeting has not reached. In this regard, nucleases have been successfully used to generate genetically modified rabbits [39–41] and monkeys [42, 43]. Most strikingly, genome editing in human embryos has been recently tried with CIRISPR/Cas9, which has triggered worldwide ethical debates [44]. Therefore, zygote injection has provided a promising, common platform for genome editing in various mammalian species. This is reminiscent of zygote injection-based transgenic technology that was commonly used more than three decades ago. However, even compared to injection of DNA into zygotic pronucleus, which is used for transgenics, cytoplasmic injection of zygotes with nuclease mRNAs is both technically simple and efficient. This is particularly important for large animals, such as cattle and pigs, as pronuclei of these species are too opaque to be visible under the microscope. But now, it is not problematic for cytoplasmic injection of nucleases.

Despite of these advancements, there is still room for improvement in these systems. Methods are needed to enhance the efficiency of HDR over NHEJ, as HDR-mediated precise genome editing is less efficient than NHEJ-induced mutagenesis. Besides, off-target effect is one of main concerns with nuclease-induced gene editing. Efforts are being made to further improve the efficiency, specificity and safety of these newly developed technologies. The technology will continue to extend our ability to explore and alter the genomes of valuable animals for developing biomedical and agricultural applications.

Keywords: Genome editing, Genetic modifications, Mammalian species, Homologous recombination, Livestock species, *Xanthomonas* bacteria

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Conflict of Interest

Authors declare no conflict of interest.

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