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Germline modification of domestic animals

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Abstract

Genetically-modified domestic animal models are of increasing significance in biomedical research and agriculture. As authentic ES cells derived from domestic animals are not yet available, the prevailing approaches for engineering genetic modifications in those animals are pronuclear microinjection and somatic cell nuclear transfer (SCNT, also known as cloning). Both pronuclear microinjection and SCNT are inefficient, costly, and time-consuming. In animals produced by pronuclear microinjection, the exogenous transgene is usually inserted randomly into the genome, which results in highly variable expression patterns and levels in different founders. Therefore, significant efforts are required to generate and screen multiple founders to obtain animals with optimal transgene expression. For SCNT, specific genetic modifications (both gain-of-function and loss-of-function) can be engineered and carefully selected in the somatic cell nucleus before nuclear transfer. SCNT has been used to generate a variety of genetically modified animals such as goats, pigs, sheep and cattle; however, animals resulting from SCNT frequently suffer from developmental abnormalities associated with incomplete nuclear reprogramming. Other strategies to generate genetically-modified animals rely on the use of the spermatozoon as a natural vector to introduce genetic material into the female gamete. This sperm mediated DNA transfer (SMGT) combined with intracytoplasmatic sperm injection (ICSI) has relatively high efficiency and allows the insertion of large DNA fragments, which, in turn, enhance proper gene expression. An approach currently being developed to complement SCNT for producing genetically modified animals is germ cell transplantation using genetically modified male germline stem cells (GSCs). This approach relies on the ability of GSCs that are genetically modified *in vitro* to colonize the recipient testis and produce donor derived sperm upon transplantation. As the genetic change is introduced into the male germ line just before the onset of spermatogenesis, the time required for the production of genetically modified sperm is significantly shorter using germ cell transplantation compared to cloning or embryonic stem (ES) cell based technology. Moreover, the GSC-mediated germline modification circumvents problems associated with embryo manipulation and nuclear reprogramming. Currently, engineering targeted mutations in domestic animals using GSCs remains a challenge as GSCs from those animals are difficult to maintain *in vitro* for an extended period of time. Recent advances in genome editing techniques such as Zinc-Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) greatly enhance the efficiency of engineering targeted genetic change in domestic animals as demonstrated by the generation of several gene knock-out pig and cattle models using those techniques. The potential of GSC-mediated germline modification in making targeted genetic modifications in domestic animal models will be maximized if those genome editing techniques can be applied in GSCs.

Keywords: genetic engineering, large animal models, male germline, transgenesis.

Introduction

Genetic manipulation and public acceptance

Genetic modification of an animal involves altering its genetic material by adding, changing or removing certain DNA sequences in a way that does not occur naturally (European Food Safety Authority; www.efsa.europa.eu). While the selection of certain animal traits has been achieved by selective breeding since the beginning of animal domestication, the advent of molecular techniques has allowed to shortcut the long process of natural breeding for editing animal genomes. Genetically-modified domestic animal models are of increasing significance in biomedical research and agriculture. More progress has been made in the field of biomedicine than for agricultural purposes due to better public acceptance of biotechnology when the intention is to generate health benefits, followed by benefits for the environment but more concern is generated by food technology (Einsiedel, 2005). The public opinion also reflected more objections to the genetic manipulation of animals than plants or microorganisms (Einsiedel, 2005). It is expected that the number of genetically modified animals and their applications will increase in the near future and so it will the public concern and awareness, which influences policies by the regulatory agencies.

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The regulatory agencies for genetically modified animals include the United States Food and Drug Administration (FDA; www.fda.gov), the European Medicine Agency (EMA; www.ema.europa.eu); the European Food Safety Authority (EFSA; www.efsa.europa.eu) and the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO).

Use of large animal models in Biomedicine and Agriculture

The availability of suitable animal models is essential for the development of new therapies. Certainly the use of rodents has been very useful in understanding the function of many genes by means of targeted mutagenesis. However, although they can be very useful for the development of new technologies, rodent models do not always faithfully reflect the patient situation and many scientific discoveries in rodent models do not translate into the clinical situation.

Large animals are genetically outbred and more closely related to humans than rodents and the pig has become a very good model for translational biomedical research because it resembles humans more closely than rodents from a physiologic, anatomic and genetic point of view (Aigner *et al*., 2010; Bode *et al*., 2010; Luo *et al*., 2012). Genetically modified domestic animals are currently available as models for diabetes, cystic fibrosis, cardiovascular diseases and neurodegenerative diseases, among others (Aigner *et al*., 2010; Luo *et al*., 2012). Other applications of farm animals include their use for xenotransplantation, as bioreactors for the production of enzymes, hormones, antibodies or other compounds of pharmaceutical interest (Niemann and Kues, 2007; Tan *et al*., 2012). In the field of agriculture, genetically modified animals can result in improved production traits, resistance to diseases, improved adaptation to the production systems or the environment and the creation of more environmentally friendly livestock (Golovan *et al*., 2001; Donovan *et al*., 2005; Niemann and Kues, 2007; Richt *et al*., 2007; Aigner e*t al*., 2010; Kues and Niemann, 2011; Piedrahita and Olby, 2011; Luo *et al*., 2012). However, although the first genetically modified animals were generated over three decades ago (Gordon and Ruddle, 1981; Hammer *et al*., 1985), the technology has not yet fulfilled the original expectations. So far, the European Medicine Agency approved in 2006 the first biological product derived from genetically engineered animals, ATryn, an alternative to antithrombin derived from human plasma (GTC Biotherapeutics UK Limited). The same product was later authorized for marketing by the US FDA in 2009.

The knowledge at the molecular level has greatly increased in recent years for domestic species which certainly has helped making advancements in the field. Moreover, the biotechnology for generating genetically modified animals is currently available, but especially in the case of livestock, for many laboratories, the cost is still extremely prohibitive (Kind and Schnieke, 2008). Even though, the costs of establishing a cell-based manufacture are greater than the costs for the development of genetically modified animals (Kind and Schnieke, 2008). Considering that many of the cell culture-derived products are retired during phase 1 or during preclinical investigations (Kind and Schnieke, 2008), it would be useful to invest in generating genetically engineered animals.

Traditional approaches to generate genetically modified animals

Pronuclear (PN) injection

Pioneer experiments demonstrated that foreign DNA can be introduced into fertilized oocytes by microinjection of DNA molecules into one of the pronuclei (Gordon *et al*., 1980; Gordon and Ruddle, 1981; Hammer *et al*., 1985). Using this technique, genetically modified rabbits, sheep and pigs were generated harbouring the MT-hGH gene (Hammer *et al*., 1985). However, PN microinjection is a technically challenging option that requires highly trained personnel. Moreover, the opacity of the ooplasm in domestic species is a key feature that contributes technical difficulties to the detection of the PN and thus, to the efficiency of the procedure. One of the main limitations of PN injection is the low efficiency, reaching success rates of 1-2% or up to 5-10% in livestock in comparison to up to 20-25% in mice (Clark, 2002; Garrels *et al*., 2011). Another drawback is the variable expression of the transgene due to random integration into the genome and the variability in the number of inserted copies. The random integration can cause position effect variegation which can lead to gene silencing (Opsahl *et al*., 2002; Ohtsuka *et al*., 2012). Despite of the inherent disadvantages of PN, this approach prevails as one of the most commonly used methods for generating genetically modified mice. Although PN injection has been successfully used to generate genetically modified livestock (Robl *et al*., 2007; Luo *et al*., 2012), the high cost of screening and maintaining founders in addition to the inefficiency of the technique, have prevented its use on a large scale in livestock or other domestic species. Recent approaches employing PN injection with the implementation of recombinases or integrases for targeted mutagenesis have been described holding more promise than the conventional PN injection-based transgenesis (Ohtsuka *et al*., 2012).

Embryonic stem (ES) cells-based targeted transgenesis

Embryonic stem cells are derived from the inner cell mass of the blastocyst. ES cells are pluripotent and can be maintained *in vitro* in an undifferentiated

stage.ES cells can be genetically modified *in vitro* and injected to an embryo where ES cells contribute to all cell types including the germ line.

ES cell-based targeted mutagenesis can overcome the aforementioned position effect variegation and repeat-induced gene silencing. With the use of ES cells, a single copy of the transgene is integrated into a determined locus by homologous recombination (HR) allowing the generation of knock-outs, knock-ins or the exchange of genes or large chromosomal regions (Capecchi, 2005; Laible and Alonso-Gonzalez, 2009; Ohtsuka *et al*., 2012). Unfortunately, this technology is only available in mice. Despite many efforts by multiple laboratories to generate ES cells in livestock, to date, there are no true established ES cell lines. So far, the ES-like cells generated in domestic species do not fulfill the more stringent *in vivo* test of chimera formation with germline transmission (Nowak-Imialek *et al*., 2011; Piedrahita and Olby, 2011).

An important breakthrough was the reprogramming of terminally differentiated fibroblasts into the pluripotent state by ectopic expression of several transcription factors, leading to the establishment of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Takahashi *et al*., 2007). The iPS cells share common characteristics with ES cells, but they differ in their epigenetic signature and they display epigenetic memory (Nowak-Imialek *et al*., 2011). Given their great potential in regenerative medicine, gene targeting has been successfully performed in iPS cells to correct disease related genetic mutations (Zou *et al*., 2009; Yusa *et al*., 2011). The generation of iPS cells has been reported in several non-rodent species, but their germ line competence has not been described so far (Nowak-Imialek *et al*., 2011).

Cloning: somatic cell nuclear transfer (SCNT) and handmade cloning (HMD)

The advent of new technologies to clone animals by the transfer of nuclei from somatic cells (Campbell *et al*., 1996; Wilmut *et al*., 1997) opened new avenues to generate genetically modified livestock animals. Somatic cell nuclear transfer involves the enucleation of matured oocytes, followed by the injection or fusion of the donor cell and activation of the reconstructed embryo. It has been successful in many species, including laboratory, domestic and wildlife species (Galli *et al*., 2012). The technical and biological aspects for successful SCNT have been reviewed elsewhere (Galli *et al*., 2012). Once SCNT became available, the first genetically modified animals were generated shortly afterwards (Schnieke *et al*., 1997) and the first knock out livestock was generated by HR in fibroblasts (McCreath *et al*., 2000; Denning *et al*., 2001).

Currently, SCNT is the preferred approach for generating genetically modified large animals (Schnieke *et al*., 1997; McCreath *et al*., 2000; Wang *et al*., 2008; Yin *et al*., 2008; Gomez *et al*., 2009; Hong *et al*., 2009; Kim *et al*., 2011; Yang *et al*., 2011a; Jeong *et al*., 2012; Luo *et al*., 2012; Zhang *et al*., 2014). Without the establishment of true ES cells in livestock, gene targeting has been done primarily in fibroblasts for subsequent SCNT. Somatic cells, however, have a low frequency of HR in comparison to mouse ES cells and have a limited life span in culture which hampers the establishment of cell lines with the desired genetic modification. Furthermore, transfection of primary cells might affect their growth and induce senescence before they can be fully characterized (Laible and Alonso-Gonzalez, 2009). Some strategies have been used in an attempt to circumvent these problems, such as the introduction of the human telomerase catalytic subunit (hTERT) to increase the longevity of the primary somatic cells, but it appears that the constant expression of hTERT is not compatible with the production of live animals by SCNT (Laible and Alonso-Gonzalez, 2009). Other options aim to rejuvenate genetically modified cell lines by serial cloning, re-deriving the cell lines from cloned fetuses (Robl *et al*., 2007; Laible and Alonso-Gonzalez, 2009), but the rejuvenation process can increase the accumulation of epigenetic/genetic errors that eventually may lead to loss of the cloning potential (Laible and Alonso-Gonzalez, 2009; Liu *et al*., 2011). Sequential targeting has been done successfully in cattle (Kuroiwa *et al*., 2004) and calves have been obtained after four rounds of genetic modifications (Robl *et al*., 2007).

Somatic cell nuclear transfer requires micromanipulation, and therefore, highly skilled technicians and sophisticated equipment. One of the main drawbacks of the technique is the low developmental competence of the reconstructed embryos to produce normal offspring. Usually, SCNT-generated embryos are associated with higher pregnancy losses than their *in vitro* fertilized counterparts, due to placental abnormalities. However, the cloned animals that survive to adulthood are normal and are able to reproduce. The abnormalities observed in SCNT-produced animals are mainly of epigenetic origin and have been reviewed by others (Galli *et al*., 2012).

A simplified methodology for cloning by SCNT, namely, hand-made cloning (HMC), has been proposed (Vajta *et al*., 2005) in order to avoid micromanipulation and its inherent difficulties to make it a more user-friendly system. With this technique, the zona pellucida is first digested and the nucleus of the oocyte is removed using blades. The enucleated cytoplast is afterwards electrofused with the desired somatic cell to make the reconstructed embryo. HMC allows the fusion of several cytoplasts to increase the volume of the reconstructed embryo which increases the average number of cells in the resulting blastocysts and thus, their developmental competence. Important progress has been made improving blastocyst yield and pregnancy

rates after HMC, genetically modified animals have been generated by this method, such as pigs (Kragh *et al*., 2009; Luo *et al*., 2011, 2012) and sheep (Zhang *et al*., 2013) and it is expected that this approach will become more used in the near future.

Transgenesis through the male germ cell line

Spermatogenesis is a very complex and coordinated process that occurs in the testis, by which spermatogonial stem cells undergo mitotic and meiotic divisions and differentiate leading to the continuous production of spermatozoa throughout the reproductive life of a male (Kerr *et al*., 2006). Spermatogenesis is an extremely efficient process, therefore, manipulations of the male germ line provides an attractive approach for the generation of genetically modified animals. Different spermatogenic cell types have been targeted for introducing genetic manipulations in the male germ line. Mainly the end product of spermatogenesis, the male gamete that eventually will be used for *in vitro* or *in vivo* fertilization; or the stem cells that will be able to establish donor-derived spermatogenesis after transplantation into the testes of recipient animals.

Sperm mediated gene transfer (SMGT) and intracytoplasmic sperm injection mediated gene transfer (ICSI-MGT)

The option of SMGT relies on the use of the spermatozoon as a natural vector for transferring genetic material into the oocyte. The mechanism of action is controversial, but exogenous DNA can specifically bind to the head of the spermatozoon through binding proteins (Parrington *et al*., 2011) which are normally blocked by inhibitory factors (Carballada and Esponda, 2001) in the seminal plasma. Different options have been used in combination with SMGT to increase its efficiency, such as liposomes, electroporation or restriction enzyme mediated integration (REMI; Parrington *et al*., 2011). Advantages of SMGT are its low cost and simplicity, together with the possibility of harvesting high numbers of spermatozoa, thus, being feasible in numerous species. The main disadvantages of this passive technique are the relatively low efficiency, random integration of DNA and mosaicism. This approach provoked interest in the scientific community for its simplicity, but has not been widely adopted for creating genetically modified animals for its variable outcomes. The use of SMGT alone or in combination with other methods to facilitate the introduction of exogenous DNA through the membrane of the sperm resulted in the production of transgenic animals (Wang *et al*., 2001; Zhang *et al*., 2012)

Another approach to overcome the difficulties of PN microinjection in large animals is the use of intracytoplasmic sperm injection mediated gene transfer (ICSI-GMT), which can also be considered as an extension of SMGT. The procedure is usually carried out using spermatozoa treated with a detergent, NaOH, lysolecithin, after freeze-thawing or other methods that cause damage to the membrane of the spermatozoa (Moisyadi *et al*., 2009; Parrington *et al*., 2011). Afterwards, the spermatozoa are incubated with the DNA and the spermatozoon-DNA complex is injected into matured (metaphase II) oocytes. This tool has mainly been used in mice (Moisyadi *et al*., 2009), but it has been also successfully applied to the pig (Umeyama *et al*., 2009). The main drawback of ICSI-MGT is that is technically complex, requiring skillful and trained personnel and ICSI in large animals is not as well established as in the mouse. Nonetheless, it allows the introduction of very large DNA fragments favouring proper gene expression. The efficiency of ICSI-MGT has been improved by the use of transposons using fresh sperm in the mouse (Moisyadi *et al*., 2009). Additionally, the use of transposons together with the injection of round spermatids has resulted in the production of genetically modified mice (Suganuma *et al*., 2005; Moisyadi *et al*., 2009).

Germ cell transplantation &male germline stem cells (GSCs)

Male GSCs are unipotent adult stem cells in the testis that self-renew and undergo differentiation to eventually form sperm (Dym, 1994). The ability of GSCs to transmit genetic information to the next generation makes them an attractive vehicle for passing on genetic modifications. The germ cell transplantation technique developed by Brinster and coworkers in 1994 laid the foundation for using GSCs as a transgene carrier as it demonstrated that GSCs from a donor mouse testis, when transplanted into the seminiferous tubules of an infertile recipient testis, were able to colonize the recipient testis and re-established long-term donor-derived spermatogenesis (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994).

As GSCs are difficult to maintain *in vitro* and proliferate very slowly, the prevailing method of introducing exogenous genes into GSCs relies on viral vector-mediated transduction. The resulted genetic modification is random insertion of the transgene into the genome. Transduction of rodent GSCs by lentiviral or retroviral vectors proved to be successful and resulted in transgenic mouse and rat offspring after germ cell transplantation (Nagano *et al*., 2001, 2002; Hamra *et al*., 2002; Ryu *et al*., 2007; Takehashi *et al*., 2007). Viral vectors have also been used to transduce GSCs from large animals to make transgenic gametes and embryos (Honaramooz *et al*., 2008; Zeng *et al*., 2013).

Due to the saftey concerns and the limited packaging size of the viral vectors, an alternative method, namely nucleofection has been applied to deliver transgenes into GSCs. Nucleofection presents a technical advancement over traditional electroporation

technology with improved transfection efficiency and survival rate. It also allows delivery of large transgene constructs and transfection of non-dividing cells (Trompeter *et al*., 2003; Lorenz *et al*., 2004; Zeitelhofer *et al*., 2007). Nucleofection has been used successfully to transfect rodent and goat GSCs prior to transplantation (Kanatsu-Shinohara *et al*., 2006; Izsvak *et al*., 2010; Zeng *et al*., 2012).

There are several advantages of GSC mediated genetic engineering. First, introduction of genetic modifications in GSCs can circumvent problems associated with embryo manipulation and nuclear reprogramming (Niemann *et al*., 2005; Bacci, 2007; Galli *et al*., 2012). Secondly, spermatogenesis *in vivo* also provides a natural selection scheme to eliminate undesired mutations that disrupt fundamental cellular processes, and the mutations that are detrimental to spermatogenesis. In addition, once transgenic GSCs re-colonize the seminiferous tubules of the recipient testis, they can continuously produce transgenic sperm over the life course of the recipient. Finally, by transplanting transduced GSCs into prepubertal recipients, the time to production of transgenic sperm is shortened compared to production of a transgenic founder animal by somatic cell nuclear transfer or by ES cell-based germline transmission (Fig. 1).

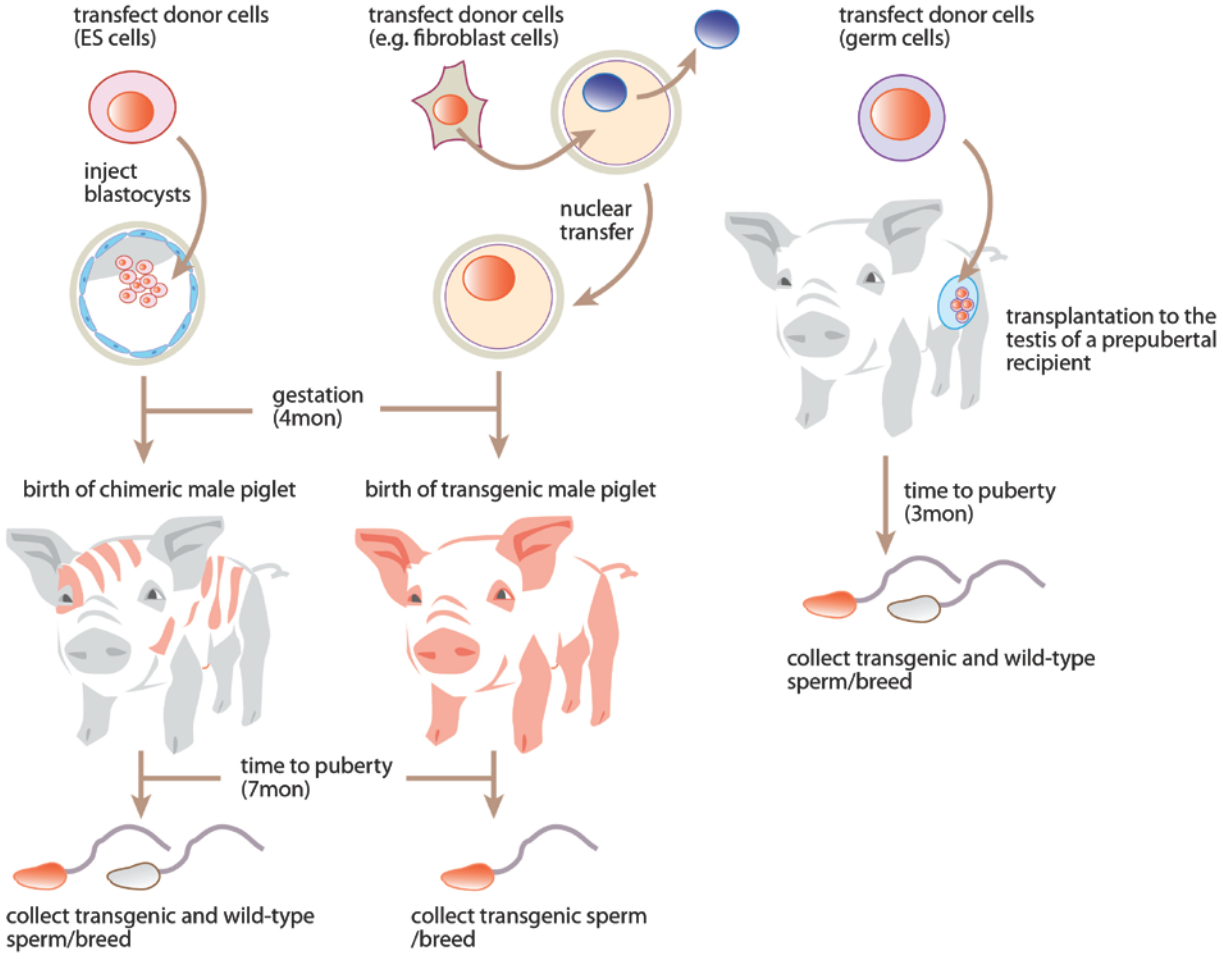

Figure 1. Approaches to generate transgenic large animals. If authentic ES cells from large animals become available, genetic modifications can be engineered in ES cells which mediate the germline transmission of the genetic modifications (left). The prevailing approach to make transgenic large animals is via SCNT (middle). Germ cell transplantation using genetically modified germline stem cells (right) emerges as an alternative approach for transmitting genetic modifications because the time required to obtain genetically modified gametes is shortened via germ cell transplantation compared to the other two approaches.

Random integration *vs*. Targeted mutagenesis

The applications of genetically modified large animals generally fall into two categories (Laible and Alonso-Gonzalez, 2009; Aigner *et al*., 2010; Tan *et al*., 2012): 1) over expression of an exogenous gene to confer certain characteristics (such as a reporter), to enhance certain traits (such as growth hormones for animal production), or to be used as bioreactors for the production of bioactive materials (such as Factor VIII and alpha-1 antitrypsin). In those scenarios, the exogenous DNA is usually integrated into the genome in a random fashion and extensive screening of the transgenic animals is desired for stable, prolonged and trans-generational expression of the transgene; 2) engineering genetic changes in a specific location in the genome for the purpose of disease modeling, trait modification or precise spatial/temporal expression of an exogenous gene, or gene silencing. In this case, the exogenous DNA carrying the desired genetic modifications is "targeted" to a specific locus by homologous recombination (Capecchi, 2005). Alternatively, double strand (ds) breaksare created in a specific locus by engineered nucleases and genetic modifications are brought about as a result of DNA repaireither by Non-Homologous End Joining (NHEJ) or homologous recombination (HR).

Transgenesis through random integration

The majority of transgenic large animals were produced by random integration of the exogenous transgene into the genome. There are a few concerns associated with random integration of the transgene. The transgene is usually carried on a plasmid or a virus based vector, which results in the presence of undesired vector sequences (bacterial or viral) upon integration into the genome. The linear transgene tends to form concatemers upon insertion into one or more loci, resulting in potential structural instability and transgene silencing by the host. Random integration can also lead to insertional mutagenesis when the transgene is inserted into a functional gene. This represents a particular concern on viral vectors as analysis on the integration site of several viruses in the human genome indicates that some viruses have integration preferences towards active genes and their regulatory motifs (Schroder *et al*., 2002; Wu *et al*., 2003; Berry *et al*., 2006). By design, the expression of a transgene is usually regulated by a promoter engineered in the expression cassette; however, the site of integration may affect the spatial and/or temporal expression of the transgene as a result of position effects or epigenetic silencing over time.

Compared to un-facilitated integration of exogenous DNA into the host genome, DNA transposons allow more efficient integration of the exogenous transgene without the contamination of vector sequences and are less prone to integrate in the form of concatemers. Those features promoted transposon-based insertional mutagenesis to become an important tool of large-scale functional genomics in several species (Kumar and Snyder, 2001; Carlson *et al*., 2003; Bessereau, 2006; Largaespada, 2009; Furushima *et al*., 2012; Yergeau *et al*., 2012). The most common transposon system used for genetic engineering is class II DNA transposons which use a "cut-and-paste" transposition mechanism to excise a precise DNA segment from a DNA sequence (i.e. from a vector) and insert it into another DNA sequence (i.e. the host genome; Ammar *et al*., 2012). The transgene expression cassette to be transposed is carried on a transposon vector and flanked by inverted terminal repeats (ITRs) of the transposons. The transposase enzyme, which is provided together with the vector, recognizes the ITRs and drives the transposition reaction.

Several transposon systems such as *Sleeping Beauty*, *Tol2* and *piggyBac* have been used as gene or enhancer trap vectors for functional genomics as well as for transgenesis in fly, fish and rodents (Carlson *et al*., 2003; Davidson *et al*., 2003; Balciunas *et al*., 2004; Bonin and Mann, 2004; Parinov *et al*., 2004; Largaespada, 2009; Nakanishi *et al*., 2010). Pronuclear injection of a transposon vector and the hyperactive *Sleeping Beauty* transposase into fertilized rabbit oocytes resulted in stable transgenic rabbits (Ivics *et al*., 2014). Through the approaches such as cytoplasmic injection into zygotes or transfection of porcine fibroblasts followed by SCNT, the *Sleeping Beauty* transposon system has also been used to generate transgenic pigs that carry a fluorescent reporter (GFP, YFP or Venus) ora human ApoBEC3G gene (Carlson *et al*., 2011, Garrels *et al*., 2011, Jakobsen *et al*., 2011). Genome-wide mutagenesis has been achieved in rat germline stem cells (GSCs) by using *Sleeping Beauty* (Izsvak *et al*., 2010). Transplantation of a polyclonal library of transfected GSCs or individually picked monoclonal GSC lines into the recipient rat testis resulted in germline transmission of the mutations and generation of knockout rat offspring.

Targeted mutagenesis through engineered nucleases

Precise gene alteration via homologous recombination (gene targeting) in mouse ES cells and subsequent germline transmission of the mutations revolutionized the field of functional genetics and became the gold standard for creating mouse models for biomedical and pharmaceutical studies. For large animals where germline-competent ES cells are not readily available, production of large animals with precise genetic modifications relies on gene targeting in somatic cells followed by somatic cell nuclear transfer (Laible and Alonso-Gonzalez, 2009). Due to the low efficiency of gene targeting in somatic cells, developmental problems associated with SCNT, and the

high cost in large animal husbandry, knockout domestic animals available to the biomedical community are scarce.

A recent advent of engineered chimeric nucleases greatly advances the process of site-specific genetic engineering in large animals. Zinc finger nucleases (ZFN) were the first nucelases described for this purpose (Kim *et al*., 1996). ZFNs are composed of zinc finger DNA recognition domains tethered to a *FokI* endonuclease domain. When two ZFN monomers recognize and bind to their DNA target in the correct orientation, the FokI endonuclease domains dimerize and induce double strand (ds) breaks in DNA. The ZFN-induced ds breaks can be repaired either via non-homologous end joining (NHEJ) or via homologous recombination (HR; Porteus and Carroll, 2005). NHEJ is error-prone and imperfect repairs result in insertions and deletions (indels) at the break sites. Some indels can lead to gene inactivation by frame shift without integration of any exogenous sequences; however, the variable nature of indels does not allow specific engineering of the target sequence. Alternatively, if a homologous sequence with desired genetic changes is provided *in trans*, HR allows the changes to be incorporated into the genome as a result of ds breaks repair.

ZFNs have been successfully used for gene targeting in primary cells, transformed cells, stem cells as well as iPS cells (Hockemeyer *et al*., 2009; Hockemeyer and Jaenisch, 2010). Moreover, knockout rodents and fish have been generated by embryonic injection of ZFNs (Meng *et al*., 2008; Geurts *et al*., 2009; Carbery *et al*., 2010; Cui *et al*., 2011). The first application of ZFNs in domestic animals was to produce eGFP knockout pigs by ZFN-facilitated targeting of the eGFP gene in GFP transgenic porcine fibroblasts and SCNT (Whyte *et al*., 2011). This study provided a proof-of-principle for the application of ZFNs in genetic engineering of domestic animals. Soon after, the same approach was used to generate knock out pigs and cattle (Hauschild *et al*., 2011; Yang *et al*., 2011b; Yu *et al*., 2011; Bao *et al*., 2014; Luo *et al*., 2014). In some of those studies, bi-allelic mutations were identified in somatic cells prior to SCNT and used to produce homozygous KO animals (Hauschild *et al*., 2011; Bao *et al*., 2014; Luo *et al*., 2014). Bi-allelic modification greatly reduces the time and cost needed to generate homozygous KO animals given the long generation intervals of domestic animals.

Transcription Activator-like Effector Nucleases (TALENs) came to the center of the stage as a superior alternative to ZFNs with their much simpler design and assembly and boarder targeting range (Joung and Sander, 2013). Similar to ZFNs, TALENs are sequence-specific nucleases consisting of the FokI endonuclease domain and themodular DNA binding domain of TALEs (Christian *et al*., 2010). At the target site, TALENs create ds DNA breaks to generate genetic modifications via NHEJ or HR. TALENs have been used for gene modification in a wide range of cell types and species including domestic animals (Carlson *et al*., 2013; Joung and Sander, 2013). Mono-allelic and bi-allelic modifications can be generated by TALENs when delivered into porcine or bovine zygotes or primary fibroblasts (Carlson *et al*., 2012). In addition, large chromosome deletions and inversions can be induced in livestock fibroblasts when two TALENs targeting the same chromosome were delivered into cells simultaneously (Carlson *et al*., 2012). Cloning with TALENs-targeted porcine primary fibroblast resulted in KO pigs with mono-allelic and bi-allelic mutations in the LDL receptor gene (Carlson *et al*., 2012). Very recently, microinjection of TALENs and ZFNs into pig zygotes resulted in production of live piglets with targeted mutations (Lillico *et al*., 2013). Although the efficiency of nucleases-mediated gene targeting is relatively high, gene mutations created by NHEJ are unpredictable due to the nature of the repair. HR-directed repair is desired to engineer specific gene modifications. This has recently been demonstrated in generation of specific KO mice and pigs by simultaneous delivery of TALENs and DNA oligonucleotides (Tan *et al*., 2013; Wefers *et al*., 2013). The HR-directed repair has also been achieved in pig, goat and cattle fibroblasts with both mono-allelic and bi-allelic modifications (Tan *et al*., 2013).

A recent addition to the designer nucleases family is a RNA-guided nuclease system named Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs)/CRISPR-associated (Cas) 9 (Jinek *et al*., 2012). The CRISPRs/Cas9 system was adopted from the adaptive immune system of bacteria and archaea against foreign DNA in which CRISPR RNAs (crRNAs), with the help of trans-activating crRNA (tracrRNA), guide the endonucleases Cas9 to induce ds breaks in target DNA (Jinek *et al*., 2012). Instead of having a DNA binding domain for DNA recognition as in ZFNs and TALENs, CRISPRs/Ca9 uses RNAs as a guide for site specific DNA recognition and recruits Cas9 endonucleases for DNA cleavage. Ever since 2012, the CRISPRs/Cas9 system has been successfully adapted for engineering mono-allelic and bi-allelic gene modifications in cells and multicellular organisms (Wilkinson and Wiedenheft, 2014). In addition, multiple guide RNAs can be encoded into a single CRISPR array to allow simultaneous multiple gene targeting in one step (Cong *et al*., 2013; Ma *et al*., 2014). This presents an exciting opportunity to knock out redundant genes, multiple members of a gene family or multiple players in signaling pathways. The most recent breakthrough in large animal genome modification was achieved in cynomolgus monkeys using CRISPRs/Cas9 (Niu *et al*., 2014). Two genes (Ppar-γ and Rag1) were disrupted simultaneously in KO monkeys (Niu *et al*., 2014).

Conclusion and perspectives

Genetically modified large animals provide important models for the study of human and animal disease, as pre-clinical platforms for the development and testing of new treatments and hold potential to improve efficiency and animal well-being in production agriculture. Transgenesis through SCNT is currently the prevailing technology to generate transgenic large animals; however, other strategies such as genetic modification through the male germ line provide alternative approaches. While the majority of existing models have involved random insertion and gain-of-function, the availability of designer nuclease technology, when combined with SCNT or germ line modification, now opens new avenues for targeted modifications and generation of loss-of-function (knockout) models.

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