REVIEW

The development and application of genome editing technology in ruminants: a review

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Abstract Transgenic ruminants are a valuable resource for both animal breeding and biomedical research. The development of transgenic breeding is proceeding slowly, because it suffers from low efficiency of gene transfer and possible safety problems from uncontrolled random integration. However, new breeding methods combined with genome editing and somatic cell nuclear transfer or microinjection can offer an economic and efficient way to produce gene-edited ruminants, which can serve as bioreactors or have improved disease resistance, animal welfare and product quality. Recent advances in precise genome editing technologies, especially clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 nucleases, are enabling the systematic development of gene-edited ruminant production. This review covers the development of gene-edited ruminants, the particulars of site-specific engineered nucleases and the state of the art and new insights into practical applications and social acceptance of genome editing technology in ruminants. It is concluded that the production of gene-edited ruminants is feasible and through improvements in genome editing technology it is possible to help feed the world.

Keywords bioreactors, breeding, engineered endonucleases, genome editing, ruminants

1 Introduction

Gene-edited ruminants have a potentially broad range of applications in the improvement of product quality and animal welfare, the enhancement of disease resistance and the production of biomedical materials[1–4]. Previously, transgenic ruminants were obtained by microinjection transferring the exogenous DNA into the cytoplasm of zygotes[5]. However, suffering from high rate of embryo chimerism and low gene-edited animal birth rates, microinjection was and remains inefficient for gene targeting, especially in monotocous species, such as cattle[6,7].

For rapidly-reproducing polytocous animals, such as mice, many progenies stably expressing the intended gene can be selected from the offspring generated by micro-injection of pluripotent embryonic stem cells (ESCs) containing the exogenous gene into blastocysts and transferring the embryos to foster mothers[8]. While for ESC lacking ruminants (goats[9], cattle[10,11] and sheep[12,13]), somatic cell nuclear transfer (SCNT) technology is available. SCNT based cloning technology transfers a somatic cell known to carry and/or silence the gene of interest to an enucleated oocyte, and then through electrofusion and chemical activation to obtain reconstructed embryos with a predictable offspring genotype and reliable efficiency[14].

The latest genome editing technology exploited as a powerful tool for identifying gene function and curing genetic diseases[15] relies on site-specific engineered endonucleases (EENs), including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 nucleases, triggering efficient DNA double-strand breaks (DSBs) at target sites[16] and DSBs mediated gene knockin (KI), knockout (KO), or substitution via intracellular DNA damage repair pathways[17]. In recent years, type II bacterial CRISPR/Cas9 has come to represent the most efficient tool for the further advancement of gene targeting strategies due to the high efficiency and simplicity of targeting any locus for cleavage with a single protein and a programmable single-guide RNA (sgRNA).

Here we review research progress in genome editing in

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ruminants and the structure and characteristics of EENs and provide an overview in detail of the application of genome editing technology to inspire further solutions for efficient and precise gene modification in ruminants.

2   Research progress in genome editing in ruminants

Dolly, the first sheep somatically cloned via SCNT, was born in 1996 in Scotland and demonstrated that mammals could be cloned from adult cells, especially cultured somatic cells containing the intended gene modification[18]. This showed that researchers can obtain gene-edited ruminants with predictable offspring genotype and reliable efficiency[19]. The first gene-edited ruminant offspring was produced via SCNT in 2000[20]. A common strategy for the production of gene-edited ruminants was to deliver sufficient donors, containing the intended gene or a DNA fragment flanked by homologous arms, as templates into the intended cells. Intracellular spontaneously created DSBs are principally repaired by the non-homologous end-joining (NHEJ) pathway and the competing homologous recombination (HR) pathway[21]. Therefore, the intended gene or the DNA fragment could be integrated into the genome at DSB sites by the HR pathway to achieve target gene KI or KO[16]. However, this method was limited in the production of gene-edited ruminants, due to the low frequency of DSBs and HR repair.

The next major advance was the advent of site-specific EENs, including ZFNs, TALENs and CRISPR/Cas9, which enabled direct targeting of a sequence for mutation, KO or site-specific KI in the genome[16]. The first ruminants produced using EENs were cattle with a β-lactoglobulin (*BLG*) gene modified via ZFNs in 2011[22]. During the process of NHEJ repair, small inserts and/or deletions (indels) are introduced at the DSB sites and may result in frameshifts and corresponding functional gene KO[16]. Gene-edited ruminants with biallelic KO of the intended gene were obtained, combined with SCNT technology, owing to the high frequency of NHEJ repair[23,24]. Also, researchers utilized microinjection by transferring the EENs mRNA or plasmids into the cytoplasm of zygotes, to produce individuals with the intended gene KO[25,26].

The ability of homology-directed repair (HDR)-based gene editing to precisely integrate interested genes of the same or different species into the genome of targeted animal endows scientists with a powerful tool to directionally and quickly generate gene-edited varieties free from the limitations of painstaking traditional breeding methods, especially for disease resistance breeding[1,7,27]. Through the introduction of DSBs at target sites, site-specific EENs enormously stimulate HR[28] and significantly improve HDR-mediated gene KI efficiency, which is the key to produce gene-edited ruminants lacking ESC and induced pluripotent stem cell[14,29].

Gene-edited ruminants have been obtained mainly by these technologies. For gene-edited cows, the main production steps (Fig. 1) and the main advantages and disadvantages of SCNT and microinjection technology are presented in Table 1.

3   Structure and characteristics of EENs

3.1   ZFNs and TALENS

Derived from eukaryotic transcription factors, ZFNs and TALENS are composed of one FokI nuclease domain and individual DNA recognition units assembled DNA binding domain[30]. Every ZFN unit (about 30 amino acids)
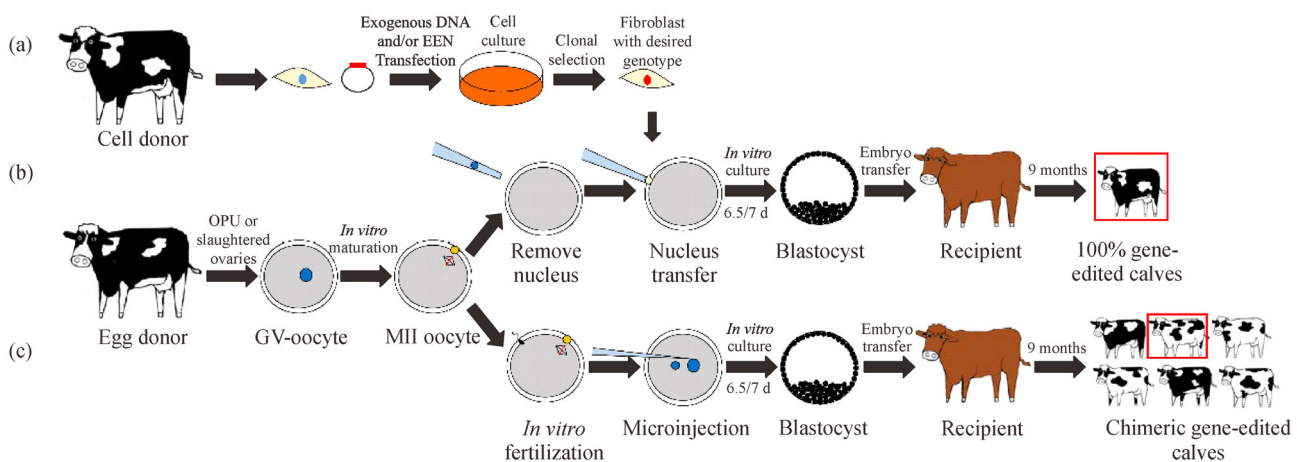
Fig. 1   Simplified diagram of the two methods for generating gene-edited cows via somatic cell nuclear transfer (SCNT) (a, b) and microinjection (c). OPU, ovum pick-up; GV, germinal vesicle; MII, metaphase II.

Table 1   Advantages and disadvantages of somatic cell nuclear transfer (SCNT) and microinjection technology for gene-edited cows

Technology	Advantages	Disadvantages
SCNT	Predictable offspring genotype	Higher rate of midwifery
	Consistent expression of target gene	Lower production efficiency* (1%–20%)
	100% gene editing efficiency	Not induced at term
	Saving time and costs	Increased developmental defects
	Allows selection of gender and elite genetic background	Greater risk of recessive genetic diseases
Microinjection	Relatively lower rate of midwifery	Chimerism
	15%–40% higher production efficiency	Not particularly useful for insertion of exogenous gene
	Easy to perform micromanipulation	Low number of zygotes
		Modified genes nonhereditary

Note: *Pregnancy rate is low but the rate of abortion is high.

combines with a single zinc atom and recognizes a 3-bp DNA sequence[31], while a TALE unit is composed of engineered TALE repeat arrays (33–35 amino acids) and recognizes a nucleotide, namely, NN, NI, HD and NG, recognizing guanine, adenine, cytosine and thymine, respectively[32]. Once ZFNs or TALENs bind to the target sites, the dimerized FokI nuclease domains forcefully induce two nicks 5- or 6-bp apart on the individual strands of DNA to form a precise DSB at the target site[33], and then the endocellular DNA damage repair pathway could be triggered to KO endogenous functional genes[34] or KI exogenous genes[35]. However, designing, constructing and screening efficient ZFNs and TALENs is expensive and time consuming for most molecular biology laboratories[36] and has relatively low efficiency[37,38]. Serious off-target effects[39–41] of ZFNs and TALENs were also reported in a few studies, which limited their development and application in the production of gene-edited ruminants.

3.2   CRISPR/Cas9

Unlike the other technologies, the type II CRISPR/Cas9 system has been most widely exploited for gene editing due to the simple requirements for a crRNA, tracRNA and a Cas9 protein for successful *in vitro* DNA cleavage[42,43]. In addition, the crRNA and tracRNA can be artificially fused as a sgRNA to further simplify this system[44]. The natural crystal structure of *Streptococcus pyogenes* Cas9 (SpCas9)-sgRNA-DNA complex indicate that the sgRNA-DNA heteroduplex divides the Cas9 protein into two lobes (a recognition lobe and a nuclease lobe) connected by a bridge helix[45]. The recognition lobe comprises Rec1 and Rec2 domains, while the nuclease lobe includes RuvC, HNH and PID domains. The recognition of target DNA is initiated through Cas9 protein binding to the protospacer adjacent motif (PAM) sequence, but the sgRNA-DNA complex is crucial for the progressive recognition process. Once the guide sequence in sgRNA is complementary to the target DNA, the RuvC and HNH nuclease domains will be allosterically activated to cleave the target DNA at a site

three base-pairs downstream of PAM and subsequently produce a DSB[15,44]. The CRISPR/Cas9 system is cheap, highly efficient and easy to use even for novices, and its cleavage site can be as accurate as a single base[44]. Therefore, since the first CRISPR/Cas9 mediated gene editing in mammalian cells[46,47], this well-established system has been a widely utilized platform for gene-edited ruminant production[1,48].

4   Applications of genome editing technology in ruminants

Traditional selective breeding and crossbreeding are time consuming[4] and only improve existing production traits, whereas gene-edited ruminants breeding offers particularly attractive possibilities for generating characteristics that do not exist in nature. The development of EEN-mediated genome editing technology was essential for increasing the efficiency of gene targeting, which promises to greatly improve the traits of disease resistance, animal welfare and product quality in ruminants, and increase the safety, control and environmental benefits of gene-edited animal breeding. Gene-edited ruminants using EENs also have been widely adopted as bioreactors, thereby greatly contributing to the improvement of human health and alleviation of disease (Table 2).

4.1   Bioreactors

Bioreactors based on gene-edited ruminants were developed for biomedical materials, such as enzymes and hormones[54]. Ruminants have huge mammary glands and secrete large quantities of milk, with milking cows producing up to 8000 $L \cdot yr^{-1}$ (cf.800 $L \cdot yr^{-1}$ for goats and 500 $L \cdot yr^{-1}$ for sheep). Due to the similarities in glycosylation patterns between the proteins secreted by the mammary gland tissue of ruminants and human native proteins[54], the development of technologies related to the purification of recombinant proteins from milk provides a

Table 2   Application of genome editing in ruminants

Application	Species	Target	Trait/Goal	Methods	Time	Reference
Bioreactors	Sheep	Insertion of *AAT*	Treatment of α1-antitryp-sin (AAT) deficiency	HR/SCNT	2000	[20]
	Sheep	Insertion of *AANAT* and *ASMT*	Melatonin-enriched milk	CRISPR/Cas9 and zygote injection	2017	[48]
Disease resistance	Cows	Insertion of lysostaphin	Lysostaphin milk	ZFNickase/SCNT	2013	[27]
	Cattle	KO of *PrP*	Lacking prion protein	HR/SCNT	2007	[49]
	Sheep	KO of *PrP*	Lacking prion protein	HR/SCNT	2001	[50]
	Cattle	Substitution of CD18	Abolish cytolysis of leukocytes	ZFNs/SCNT	2016	[51]
	Cattle	Insertion of *NRAMP1*	Tuberculosis-resistant	Cas9 nickase/SCNT	2017	[1]
	Cattle	Insertion of *SP110*	Tuberculosis-resistant	TALE nickase/SCNT	2015	[7]
Animal welfare	Cattle	Introgression of POLLED allele	Polled dairy cattle	TALEN/SCNT	2016	[3]
Milk	Cattle	*BLG* biallelic mutations	Modification of *BLG*	ZFNs/SCNT	2011	[22]
	Cows	*BLG* biallelic KO	BLG-free milk	ZFNs/SCNT	2018	[23]
	Cattle	*BLG* KO	Milk free of mature BLG	TALEN and zygote injection	2018	[26]
	Goats	*BLG* KO	BLG abolished	CRISPR/Cas9 and zygote injection	2017	[25]
	Goats	*BLG*-KO and *hLF*-KI	BLG-free and high-hLF milk	TALEN/SCNT	2015	[35]
Meat	Cattle	*MSTN* biallelic mutations	Double-muscled phenotype	ZFNs/SCNT	2014	[4]
	Goats	*MSTN* biallelic KO	Disruption of MSTN expression	CRISPR/Cas9 and SCNT	2014	[24]
	Goats	*MSTN*-KO and fat-1 KI	Muscle fibers stronger	CRISPR/Cas9 and SCNT	2018	[52]
	Sheep	*MSTN*-KO	Muscle fibers stronger	CRISPR/Cas9 and SCNT	2018	[53]

mature and widely used system for recombinant proteins and makes ruminants most suitable species for recombinant proteins production[54,55]. Researchers have introduced a therapeutic gene encoding human α1-antitrypsin (AAT) into α1(I) procollagen locus in primary ovine fetal fibroblasts by HR-mediated gene editing[20]. Nuclear transfer with four independent donor cell lines resulted in the production of 14 gene-edited sheep, three of which lived for over one year. The analysis of hormonally induced milk of one of the three lambs showed the expression of AAT at a concentration of 650 μg·mL^{-1}. In contrast, previous research reported the highest level of AAT production observed in milk of gene-edited sheep produced by random integration only reached 18 μg·mL^{-1}. AAT is available for the treatment of AAT deficiency in patients with clinically evident emphysema[56]. Compared with random integration, gene-edited sheep produced by HR-mediated AAT gene integration showed a dramatic increase in yield.

Melatonin is a potent antioxidant widely used for medicinal purposes. In 2017, researchers report the first functional arylalkylamine N-acetyltransferase (*AANAT*) and acetylserotonin methyltransferase (*ASMT*) gene-edited animal model for high level of melatonin production in the milk of gene-edited sheep[48]. Thirty-four gene-edited lambs were liveborn: seven contained *AANAT*, two contained *ASMT*, whereas 25 contained both *AANAT* and *ASMT*. CRISPR/Cas9 and microinjection technologies were combined to generate gene-edited sheep at a rate of up to approximately 35%, compared with 5% without EENs[57].

4.2   Disease resistance

Using genome editing technology, biomedical materials can be produced in ruminants. However, there are safety concerns about the products of ruminants infected with certain diseases, especially mastitis and prion diseases. Both diseases also affect animal health and welfare, causing substantial financial strain to the dairy industry worldwide.

Mastitis is one of the most costly diseases in the dairy industry and has a great influence on ruminants' health and welfare. The industry suffers from high costs brought

about by extended calving intervals, extra work for veterinarian and herdsman, increased culling rate and mortality risk, and reduced milk yield and quality[58]. The disease, caused by *Staphylococcus aureus*, has a low cure rate with antibiotics (often less than 25%) and there is currently no effective vaccine[59]. However, lysostaphin enables specific targeting of *S. aureus* through cleaving the pentaglycine bridge of the peptidoglycan of the bacterium[59]. Researchers had previously reported the generation of eight transgenic heifers, five of which survived into adulthood and secreted lysostaphin[60]. The highest expressing cows were not infected by intramammary infusion of *S. aureus*. However, the lysostaphin gene was randomly integrated into the bovine genome. Researchers reported ZFNickases-mediated gene-edited cows with addition of the lysostaphin gene at the endogenous β-casein locus. The milk contained lysostaphin that was analyzed to confirm its ability to kill *S. Aureus*[27]. Although comparison of treatment of *S. aureus* bovine mastitis with lysostaphin and a commonly used antibiotic revealed similar cure rates[61], it is feasible to protect gene-edited cows from mastitis by high expression of lysostaphin in milk.

Prion diseases are transmissible and fatal neurodegenerative disease[62] (such as bovine spongiform encephalopathy in cattle[63], and scrapie in sheep and goats[64]) that occur because of the conversion of the physiological cellular prion protein (PrP^C) into the pathogenic β-sheets enriched isoform designated PrP^{Sc} and the propagation of PrP^{Sc} by recruiting PrP^C[65]. It has been reported that mice devoid of PrP^C were resistant to scrapie[66]. To test the feasibility of this strategy in cattle, 12 PrP^C-deficient cattle were produced by HR-mediated KO method and SCNT technology[49]. Two PrP^C-deficient cattle were subjected to histopathological analyses and showed no obvious abnormalities or significant lesions in any tissues. Protein misfolding cyclic amplification assay demonstrated the ability of the brain homogenates from one gene-edited individual to resist prion propagation. At over 20 months of age, the other eight cattle were still healthy. In addition, four PrP$^{-/+}$ lambs were born, unfortunately three died soon after birth and one survived for 12 days[50]. The production of PrP^C-deficient ruminants has assumed tremendous importance for disease resistance breeding and the production of prion-free biomedical materials.

Leukotoxin secreted by *Mannheimia hemolytica* causes acute inflammation and lung tissue damage[67]. Researchers using CRISPR/Cas9 introduced Q(−5)G substitution in both alleles of CD18 in bovine fetal fibroblast, which impacted on leukotoxin binding to signal peptide of ruminant CD18 and abolished cytolysis of leukocytes in gene-edited cattle[51]. Also, researchers reported on the site-specific KI of the Cas9 nickase-mediated *NRAMP1* gene and TALE nickase-mediated SP110 nuclear body protein gene to produce tuberculosis-resistant cattle[1,7]. These disease-resistant gene-edited cattle enabled the

significant decrease in economic losses, maintaining animal health and reducing the use of antibiotics and prophylactics[60].

4.3 Animal welfare

Physical dehorning is a common method used to protect dairy cattle and humans from harm but results in reduced animal welfare and high cost. The development of SCNT and EENs mediated genome editing technology enabled the generation of hornless dairy cattle. In 2016, researchers achieved introgression of a putative POLLED allele into the genome of bovine embryo fibroblasts through TALEN-mediated integration and produced two healthy, homozygous polled dairy cattle by SCNT[3]. Furthermore, researchers simulated introgression of the POLLED allele into cattle populations via conventional breeding or gene editing over the course of 20 years in the USA[68]. In comparison to crossbreeding, gene editing enabled fast or faster decrease of frequency of the horned allele to < 0.1, a significant improvement of lifetime net merit and less inbreeding, which provided scientists with a powerful tool to provide polled genetics to the dairy industry. These reports illustrate the potential of genome editing in the production of polled cattle. However, a recent report by US Food and Drug Administration (FDA) scientists revealed the undesired heterozygous integration of bacterial DNA and a second copy of the repair template sequence in the genome of one of the edited polled cattle, although it had been claimed to be 100% bovine[69,70]. To reduce the risk of unintended consequences from gene-edited animals, it is thus important to improve the specificity of EENs[71,72] and select appropriate methods, such as long-range PCR, quantitative PCR, Southern blot, long-read sequencing[70] and unbiased genome-wide off-target detection[73,74], to measure plasmid integration events and off-target mutagenesis.

4.4 Milk and meat

Currently, we face the situation where the demand for animal products outstrips supply. Through EENs, gene-edited ruminants could improve the quality of milk and meat that are indispensable as a source of food for humans. BLG, considered to be one of the most important allergens in milk, cannot be abolished by heat treatment or fermentation[75,76]. In addition, BLG can cause allergic reactions even after gastrointestinal digestion due to its resistance to acid and protease hydrolysis[23]. The first report of gene-edited ruminants produced using EENs involved a cow containing biallelic mutations of the *BLG* gene which survived for over six months[22]. In 2018, researchers generated a ZFN-mediated marker-gene-free cloned cow, in which the *BLG* biallelic mutations consisted of 17-bp and 16-bp indels that could lead to frameshift mutations, resulting in BLG-free milk that caused lower

allergic reactions in Balb/c mice[23]. Furthermore, researchers reported that BLG protein was abolished in cattle edited by zygote-mediated editing using TALEN[26] and goats edited by co-injection of Cas9 mRNA and sgRNAs into one-cell-stage embryos[25]. In 2015, researchers utilized TALEN to target the BLG locus to achieve KI of the *hLF* gene in fetal fibroblasts from goats[35]. Analysis of the milk from the gene-edited goats revealed large-scale hLF expression and decreased expression of BLG.

Double-muscled animals have an increase in muscle mass of about 20% because of a mutation of myostatin (MSTN)[77], which provides a direction for improving meat quality and economic benefit in ruminants. Researchers generated marker-gene-free *MSTN* biallelic mutation cattle via ZFNs combined with SCNT technology, which exhibited the double-muscled phenotype with about half the level of MSTN protein with an intact C-terminal domain estimated by ELISA analysis[4]. *MSTN*-KO goats[24,52] and sheep[53] have also been generated via CRISPR/Cas9.

5 Discussion

SCNT technology enables the high efficiency of gene editing and the consistent expression of the target gene in gene-edited offsprings[78] and permits full use of preferred germplasm for ruminant breeding[79,80]. However, it still needs to be improved to overcome some problems, probably because of inbreeding between clones and incomplete epigenetic reprogramming of the somatic donor nuclei[23]. For gene-edited cattle, compared to microinjection, SCNT requires more hours of practice and shows higher rates of midwifery and lower production efficiency. However, it is difficult to produce gene-edited calves via microinjection for monotocous cattle, because of chimerism and low numbers of zygotes. Thus, SCNT is currently the prevailing method to produce gene-edited cattle.

Random integration-mediated transgenic technology faces great safety and ethical problems because of its ability to generate uncontrolled gene transfer at unwanted sites and unregulated number of copies, which makes it easy to generate mutations of endogenous genes, to silence exogenous genes, and to integrate bacterial constituents into the target genome. HR-mediated gene editing technology could achieve exogenous DNA insertion to the target sites, which means transgenic technology is moving from an era of random integration into an era of site-specific integration. However, this is a huge challenge for large-scale production of gene-edited ruminants because of their low fertility. Hopefully, full development of the EENs mediated site-specific integration will significantly increase safety and efficiency of genome editing, which will greatly facilitate the production of gene-edited ruminants to serve human needs. Meanwhile, some optimizations have been developed for HDR-based KI technology including the quest for a double cut HDR donor[81] and the optimal frequency at which HDR takes place[82].

Relying on the bacterial expression system for the production of certain proteins is most often impractical because of misfolding, aggregation of insoluble inclusion bodies and lack of posttranslational modifications[83]. The use of insect cells, yeast and plants for therapeutic proteins also shows differences in glycosylation patterns, so are sometimes not suitable for therapeutic use in humans[84]. Mammalian expression systems provide another option for the production of biomedical materials, for example with most of the current mAbs[85]. However, low yield and high cost limit wider adoption[86]. Compared to other species, cattle, sheep and goats have higher milk yield with existing dairy infrastructure[78]. Therefore, gene-edited ruminants might be useful for efficiently and cheaply producing large and complex bioactive proteins, small unstable peptides, and proteins that require extensive posttranslational modification and are multimeric in nature[85]. In addition to gene-edited animal models as bioreactors, both disease models of cystic fibrosis[87] and human hypophosphatasia[88] have been accomplished using CRISPR/Cas9 in sheep, which revealed characteristics consistent with disease symptoms observed in human patients and provided a novel large animal platform for human diseases.

Transgenic Atlantic salmon[14] were developed by AquaBounty Technologies (Maynard, MA, USA) in 1992[89]. Although transgenic salmon represent a significant milestone, they were only recently approved in Canada, so will finally come onto the market as the first edible transgenic animal. Interestingly, three pharmaceutical proteins produced by transgenic animals (ATryn produced in goats[90], Ruconest in rabbits[91] and Kanuma in chicken eggs[92]) have been approved by the European Medicines Agency (EMA) and the FDA for medical use. Marketing transgenic ruminant products is more likely to be approved than the animals themselves, which can be limited by a series of issues, such as appropriate regulation, potential impact on the industry sector and social acceptability[93]. The social acceptance of such gene-edited ruminant would depend on the major premise that factors such as the safety, animal welfare and ethical concerns, were taken into account[94]. Public consideration of ruminant breeding by genome editing is largely based on their views on ethics, including moral standing, handling of animals, animal dignity and a critical analysis of the underlying relationship[95]. It is possible that public consideration will be an essential reference and direction to the research and government regulation of gene-edited ruminant breeding. Gene-edited ruminant breeding mediated by site-specific EENs is safer and more controlled with potentially better market acceptance, in contrast to the transgenic breeding. However, the recent

controversy over edited polled cattle[68] suggests that many people will be unlikely to accept the use of foreign plasmid in the process of genome editing. Thus, NHEJ-mediated genome editing may be considered for the production of ruminants because of the similarity to naturally occurring mutations[94].

6   Conclusions

Although the production of gene-edited ruminants has made great progress toward benefiting humans, the development of genome editing technology in ruminants has been much slower than in model organisms. However, the enormous scope for developing gene-edited ruminants holds immense promise for the future.

Compliance with ethics guidelines   Mengke Yuan, Yuanpeng Gao, Jing Han, Teng Wu, Jingcheng Zhang, Yongke Wei, and Yong Zhang declare that they have no conflicts of interest or financial conflicts to disclose.

This article is a review and does not contain any studies with human or animal subjects performed by any of the authors.

References

1. Gao Y, Wu H, Wang Y, Liu X, Chen L, Li Q, Cui C, Liu X, Zhang J, Zhang Y. Single Cas9 nickase induced generation of *NRAMP1* knockin cattle with reduced off-target effects. *Genome Biology*, 2017, **18**(1): 13

2. Miao X. Recent advances in the development of new transgenic animal technology. *Cellular and Molecular Life Sciences*, 2013, **70** (5): 815–828

3. Carlson D F, Lancto C A, Zang B, Kim E S, Walton M, Oldeschulte D, Seabury C, Sonstegard T S, Fahrenkrug S C. Production of hornless dairy cattle from genome-edited cell lines. *Nature Biotechnology*, 2016, **34**(5): 479–481

4. Luo J, Song Z, Yu S, Cui D, Wang B, Ding F, Li S, Dai Y, Li N. Efficient generation of myostatin (*MSTN*) biallelic mutations in cattle using zinc finger nucleases. *PLoS One*, 2014, **9**(4): e95225

5. Hammer R E, Pursel V G, Rexroad C E Jr, Wall R J, Bolt D J, Ebert K M, Palmiter R D, Brinster R L. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature*, 1985, **315**(6021): 680–683

6. Lillico S G, Proudfoot C, Carlson D F, Stverakova D, Neil C, Blain C, King T J, Ritchie W A, Tan W, Mileham A J, McLaren D G, Fahrenkrug S C, Whitelaw C B A. Live pigs produced from genome edited zygotes. *Scientific Reports*, 2013, **3**(1): 2847

7. Wu H, Wang Y, Zhang Y, Yang M, Lv J, Liu J, Zhang Y. TALE nickase-mediated *SP110* knockin endows cattle with increased resistance to tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, 2015, **112**(13): E1530–E1539

8. Capecchi M R. How close are we to implementing gene targeting in animals other than the mouse? *Proceedings of the National Academy of Sciences of the United States of America*, 2000, **97** (3): 956–957

9. Baguisi A, Behboodi E, Melican D T, Pollock J S, Destrempes M M, Cammuso C, Williams J L, Nims S D, Porter C A, Midura P, Palacios M J, Ayres S L, Denniston R S, Hayes M L, Ziomek C A, Meade H M, Godke R A, Gavin W G, Overström E W, Echelard Y. Production of goats by somatic cell nuclear transfer. *Nature Biotechnology*, 1999, **17**(5): 456–461

10. Cibelli J B, Stice S L, Golueke P J, Kane J J, Jerry J, Blackwell C, Ponce de León F A, Robl J M. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science*, 1998, **280**(5367): 1256–1258

11. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science*, 1998, **282**(5396): 2095–2098

12. Wilmut I, Schnieke A E, McWhir J, Kind A J, Campbell K H S. Viable offspring derived from fetal and adult mammalian cells. *Nature*, 1997, **385**(6619): 810–813

13. Schnieke A E, Kind A J, Ritchie W A, Mycock K, Scott A R, Ritchie M, Wilmut I, Colman A, Campbell K H S. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science*, 1997, **278**(5346): 2130–2133

14. Garas L C, Murray J D, Maga E A. Genetically engineered livestock: ethical use for food and medical models. *Annual Review of Animal Biosciences*, 2015, **3**(1): 559–575

15. Hsu P D, Lander E S, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, 2014, **157**(6): 1262–1278

16. Gaj T, Gersbach C A, Barbas C F 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, 2013, **31**(7): 397–405

17. Kawahara A, Hisano Y, Ota S, Taimatsu K. Site-specific integration of exogenous genes using genome editing technologies in zebrafish. *International Journal of Molecular Sciences*, 2016, **17**(5): 727

18. Campbell K H, McWhir J, Ritchie W A, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature*, 1996, **380**(6569): 64–66

19. Whitelaw C B A, Sheets T P, Lillico S G, Telugu B P. Engineering large animal models of human disease. *Journal of Pathology*, 2016, **238**(2): 247–256

20. McCreath K J, Howcroft J, Campbell K H S, Colman A, Schnieke A E, Kind A J. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature*, 2000, **405**(6790): 1066–1069

21. San Filippo J, Sung P, Klein H. Mechanism of eukaryotic homologous recombination. *Annual Review of Biochemistry*, 2008, **77**(1): 229–257

22. Yu S, Luo J, Song Z, Ding F, Dai Y, Li N. Highly efficient modification of β-lactoglobulin (*BLG*) gene via zinc-finger nucleases in cattle. *Cell Research*, 2011, **21**(11): 1638–1640

23. Sun Z, Wang M, Han S, Ma S, Zou Z, Ding F, Li X, Li L, Tang B, Wang H, Li N, Che H, Dai Y. Production of hypoallergenic milk from DNA-free β-lactoglobulin (*BLG*) gene knockout cow using zinc-finger nucleases mRNA. *Scientific Reports*, 2018, **8**(1): 15430

24. Ni W, Qiao J, Hu S, Zhao X, Regouski M, Yang M, Polejaeva I A, Chen C. Efficient gene knockout in goats using CRISPR/Cas9 system. *PLoS One*, 2014, **9**(9): e106718

25. Zhou W, Wan Y, Guo R, Deng M, Deng K, Wang Z, Zhang Y, Wang F. Generation of β-lactoglobulin knock-out goats using CRISPR/Cas9. *PLoS One*, 2017, **12**(10): e0186056

26. Wei J, Wagner S, Maclean P, Brophy B, Cole S, Smolenski G, Carlson D F, Fahrenkrug S C, Wells D N, Laible G. Cattle with a precise, zygote-mediated deletion safely eliminate the major milk allergen β-lactoglobulin. *Scientific Reports*, 2018, **8**(1): 7661

27. Liu X, Wang Y, Guo W, Chang B, Liu J, Guo Z, Quan F, Zhang Y. Zinc-finger nickase-mediated insertion of the lysostaphin gene into the β-casein locus in cloned cows. *Nature Communications*, 2013, **4**(1): 2565

28. Rouet P, Smih F, Jasin M. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, 1994, **91**(13): 6064–6068

29. Murray J D, Maga E A. Genetically engineered livestock for agriculture: a generation after the first transgenic animal research conference. *Transgenic Research*, 2016, **25**(3): 321–327

30. Li L, Wu L P, Chandrasegaran S. Functional domains in Fok I restriction endonuclease. *Proceedings of the National Academy of Sciences of the United States of America*, 1992, **89**(10): 4275–4279

31. Pavletich N P, Pabo C O. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science*, 1991, **252**(5007): 809–817

32. Joung J K, Sander J D. TALENs: a widely applicable technology for targeted genome editing. *Nature Reviews. Molecular Cell Biology*, 2013, **14**(1): 49–55

33. Händel E M, Alwin S, Cathomen T. Expanding or restricting the target site repertoire of zinc-finger nucleases: the inter-domain linker as a major determinant of target site selectivity. *Molecular Therapy*, 2009, **17**(1): 104–111

34. He J, Li Q, Fang S, Guo Y, Liu T, Ye J, Yu Z, Zhang R, Zhao Y, Hu X, Bai X, Chen X, Li N. *PKD1* mono-allelic knockout is sufficient to trigger renal cystogenesis in a mini-pig model. *International Journal of Biological Sciences*, 2015, **11**(4): 361–369

35. Cui C, Song Y, Liu J, Ge H, Li Q, Huang H, Hu L, Zhu H, Jin Y, Zhang Y. Gene targeting by TALEN-induced homologous recombination in goats directs production of β-lactoglobulin-free, high-human lactoferrin milk. *Scientific Reports*, 2015, **5**(1): 10482

36. Moore F E, Reyon D, Sander J D, Martinez S A, Blackburn J S, Khayter C, Ramirez C L, Joung J K, Langenau D M. Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). *PLoS One*, 2012, **7**(5): e37877

37. Xiao A, Wu Y, Yang Z, Hu Y, Wang W, Zhang Y, Kong L, Gao G, Zhu Z, Lin S, Zhang B. EENdb: a database and knowledge base of ZFNs and TALENs for endonuclease engineering. *Nucleic Acids Research*, 2013, **41**(Database Issue): D415–D422

38. Cade L, Reyon D, Hwang W Y, Tsai S Q, Patel S, Khayter C, Joung J K, Sander J D, Peterson R T, Yeh J R J. Highly efficient generation of heritable zebrafish gene mutations using homo- and hetero-dimeric TALENS. *Nucleic Acids Research*, 2012, **40**(16): 8001–8010

39. Pattanayak V, Ramirez C L, Joung J K, Liu D R. Revealing off-

40. Gabriel R, Lombardo A, Arens A, Miller J C, Genovese P, Kaeppel C, Nowrouzi A, Bartholomae C C, Wang J, Friedman G, Holmes M C, Gregory P D, Glimm H, Schmidt M, Naldini L, von Kalle C. An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nature Biotechnology*, 2011, **29**(9): 816–823

41. Grau J, Boch J, Posch S. TALENoffer: genome-wide TALEN off-target prediction. *Bioinformatics*, 2013, **29**(22): 2931–2932

42. Sontheimer E J, Barrangou R. The bacterial origins of the CRISPR genome-editing revolution. *Human Gene Therapy*, 2015, **26**(7): 413–424

43. Deltcheva E, Chylinski K, Sharma C M, Gonzales K, Chao Y, Pirzada Z A, Eckert M R, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, 2011, **471**(7340): 602–607

44. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna J A, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 2012, **337**(6096): 816–821

45. Bolukbasi M F, Gupta A, Wolfe S A. Creating and evaluating accurate CRISPR-Cas9 scalpels for genomic surgery. *Nature Methods*, 2016, **13**(1): 41–50

46. Cong L, Ran F A, Cox D, Lin S, Barretto R, Habib N, Hsu P D, Wu X, Jiang W, Marraffini L A, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*, 2013, **339**(6121): 819–823

47. Mali P, Yang L, Esvelt K M, Aach J, Guell M, DiCarlo J E, Norville J E, Church G M. RNA-guided human genome engineering via Cas9. *Science*, 2013, **339**(6121): 823–826

48. Ma T, Tao J, Yang M, He C, Tian X, Zhang X, Zhang J, Deng S, Feng J, Zhang Z, Wang J, Ji P, Song Y, He P, Han H, Fu J, Lian Z, Liu G. An *AANAT/ASMT* transgenic animal model constructed with CRISPR/Cas9 system serving as the mammary gland bioreactor to produce melatonin-enriched milk in sheep. *Journal of Pineal Research*, 2017, **63**(1): e12406

49. Richt J A, Kasinathan P, Hamir A N, Castilla J, Sathiyaseelan T, Vargas F, Sathiyaseelan J, Wu H, Matsushita H, Koster J, Kato S, Ishida I, Soto C, Robl J M, Kuroiwa Y. Production of cattle lacking prion protein. *Nature Biotechnology*, 2007, **25**(1): 132–138

50. Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie W A, Rollo M, de Sousa P, Travers A, Wilmut I, Clark A J. Deletion of the α(1,3)galactosyl transferase (*GGTA1*) gene and the prion protein (*PrP*) gene in sheep. *Nature Biotechnology*, 2001, **19**(6): 559–562

51. Shanthalingam S, Tibary A, Beever J E, Kasinathan P, Brown W C, Srikumaran S. Precise gene editing paves the way for derivation of *Mannheimia haemolytica* leukotoxin-resistant cattle. *Proceedings of the National Academy of Sciences of the United States of America*, 2016, **113**(46): 13186–13190

52. Zhang J, Cui M L, Nie Y W, Dai B, Li F R, Liu D J, Liang H, Cang M. CRISPR/Cas9-mediated specific integration of fat-1 at the goat *MSTN* locus. *FEBS Journal*, 2018, **285**(15): 2828–2839

53. Zhang Y, Wang Y, Yulin B, Tang B, Wang M, Zhang C, Zhang W, Jin J, Li T, Zhao R, Yu X, Zuo Q, Li B. CRISPR/Cas9-mediated sheep *MSTN* gene knockout and promote sSMSCs differentiation. *Journal of Cellular Biochemistry*, 2018, **120**(2): 1794–1806

54. Bertolini L R, Meade H, Lazzarotto C R, Martins L T, Tavares K C, Bertolini M, Murray J D. The transgenic animal platform for biopharmaceutical production. *Transgenic Research*, 2016, **25**(3): 329–343

55. Houdebine L M. Production of pharmaceutical proteins by transgenic animals. *Comparative Immunology, Microbiology and Infectious Diseases*, 2009, **32**(2): 107–121

56. Yamasaki M, Sendall T J, Pearce M C, Whisstock J C, Huntington J A. Molecular basis of α1-antitrypsin deficiency revealed by the structure of a domain-swapped trimer. *EMBO Reports*, 2011, **12** (10): 1011–1017

57. Wang B, Baldassarre H, Tao T, Gauthier M, Neveu N, Zhou J F, Leduc M, Duguay F, Bilodeau A S, Lazaris A, Keefer C, Karatzas C N. Transgenic goats produced by DNA pronuclear microinjection of *in vitro* derived zygotes. *Molecular Reproduction and Development*, 2002, **63**(4): 437–443

58. Esslemont D, Kossaibati M. Mastitis: how to get out of the dark ages. *Veterinary Journal*, 2002, **164**(2): 85–86

59. Rainard P. Tackling mastitis in dairy cows. *Nature Biotechnology*, 2005, **23**(4): 430–432

60. Wall R J, Powell A M, Paape M J, Kerr D E, Bannerman D D, Pursel V G, Wells K D, Talbot N, Hawk H W. Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nature Biotechnology*, 2005, **23**(4): 445–451

61. Oldham E R, Daley M J. Lysostaphin: use of a recombinant bactericidal enzyme as a mastitis therapeutic. *Journal of Dairy Science*, 1991, **74**(12): 4175–4182

62. Lloyd S E, Mead S, Collinge J. Genetics of prion diseases. *Current Opinion in Genetics & Development*, 2013, **23**(3): 345–351

63. Wells G A, Scott A C, Johnson C T, Gunning R F, Hancock R D, Jeffrey M, Dawson M, Bradley R. A novel progressive spongiform encephalopathy in cattle. *Veterinary Record*, 1987, **121**(18): 419–420

64. Jeffrey M, González L. Classical sheep transmissible spongiform encephalopathies: pathogenesis, pathological phenotypes and clinical disease. *Neuropathology and Applied Neurobiology*, 2007, **33** (4): 373–394

65. Aguilar-Calvo P, García C, Espinosa J C, Andreoletti O, Torres J M. Prion and prion-like diseases in animals. *Virus Research*, 2015, **207**: 82–93

66. Prusiner S B, Groth D, Serban A, Koehler R, Foster D, Torchia M, Burton D, Yang S L, DeArmond S J. Ablation of the prion protein (*PrP*) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proceedings of the National Academy of Sciences of the United States of America*, 1993, **90**(22): 10608–10612

67. Breider M A, Walker R D, Hopkins F M, Schultz T W, Bowersock T L. Pulmonary lesions induced by *Pasteurella haemolytica* in neutrophil sufficient and neutrophil deficient calves. *Canadian Journal of Veterinary Research*, 1988, **52**(2): 205–209

68. Mueller M L, Cole J B, Sonstegard T S, Van Eenennaam A L. Comparison of gene editing versus conventional breeding to introgress the POLLED allele into the US dairy cattle population. *Journal of Dairy Science*, 2019, **102**(5): 4215–4226

69. Regalado A. Gene-edited cattle have a major screwup in their DNA. *MIT Technology Review*, 2019 [Published Online] https://www.technologyreview.com/s/614235/recombinetics-gene-edited-hornless-cattle-major-dna-screwup/

70. Norris L A, Lee S S, Greenlees K J, Tadesse D A, Miller M F, Lombard H. Template plasmid integration in germline genome-edited cattle. *BioRxiv*, 2019 [Published Online] doi:10.1101/715482

71. Kleinstiver B P, Pattanayak V, Prew M S, Tsai S Q, Nguyen N T, Zheng Z, Joung J K. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*, 2016, **529**(7587): 490–495

72. Slaymaker I M, Gao L, Zetsche B, Scott D A, Yan W X, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. *Science*, 2016, **351**(6268): 84–88

73. Tsai S Q, Nguyen N T, Malagon-Lopez J, Topkar V V, Aryee M J, Joung J K. CIRCLE-seq: a highly sensitive *in vitro* screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nature Methods*, 2017, **14**(6): 607–614

74. Tsai S Q, Zheng Z, Nguyen N T, Liebers M, Topkar V V, Thapar V, Wyvekens N, Khayter C, Iafrate A J, Le L P, Aryee M J, Joung J K. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology*, 2015, **33**(2): 187–197

75. Ehn B M, Ekstrand B, Bengtsson U, Ahlstedt S. Modification of IgE binding during heat processing of the cow's milk allergen β-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 2004, **52**(5): 1398–1403

76. Ehn B M, Allmere T, Telemo E, Bengtsson U, Ekstrand B. Modification of IgE binding to β-lactoglobulin by fermentation and proteolysis of cow's milk. *Journal of Agricultural and Food Chemistry*, 2005, **53**(9): 3743–3748

77. Grobet L, Martin L J, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Ménissier F, Massabanda J, Fries R, Hanset R, Georges M. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nature Genetics*, 1997, **17**(1): 71–74

78. Dove A. Milking the genome for profit. *Nature Biotechnology*, 2000, **18**(10): 1045–1048

79. Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G. Cloned transgenic cattle produce milk with higher levels of β-casein and κ-casein. *Nature Biotechnology*, 2003, **21**(2): 157–162

80. Colman A. Somatic cell nuclear transfer in mammals: progress and applications. *Cloning*, 1999–2000, **1**(4): 185–200

81. Zhang J P, Li X L, Li G H, Chen W, Arakaki C, Botimer G D, Baylink D, Zhang L, Wen W, Fu Y W, Xu J, Chun N, Yuan W, Cheng T, Zhang X B. Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. *Genome Biology*, 2017, **18**(1): 35

82. Song J, Yang D, Xu J, Zhu T, Chen Y E, Zhang J. RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. *Nature Communications*, 2016, **7**(1): 10548

83. Kipriyanov S M, Moldenhauer G, Braunagel M, Reusch U, Cochlovius B, Le Gall F, Kouprianova O A, Von der Lieth C W, Little M. Effect of domain order on the activity of bacterially produced bispecific single-chain Fv antibodies. *Journal of Molecular Biology*, 2003, **330**(1): 99–111

84. Little M, Kipriyanov S M, Le Gall F, Moldenhauer G. Of mice and men: hybridoma and recombinant antibodies. *Immunology Today*,

2000, **21**(8): 364–370

85. Walsh G. Biopharmaceutical benchmarks 2014. *Nature Biotechnology*, 2014, **32**(10): 992–1000

86. Grosse-Hovest L, Müller S, Minoia R, Wolf E, Zakhartchenko V, Wenigerkind H, Lassnig C, Besenfelder U, Müller M, Lytton S D, Jung G, Brem G. Cloned transgenic farm animals produce a bispecific antibody for T cell-mediated tumor cell killing. *Proceedings of the National Academy of Sciences of the United States of America*, 2004, **101**(18): 6858–6863

87. Fan Z, Perisse I V, Cotton C U, Regouski M, Meng Q, Domb C, Van Wettere A J, Wang Z, Harris A, White K L, Polejaeva I A. A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the *CFTR* gene. *JCI Insight*, 2018, **3**(19): e123529

88. Williams D K, Pinzón C, Huggins S, Pryor J H, Falck A, Herman F, Oldeschulte J, Chavez M B, Foster B L, White S H, Westhusin M E, Suva L J, Long C R, Gaddy D. Genetic engineering a large animal model of human hypophosphatasia in sheep. *Scientific Reports*, 2018, **8**(1): 16945

89. Du S J, Gong Z Y, Fletcher G L, Shears M A, King M J, Idler D R, Hew C L. Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. *Bio-Technology*, 1992, **10**(2): 176–181

90. Kling J. First US approval for a transgenic animal drug. *Nature Biotechnology*, 2009, **27**(4): 302–304

91. van Veen H A, Koiter J, Vogelezang C J M, van Wessel N, van Dam T, Velterop I, van Houdt K, Kupers L, Horbach D, Salaheddine M, Nuijens J H, Mannesse M L M. Characterization of recombinant human C1 inhibitor secreted in milk of transgenic rabbits. *Journal of Biotechnology*, 2012, **162**(2–3): 319–326

92. Sheridan C. FDA approves 'farmaceutical' drug from transgenic chickens. *Nature Biotechnology*, 2016, **34**(2): 117–119

93. Bruce A. Genome edited animals: learning from GM crops? *Transgenic Research*, 2017, **26**(3): 385–398

94. Ishii T. Genome-edited livestock: ethics and social acceptance. *Animal Frontiers*, 2017, **7**(2): 24–32

95. Schicktanz S. Ethical considerations of the human-animal-relationship under conditions of asymmetry and ambivalence. *Journal of Agricultural & Environmental Ethics*, 2006, **19**(1): 7–16