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Genome Engineering in Livestock: Recent Advances and Regulatory Framework

Dharmendra Kumar[1] and Wilfried A. Kues[2,*]

[1]Animal Physiology and Reproduction Division, ICAR-Central Institute for Research on Buffaloes, Hisar-125001, Haryana, India
[2]Friedrich-Loeffler-Institut, Federal Research Institute of Animal Health, Institute of Farm Animal Genetics, Department of Biotechnology, Stem Cell Physiology, Höltystr 10, 31535 Neustadt, Germany

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ABSTRACT

Since the domestication of animals, livestock species are an important source of protein-rich food and other animal products. The very recent progress in genetic engineering allows to modify the genomes of livestock species in an unprecedented way to improve production traits, disease resistance, adaptation to climate changes, and animal welfare aspects and for the development of large animal models for developmental biology and biomedicine. Here, we concisely summarize the recent progress of genome-editing technologies, with a particular focus on the CRISPR/Cas9 designer nuclease, in livestock. Currently, precision-modified livestock lines with disease resistance and production traits are ready to be introduced into commercial production. On a scientific basis, these lines are considered safe for human consumption, especially for genome edits implementing only a single nucleotide change, which mimics ´natural´ point mutations. Internationally, however, there are clear differences in the interpretation of the legal framework on whether genome-edited animals or their products need to be regulated.

Introduction

Humankind faces a major challenge to feed its growing population, which is expected to reach nearly 10 billion people by 2050, and in parallel coping with climate change. Agriculture is the main source of food for the world population, but its share in total food production is stagnating due to high-input and resource-intensive farming systems, massive deforestation, soil depletion, water scarcity, and high levels of greenhouse gas emissions. Livestock can contribute an instrumental role in achieving sustainable food security (Godber and Wall, 2014; Nabarro and Wannous, 2014; Selokar and Kues, 2018). The rising demand of animal-based foods has already led to an increase in livestock production, and this demand will surge in the coming years (Nabarro and Wannous, 2014). The classical approaches enhance animal productivity through the intervention of animal health, nutrition, genetics, repro-

*Corresponding author.
E-mail address: Wilfried.kues@fli.de (Wilfried A. Kues)
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duction and management, but these are unlikely to be sufficient to meet the required productivity (FAO. 2018) This scenario necessities novel ideas and technologies, and genome engineering appears as a powerful contribution to transforming global livestock production in a sustainable manner. Genome engineering or genome editing is a type of genetic manipulation to change the genetic makeup of cells using highly specific enzymes, such as programmable nucleases, and enable the targeted modification of selected DNA sequences and expression of genes. Recently developed genome editing technologies facilitated the introduction of targeted modifications and the production of genetically engineered livestock. These technological advancements significantly improved the productivity of livestock for agriculture purposes, and strengthen the field of biomedicine by providing animal models that are more accurately representing human diseases (Whitelaw et al., 2016; Hamernik, 2019; Menchaca et al., 2020; Lee et al., 2020; Perisse et al., 2021).

Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) systems emerged as the most efficient, accurate, repeatable, and straightforward genome editing technology (Doudna and Charpentier, 2014). CRISPR/Cas9 has a nuclease activity guided by a small RNA following Watson-Crick base pairing with target DNA, and represents a system that is easy to design, highly specific, efficient, and well-suited for high-throughput and multiplexed gene editing for a variety of cell types and organisms (Hsu et al., 2014). It allows modification of a target gene without the addition of foreign DNA to the animal's genome. Currently, precision-modified livestock lines with disease resistance and production traits are ready to be introduced into commercial production. On a scientific basis, these lines are considered safe for human consumption, especially for genome edits implementing only a single nucleotide change. Here, we summarize the recent progress of genome-editing technologies in livestock, with a focus on the CRISPR/Cas9 designer nuclease, put it in the context of classical transgenesis, and discuss the regulatory framework worldwide.

"Classic" Transgenesis in Livestock

Transgenesis is the process of introducing a gene of interest obtained from one organism into the genome of another organism, which expresses the gene and exhibits some new property or characteristic. This is made possible by the fact that the genetic code is universal for all living things. The first transgenic livestock were produced via microinjection of foreign DNA into pronuclei of fertilized eggs in 1985 (Hammer et al., 1985) and after that various methods such as viral vectors, sperm-mediated gene transfer (SMGT) and somatic cell cloning have been applied for the generation of transgenic livestock, including cattle, sheep, goats, and pigs (Kues and Niemann, 2011; Garrels et al., 2012; Amare and Ayalew, 2019). Each new method added some advancement and helped overcome the limitations of earlier methods. Commonly, transgenic livestock are being produced using established approaches, either DNA microinjection into zygotes (Garrels et al., 2016) or somatic cell nuclear transfer (SCNT) using a transgenic somatic cell as donor (Kuroiwa et al., 2020). Microinjection has several significant shortcomings, such as low efficiency, frequent incidence of mosaicism, random integration into the host genome, and a variable number of copies of the gene that integrates. SCNT allows targeted genetic modification of in vitro cultured somatic cells, which are then used to produce transgenic livestock via animal cloning (Schnieke et al., 1997; Denning et al., 2001; Park et al., 2001), but it is also associated with multiple bottlenecks (Review, Kumar et al., 2022). Using this approach, a limited number of site-specific genetically modified livestock models have been produced (Dai et al., 2002; Lai et al., 2006; Rogers et al., 2008). However, targeted modifications in somatic cells depend on the frequency of homologous recombination, which is extremely inefficient in somatic cells (Dai et al., 2002; Lai et al., 2002). The low efficiency allows for modification of only one allele at a time (Liu et al., 2015). Hence, further breeding is required to get homozygous modifications in genetically modified animals. Especially large animals, such as cattle with long gestation (9 months) and long generation intervals (22–26 months) required extended periods for the completion of breeding steps.

Recently, embryonic stem (ES) cell-mediated genetic modifications became a perfected approach for the production of transgenic and knock-out mice, but the lack of true ES cells from larger mammals prevented following this route for the generation of transgenic livestock. In addition, viral-mediated transgenesis via retrovirus, lentivirus, and adeno-associated virus, has also been used to successfully produce transgenic livestock (Lois et al., 2002; Pfeifer et al., 2002; Hofmann et al., 2003; Whitelaw et al., 2008; Pfeifer and Hofmann, 2009), but it is limited by the DNA cargo size. Another method, SMGT has also been assessed for the integration of exogenous DNA. It was demonstrated that sperm of many species showed the affinity to bind with naked DNA or liposome-DNA complex (Lavitrano et al., 1989; Horan et al., 1991; Bachiller et al., 1991; Sperandio et al., 1996), and could act as a vector for gene transfer into the oocyte. This methodology has been reported for mice (Lavitrano et al., 1989; Bachiller et al., 1991), pigs (Sperandio et al., 1996), rabbits (Kuznetsov

and Kuznetsova, 1994), chicken (Rottmann et al., 1992), and cattle (Schellander et al., 1995; Sperandio et al., 1996). However, transferring exogenous DNA to the embryo and further to progeny has been found to be difficult and currently SMGT remains an unreliable method for producing transgenic livestock (Smith and Spadafora, 2005). In summary, classical transgenesis techniques are not efficient for the production of livestock with desired genetic modifications, thus alternative approaches such as enzyme-mediated genetic engineering and genome-editing technologies have been promoted.

Enzyme-mediated Genetic Engineering in Livestock

A relative recently, transgenesis was achieved via exogenous enzymes, which provide specific gain-of-function or loss-of-function genetics and allow for precise genetic modifications in livestock (Shinohara et al., 2007; Garrels et al., 2012; Bosch et al., 2015). Mainly, the hyperactive form of transposon systems such as Sleeping Beauty (Zayed et al., 2004; Mates et al., 2009) and piggyBac (Yusa et al., 2011) are being used as exogenous enzymes to produce transgenic mammals. In addition, Cre recombinase and ΦC31 integrase gained some attention for livestock transgenesis (Jakobsen et al., 2013; Yu et al., 2013).

Transposons, or jumping genes, are discrete pieces of DNA that have the ability to move from one site to another within a genome using a 'cut and paste' mechanism (Ivancevic et al., 2013; Walsh et al., 2013; Kumar et al., 2015). The best characterized transposons are Sleeping Beauty, piggyBac, and Tol2, which originate from non-mammalian species (Kawakami et al., 2000; Miskey et al., 2003; Ivics et al., 2009; Clark et al., 2009). For genetic engineering, the Sleeping Beauty and piggyBac transposons have been developed as bicomponent transgenic systems in which the gene of interest is flanked by inverted terminal repeats (ITRs), and the transposase is delivered in trans. The transposase binds to the ITRs region forming a synaptic complex that leads to the transposon being introduced into a genomic consensus site (Ivics et al., 2009; Mun˜oz-Lo´pez and Garcı´a-Pe´rez, 2010). Sleeping Beauty and piggyBac transposon systems were used to produce transgenic livestock such as cattle (Garrels et al., 2016; Yum et al., 2016; Yum et al., 2018), sheep (Deng et al., 2017), goats (Bai et al., 2017) and pigs (Garrels et al., 2011; Carlson et al., 2011; Ivics et al., 2014). The transposon system is considered as valuable tool for the efficient genomic integration and stable expression of transgenes with germline transmission capability. These results demonstrate that transposon-mediated transgenesis is capable of increasing the transgenic efficiencies and producing antibiotic marker-free animals, which satisfy regulatory guidelines for animal transgenesis (Bosch et al., 2015).

Genome Editing in Livestock

In the past decade, engineered designer nucleases have emerged as a new approach for "genome editing". The engineered nucleases include zinc finger nucleases (ZFNs), transcription activator-like endonucleases (TALENs), and CRISPR/Cas9. CRISPR/Cas9 turned out to be the simplest and most predictable designer nuclease and has the capability of simultaneously targeting multiple genomic sites. The mechanisms of designer nucleases for genome editing of livestock have been reviewed earlier (Petersen, 2017; Kalds et al., 2019; Navarro-Serna et al., 2020; Kumar and Kues, 2020; Perisse et al., 2021), and we are focusing on recent applications of CRISPR/Cas9 for livestock genome editing and the current legal framework. The focus on CRISPR/Cas9 results from the over proportional use of this technology for livestock editing (Fig. 1).

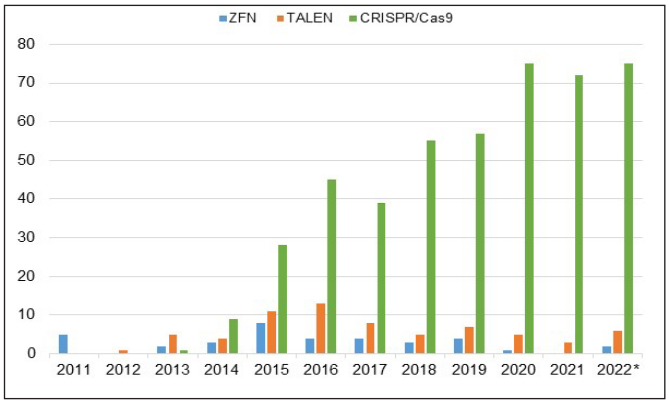

Fig. 1. Genome editing in livestock. The number of publications describing the use of a designer nuclease in livestock per year, data was extracted from PubMed (NCBI) using the terms acronym of "designer nuclease" and "livestock or farm animal" and "year", accessed 13.09.2022.

The CRISPR/Cas9-mediated genome engineering technologies allow performing precise genetic modifications, which dramatically enhance the ease and speed for producing genetically modified livestock (Zhao et al., 2019; Van Eenennaam, 2019; Menchaca et al., 2020; Lee et al., 2020; Navarro-Serna et al., 2020; Bishop and Van Eenennaam, 2020; Dua et al., 2021). CRISPR/Cas9 even allowed genome editing in non-human primates, which are hard to tackle (Niu et al., 2014; Kues et al., 2022). In the same year, genome-edited pigs were produced using the CRISPR/Cas9 system in which the von Willebrand factor (vWF) gene was targeted in order to generate a medical model with reduced activity of coagulation factor VIII leading to severe bleeding closely mimicking the human

disease (Hai et al., 2014). Thereafter, genome engineering technologies have been applied in many livestock such as cattle, sheep, goat and pig for improvement of growth performance, development of disease-resistant lines, enhancement of animal welfare, and for generation of models for human diseases (Table 1).

For improvement in the performance of livestock, knockout of the myostatin (MSTN) gene is the most prominent example due to its association with growth and skeletal muscle development. Earlier, it was demonstrated that the knockout of MSTN leads to enhanced formation of skeletal muscles (Kambadur et al., 1997), an economically important trait associated with economic meat production. CRISPR/Cas9 technology has been successfully applied to knockout the MSTN from the genomes of sheep (Zhang et al., 2018), goat (He et al., 2018) and pig (Tanihara et al., 2016). Another significant accomplishment has been the production of tuberculosis-resistant cattle and porcine reproductive and respiratory syndrome (PRRS) virus-resistant pigs, both of which are economically important in the livestock industry. Tuberculosis-resistant cattle were produced by insertion of natural resistance to infection with intracellular pathogens 1 (NRAMP1) gene into bovine fetal fibroblasts followed by SCNT (Tuggle and Waters, 2015). The genome-edited animal showed higher expression of the NRAMP1 gene and exhibited higher degree of resistance to *Mycobacterium bovis* infection (Tuggle and Waters, 2015). PRRS is considered an important infectious disease of the swine industry worldwide, affecting the production, reproduction, health, and welfare of pigs. Whitworth et al. reported the production of CD163-knockout pigs showing protection against the clinical outcome of PRRS virus infection (Whitworth et al., 2016). The CD163 knockout pigs or pigs with a deletion of the virus binding domain showed resistance towards exposure to the PRRS virus (Whitworth et al., 2016; Wells

Table 1. Examples of the CRISPR/Cas9-mediated genome-edited livestock for agricultural and biomedical purposes

Species	Gene	Cell type	Transduction Method	Mutation and repair mechanism	Purpose	Reference
Cattle	*NRAMP1*	FF, SCNT	Electroporation	HDR-KI	Disease resistance to tuberculosis	Gao et al., 2017
	IARS	FF or ear-derived fibroblast, SCNT	Electroporation	HDR- single base substitution	Disease resistance to IARS syndrome	Ikeda et al., 2017; Ishino et al., 2018
	PRNP	Zygote	CPI	HDR-KI	Resilience towards human prion pandemics	Park et al., 2020
Sheep	*MSTN*	Zygote	CPI	NHEJ-KO	Meat production, composition and quality	Crispo et al., 2015
	MSTN, ASIP and *BCO2*	Zygote	CPI	NHEJ-KO	Meat production, composition and quality	Wang et al., 2016
	FGF5	Zygote	PNI	NHEJ-KO	Negative regulator of wool length	Hu et al., 2019
	MSTN	Ear fibroblasts	Electroporation	NHEJ-KO	Meat production, composition and quality	Zhang et al., 2018
	OTOF	Zygote	CPI	ssODN induce HDR repair	Model for human deafness related to genetic disorders	Menchaca et al., 2020
Goat	*MSTN*	FF	Nucleofection	NHEJ-KO	Meat production, composition and quality	Ni et al., 2014
	MSTN	Zygote	CPI	NHEJ-KO	Meat production, composition and quality	Guo et al., 2016
	MSTN	Zygote	CPI	NHEJ-KO	Meat production, composition and quality	He et al., 2018
	GDF9	Zygote	CPI	ssODN mediated HDR-single base substitution	Increases litter size	Niu et al., 2018
	C. elegans fat-1	FF, SCNT	Electroporation	HDR-KI & KO	Convert n-6 PUFA into n-3 PUFA) into MSTN locus	Zhang et al., 2018

Species	Gene	Cell type	Transduction Method	Mutation and repair mechanism	Purpose	Reference
Pig	*CD163*	FF, SCNT	Electroporation/ Nucleofection	HDR-KI & KO	Disease resistance to PRRSV	Whitworth et al., 2014; Chen et al., 2019
	NPC1L1	Zygote	CPI	NHEJ-KO	Disease model for cardiovascular and metabolic diseases	Wang et al., 2015
	PARKIN/ DJ-1/PINK1	Zygote	CPI	NHEJ-KO	Disease model for PD	Wang et al., 2016
	MSTN	Zygote	Electroporation	NHEJ-KO	Optimization of protocol for efficient generation of genetically modified pigs	Tanihara et al., 2016
	NANOS2	Zygote	CPI	NHEJ-KO	Male model as surrogates for SSC transplantation	Park et al., 2017
	TP53	Zygote	Electroporation	NHEJ-KO	Model for cancer disease	Tanihara et al., 2018
	shRNAs	FF, SCNT	Electroporation	HDR-KI	Resistance to classical swine fever virus	Xie et al., 2018
	HTT	FF, SCNT	Electroporation	KI	Disease model for HD	Yan et al., 2018
	ZBED6	PFF, SCNT	Electroporation	NHEJ-KO	Improve lean meat percentage	Liu et al., 2019

ANPEP, Amino peptidase N; ASIP, agouti signaling protein; BCO2, β-carotene oxygenase 2; CPI, cytoplasmic injection; FF, fetal fibroblast; GDF9, growth differentiation factor 9; HD, Huntington disease; HDR, homology-directed repair; KI, knock-in; MSTN, myostatin; NHEJ, nonhomologous end-joining; Npc1l1, Niemann-Pick C1-Like 1; NRAMP1, natural resistance-associated macrophage protein-1 gene; PD, Parkinson's disease; PNI, pronuclear injection; PRNP, prion protein; PRRSV, Porcine reproductive and respiratory syndrome virus; PUFA, polyunsaturated fatty acid; SCNT, somatic cell nuclear transfer; shRNAs, antiviral small hairpin RNAs; SSC, spermatogonial stem cells; ssODN, single strand oligodeoxynucleotides; TPH2, tryptophan hydroxylase-2; ZBED6, zinc finger BED-type containing 6.

et al., 2017; Burkard et al., 2017), indicating that a small deletion of the sequence encoding the virus-attachment site could establish a disease-resistant breed.

Livestock is also assessed as a bioreactor to produce human biological products such as albumin. The blood-derived human serum albumin (HSA) is recommended for a number of severe diseases, such as liver failure and traumatic shock, and is in high demand. Due to the shortage of human blood supplies and the infection risks associated with human blood, alternative ways to produce HSA have long been sought. Success was obtained when CRISPR/Cas9 was used to knock in human albumin cDNA to the pig endogenous albumin locus, leading to transgenic piglets with human albumin in their blood (Peng et al., 2015).

Using CRISPR/Cas9, a mouse adiponectin-UCP1 gene was inserted into the endogenous UCP1 locus of pig, which showed an improved ability to maintain body temperature during acute cold exposure with normal physical activity (Zheng et al., 2017). Previously, it was reported that UCP1-knockin pigs were able to produce more lean meat with less fat deposition compared with control pigs, making them a valuable resource for the pig industry (Wang et al., 2016). Park et al. recently reported using the CRISPR/Cas tool to introduce a novel prion protein (PRNP) allelic variant in the cattle genome and produce cattle with prion disease resistance (Park et al., 2020).

Due to the similarity in anatomy, size, and physiology of porcine organs with their human counterparts, the pig is considered the most suitable model for human diseases such as atherosclerosis, Parkinson's disease, Huntington's disease, cardiovascular diseases, diabetes, ophthalmological diseases, and neuronal disorders (Garrels et al., 2016; Gün and Kues, 2014; Holm et al., 2016; Schook et al., 2016). To understand the molecular mechanism of human atherosclerosis, LDLR, and apolipoprotein E (ApoE) double knockout pigs were produced using CRISPR/Cas9, which exhibited elevated levels of low-density lipoprotein cholesterol (LDL-C), apolipoprotein B, and total cholesterol (TC) in serum (Huang et al., 2017). Researchers created CRISPR/Cas9-mediated double DJ1, PARK2/PINK1, or Parkin/DJ-1/

PINK1 triple knockout pigs mimicking Parkinson's disease (Zhou et al., 2015; Yao et al., 2015; Wang et al., 2016) and Huntingtin knock-in pig model for Huntington´s disease (Yan et al., 2018), which could help in evaluating the pathology of the disease and for development of the therapeutic intervention. Li et al. created mutant pigs for the study of behavioral and neuropsychiatric disorders of humans by deleting tryptophan hydroxylase-2 (TPH2) using CRISPR/Cas9 (Li et al., 2017). The generated pigs were able to produce reduced levels of serotonin which impaired pup survival and growth rates. In another study, tyrosinase (TYR) was biallelically mutated with CRISPR/Cas9, resulting in complete loss of pigmentation in the skin, hair, and eyes of the produced pigs that could be useful to elucidate the genetics of human skin pigmentation (Zhou et al., 2015). Recently, Han and coworkers produced a model pig by deletion of Hoxc13 gene for ectodermal dysplasia-9 (ED-9), a human disease characterized by reduced hair follicles, external hair loss, and abnormal hair follicle structure, but normal skin structure, skeleton phenotype, and growth pattern (Han et al., 2017). Recently, Menchaca et al. produced otoferlin gene-edited sheep as a model which may allow a better understanding and development of new therapies for human deafness related to genetic disorders (Menchaca et al., 2020).

Controversial Issues about Genome-edited Animals

The CRISPR/Cas9 system facilitates genetic alterations by enhancing DNA mutation frequency via the generation of a double-strand break (DSB) at a predetermined genomic site. The DSB activates the machinery for the repair of endogenous cellular DNA either by blunt-end, non-homologous end-joining (NHEJ) or by homologous dependent repair (HDR) mechanisms (Bauer et al., 2015). The NEHJ is used by most cell types, which is perhaps an error-prone mechanism that generally results in minor insertions or deletions (indels) at the site of repair. NEHJ-introduced mutations are likely to lead to a frame shift and a functional gene knockout.

On the other hand, HDR is a precise repair pathway that requires an endogenous or exogenous piece of homologous DNA as a template or "donor" for the repair of the DNA break. It enables to introduction a specific mutation ranging from single base changes to the insertion of transgenes (Urnov, 2018). Commonly, HDR-mediated genome editing relied on the co-delivery of an engineered endonuclease and a circular plasmid donor construct. More recently, it was demonstrated that single-stranded oligodeoxynucleotides (ssODNs) can serve as DNA donor templates and thus obviate the more laborious and time-consuming plasmid vector construction process (Chen et al., 2015). The ssODN-mediated gene editing efficiency critically depends on the distance between the editing site and the DSB. For high targeting efficiency, it is advantageous to place the DSBs in close proximity to the genome editing sites. In general, CRISPR/Cas9-mediated editing efficiency decreases significantly when the editing site is more than 20 bp away from the cleavage site (Boel et al., 2018; Bollen et al., 2018). The homology arm of the ssODNs may vary from 30 to 60 nucleotides on each side, but 52 nucleotides seem to be optimal for efficient integration. A few reports also suggested that asymmetric arms of ssODNs showed enhanced gene editing efficiency (Yang et al., 2013; Richardson et al., 2016). In livestock, ssODN-mediated editing efficiency for knock-in or knock-out has been shown in sheep (Williams et al., 2018; Eaton et al., 2019), goats (Niu et al., 2018) and cattle (Perota et al., 2019). Now, most of the livestock genome editing efforts has been shifted to using ssODN-mediated repair templates to reduce the frequency of unintended genomic integration (McFarlane et al., 2019). Larger insertions require plasmid or dsDNA templates with homology arms of 1-3 kb at either side of a DSB site, but its efficiency is lower than with ssODN (Chen et al., 2011).

The induction of DSBs at unintended locations leads to off-targets or undesirable mutations (Zhang et al., 2015; Doench et al., 2016; Li et al., 2018; Carey et al., 2019). The off-targets could result in unwanted side effects in edited animals (Ishii, 2017; Schultz-Bergin, 2018; de Graeff et al., 2019; Ayanoğlu et al., 2020). A recent example of off-targeting is the production of hornless cattle, which carried an unintended insertion of the HDR plasmid backbone (Young et al., 2020). However, the recent scientific intervention has significantly expanded the toolkit of CRISPR/Cas, which is able to prevent off-target activity from the genome editing systems. For example, use of Cas9-nickase, a variant of Cas9 protein which causes only a single strand break instead of DSBs, is able to reduce off-target events (Komor et al., 2018; Naeem et al., 2020). Using these approaches several genetically engineered livestock models have been produced (Gao et al., 2017; Park et al., 2017). Similarly, the use of base editor variants of Cas may result in reduced unwanted modifications, since the DNA backbone is not cut at all (Li et al., 2018; Xie et al., 2019). In addition, off-targets can either be removed in the subsequent generations through selected breeding or disappear by drift if they are neutral (Ruan et al., 2015). Apart from off-targeting, animal dignity, changes in their natural

environment and physiological needs are also concerns of gene editing technology (Manesh et al., 2014; Eriksson et al., 2018). Recently, bioethical issues associated with CRISPR/Cas9-mediated genome editing have been reviewed (Ayanoğlu et al., 2020).

The Legal Framework of Genome-edited Animals in Important Agricultural Nations

Genome engineering technologies have recently gained momentum due to their ability to precisely modify the genome, which is likely to transform animal production. The technology of genome editing differs from classical transgenesis, therefore the regulations applied to transgenics may not apply to gene-edited organisms (Ruan et al., 2015; Caplan et al., 2015). Because CRISPR/Cas9 can be used to make single base pair modifications in a mammalian genome, identifying and regulating genetically modified livestock in the market is difficult. A single base pair modification can, of course, be sequenced, but sequencing cannot distinguish between spontaneous mutations and intentionally introduced base changes through genome editing. In case the genome-edited product is labelled as such, then sequencing can be used to verify the mutation. Legally, to discriminate the products of new breeding technologies (NBT) from classical transgenesis the terminology of sited directed nuclease (SDN) action is often used for the classification of plants resulting from gene editing, but can be analogously used for animals. Organisms of the SDN-1 classification result from a DSB caused by a programmable nuclease, but without the addition of foreign repair DNA. The cellular repair can lead to a mutation or indel formation. SDN-2 involves the addition of a small nucleotide template that is complementary to the sequences around the DSB. As a result, the desired base change or addition/deletion of a few bases is produced. In principle, the outcomes of SDN-1 and SDN-2 changes are identical to point mutations or indels, which may also happen spontaneously or in forced mutagenesis approaches. In SDN-3 changes a large repair template is supplemented, which may carry a whole gene or other sequences. Thus, the SDN-3 scenario is similar to classical transgenesis approaches.

However, one has to keep in mind that these are legal classifications, which are not necessarily meaningful in scientific terms. The legal terms to classify the different approaches more or less reflect the knowledge at the time of the Asilomar Conference on Recombinant DNA in 1975; basically assumed that genomes are static and evolve essentially only by replication errors resulting in point mutations. Meanwhile, scientific advancements, particularly whole genome sequencing projects, have revealed that even eukaryotic genomes are highly dynamic and contain numerous "foreign" DNA sequences. In humans this includes LINE, SINE, retrotransposons, and DNA transposons, which together make up about 45 % of the genome. The genomes of livestock species show similar compositions. Thus emphasizing that genomes are highly dynamic in evolutionary time frames, and that large insertions and recombination events, for example by mobile DNA elements, are naturally occurring and shaping the genome. Thus, discrimination between intentionally introduced point mutations, small and large insertions (or deletions), and more complex recombination events is highly artificial, all of these genome changes occur naturally, and horizontal gene transfer between viruses, bacteria, and eukaryotes of different clades seems to be more common than previously assumed.

Several national regulatory agencies, such as the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and others, but also the European Court of Justice (ECJ), are struggling to establish legal frameworks to evaluate genome-edited animals (Ledford, 2015; Hundleby and Harwood, 2019; Menchaca et al., 2020). The main regulatory framework governing worldwide the improvement and use of genetically modified organisms is the United Nations Convention on Biological Diversity, implemented by the Cartagena and Nagoya Protocols, but key players like the USA did not ratify these treaties. Criteria are needed to be established for evaluating the safety of CRISPR/Cas-edited livestock which additionally accelerates the development of genetically modified animals, organs, and tissues for regenerative medicine, therapeutic applications, and for human consumption (Zhao et al., 2019; Bishop and Van Eenennaam, 2020). Currently, different countries have their own legal framework toward genome-edited animals and animal products, some examples are listed below.

United States of America (USA)

In the USA, several agencies regulate genetically modified organisms, such as the FDA, the US Environmental Protection Agency (EPA), and the National Institutes of Health (NIH). In 2009 FDA created the Federal Food Drug & Cosmetic Act to regulate DNA constructs in genetically-engineered animals, food, or feed coming from biotechnology. The United States Department of Agriculture (USDA) stated that products derived from genetically modified plants using genome editing tools without exogenous DNA insertion would be exempt from the regula-

tions. As consequence, several varieties of gene edited plants and fungi are not regulated by the agency (Selokar and Kues, 2018). In parallel, in 2017, the FDA drafted a guideline entitled "Regulation of Intentionally Altered Genomic DNA in Animals" and proposed to regulate all animals whose genomes have been deliberately altered using modern molecular technologies and considered them as new animal drugs. The proposed idea will prevent the progress of gene editing to solve zoonotic disease and animal welfare problems in the USA (Van Eenennaam, 2019; Mueller et al., 2019). Recently, a scientific meeting on Plant and Animal Genome Editing (https://www.gopetition.com/petitions/harmonize-us-geneedited-food-regulations.html) emphasized the harmonization of regulations on plants and food animals that could otherwise have been developed through traditional breeding techniques and suggested that they are not subject to additional pre-market regulatory requirements based solely on the fact that deliberate genomic alterations were introduced using modern biotechnologies in the breeding process.

Argentina

Recently, Argentina proposed an approach to develop a regulation and evaluation system for the biosafety of genome-edited organisms (Whelan and Lema, 2015). It was announced that the genome-edited products with no base pair insert would fall outside the regulations of the legal regulatory framework, but products having a large DNA fragment insertion should be regulated on a case-by-case basis (Araki et al., 2014). The National Advisory Commission on Agricultural Biotechnology (CONABIA) of Argentina recently received a proposal for the evaluation of gene-edited animals that do not contain any foreign DNA or a new combination of genetic material and declared them exempt from genetic modification regulation. This also seems to be applicable on gene edited fish, cattle, and horses in the country (Whelan and Lema, 2015).

European Union (EU)

In the EU, genome-edited organisms including livestock are required to undergo both environmental, food, and feed risk assessments. A guideline has been developed for the risk assessment of genetically modified animals and plants by the European Food Safety Authority (EFSA) for the environment and food and feed within the framework of European regulations (Kawall et al., 2020). The EU legal framework regulates a process-based approach while most other countries have a stronger emphasis on product-oriented regulation (Eriksson et al., 2018). In consequence, the ECJ judged in 2018 that all genome-edited organisms are genetically modified organisms (GMOs) and must be handled according to the gene law rules. However, the EU Commission considers that the current GMO legislation is not adequate for some NBTs and their products and needs to be adapted to scientific and technical progress. As a result, the EU Commission intends to launch policy measures for plants resulting from targeted mutagenesis and cisgenesis (the transfer of genetic material within a species) and has published its previously announced impact assessment in the first phase. (https://ec.europa.eu/food/plants/genetically-modified-organisms/new-techniques-biotechnology/ec-study-new-genomic-techniques_en).

China

As of now, there is no regulatory framework in China for gene editing in animals, but the Ministry of Agriculture has introduced Regulations on Administration of Agricultural Genetically Modified Organisms Safety, which govern gene-edited animals and are considered genetically modified organisms. China has strict regulations for genome-edited animals and their products, but there is extensive research being done on this aspect, so many researchers and companies believe China will decide to regulate most gene editing techniques as conventional animals (https://CRISPR-gene-editing-regs-tracker.geneticliteracyproject.org/china-animals/).

Canada

The genome editing technology in animals for research is governed by Environment and Climate Change, Canada (ECCC), which regulates the environmental and human risk assessments in genetically modified animals. Further, gene-edited food, including animals is being regulated through the Food and Drugs Act and Regulations under the umbrella of the Canadian Food Inspection Agency (CFIA), Canada. Most of the mutagenic products currently being developed are not considered organisms with novel traits, and it is likely that this will also be the case for most gene-edited organisms, which will therefore be regulated as conventional. In 2018, Canada and 12 other nations, including Argentina, Australia, Brazil, and the US, issued a joint statement to the World Trade Organization supporting relaxed regulations for genome editing, stating that governments should "avoid arbitrary and unjustifiable distinctions" between animals developed through gene editing and animals developed through conventional breeding (https://CRISPR-gene-editing-regs-tracker.geneticliteracyproject.org/canada-animals/).

Brazil

In 2018, The National Technical Biosafety Commission (CTNBio) released Normative Resolution No. 16, focusing on NBTs. It clarified that gene-edited animals that do not contain foreign DNA are regulated on a case-by-case basis and considered as conventional animals. The regulatory authority will evaluate the characteristics of the final product rather than the process used to create it. Even then, the government thoroughly evaluates the risk assessment of each newly developed animal or food derived using new genetic material or the product has already been approved for commercialization in other countries. The gene-edited hornless cow has now also been decided for regulation as a conventional organism in Brazil (https://CRISPR-gene-editing-regs-tracker.geneticliteracyproject.org/brazil-animals/).

Australia

In Australia, gene edited animals are regulated by the Gene Technology Regulator (GTR) under the Gene Technology Regulations 2001. Amendments have been made in 2019 in the GTRs on new techniques that cut the genome at a specific location, SDN-1 techniques, are not regulated because they are more like traditional mutagenesis techniques. On the other hand, gene editing techniques that involve the introduction of gene sequence using SDN-2 and SDN-3 techniques will be regulated under existing gene technology legislation. For example, gene-edited hornless cattle, which carry an introduced DNA insertion from other cattle breeds that is naturally hornless, will fall under the regulation.

In Australia, the regulations adopted for gene editing in animals are on 'the middle ground' between more lenient gene-editing rules in Brazil and Argentina and tougher measures in the EU. At the moment, no gene-edited animals have been approved in Australia (https://CRISPR-gene-editing-regs-tracker.geneticliteracyproject.org/australia-animals/).

Russia

In Russia, genetically engineered organisms are regulated by the Federal Service for Surveillance of Consumer Rights Protection and Human Welfare. This regulatory framework is responsible for developing legislation on genetically engineered products and monitoring the influence of these products on people and the environment. The Ministry of Agriculture is responsible for developing policies for the use of genetically engineered organisms in agriculture. In 2018, the Ministry of Agriculture published the first draft of a set of proposed guidelines for the required safety assessments and testing of genetically engineered animals. In 2019, a federal program announced that some gene editing techniques would be exempt from a 2016 law that banned the cultivation of genetically engineered organisms except for research purposes. In addition, a program has been launched by decree to suggest that gene editing is equivalent to conventional breeding methods. The federal program aims to develop 30 gene-edited plant and animal varieties in the next decade (https://CRISPR-gene-editing-regs-tracker.geneticliteracyproject.org/russia-animals/).

India

The genetically engineered products are being regulated using 'Rules for the Manufacture, Use, Import, Export, and Storage of Hazardous Microorganisms, Genetically Engineered Organisms or Cells", 1989 (Rules 1989), under the guidance of the Environment (Protection) Act, 1986. The new genome engineering technologies such as CRISPR/Cas9 and gene drives may also be covered under the rules. The Department of Biotechnology recently published a draught guideline for gene editing regulation, proposing strict regulation of gene-edited organisms (https://dbtindia.gov.in/sites/default/files/Draft_Regulatory_Framework_Genome_Editing_9 Jan 2020a.pdf). In India, the Genetic Engineering Appraisal Committee (GEAC) is responsible for the approval of genetically engineered organisms for research, development, and cultivation. In Rules 1989, genetic engineering defined as modification, deletion or removal of parts of heritable material which infers that all new gene editing technologies will be subject to regulation under the provision of the Rules, 1989 (Raman, 2017). In view of the present definition, it is expected that regulatory considerations for new and emerging technologies will be on a caseby-case basis based on the existing regulatory framework (https://CRISPR-gene-editing-regs-tracker.geneticliteracyproject.org/india-animals/).

Japan

Just recently, the first genome-edited animal was approved for commercialization in Japan. Recently, a startup called Regional Fish Co., together with the Kyoto University and Kinki University, has created a gene-edited red sea bream with a knockout of the MSTN gene that puts on up to about 20 percent more edible meat. At the same time, they show better feed conversion than conventional bream. CRISPR-mediated leptin receptor gene editing has recently resulted in tiger puffer fish with rapid weight gain. The developers have completed all regulatory procedures, and two

genome-edited fish were approved for commercial sale in Japan in October and November 2021 (https://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=19061). In Japan, applications for approval of organisms modified using genome editing are evaluated on a case-by-case basis. The responsible ministry (The Ministry of Health, Labor and Welfare) now approved the edited fish for food production since they do not contain extra DNA (https://www.asahi.com/ajw/articles/14445610). The company promises to label them as "genome-edited". However, labeling is not mandatory in Japan.

Conclusion

CRISPR/Cas9-mediated genome editing emerged as a breakthrough tool addressing challenges associated with livestock productivity, health and welfare, environmental preservation, and impacts on human health. CRISPR/Cas9-mediated genome editing provides revolutionary ways to change and regulate the genomes of large animals due to its high precision, which minimizes the risk of off-target mutations and increases consumer acceptance of food products derived from genome-edited livestock. Although genome editing using the CRISPR/Cas system and its expanded tools is effective and specific, the safety and ethical standards of animals and their products remain a focus of considerable debate. Some countries already deregulated genome editing in livestock equivalent to conventional breeding, whereas others consider it to result in GMOs or new animal drugs. Thus, regulations associated with genome-edited animals and their products are more political than scientifically-based in nature. Given the conflicting international assessments and the lack of reliable tracking methods, we believe there will be no agreement on this issue and that genome-edited livestock will be (de) regulated and integrated into the food chain in major agricultural countries.

Author Contributions

DK and WAK drafted and wrote the review.

References

Amare BA, Ayalew A. Animal transgenesis technology: A review. Cogent. Food & Agriculture, 2019; 5:1. doi: 10.1080/23311932.2019.1686802.

Araki M, Nojima K, Ishii T. Caution required for handling genome editing technology. Trends Biotechnol. 2014;32(5):234-7. doi: 10.1016/j.tibtech.2014.03.005.

Ayanoğlu FB, Elçin AE, Elçin YM. Bioethical issues in genome editing by CRISPR-Cas9 technology. Turk J Biol. 2020;44(2):110-120. doi: 10.3906/biy-1912-52.

Bachiller D, Schellander K, Peli J, Rüther U. Liposome-mediated DNA uptake by sperm cells. Mol Reprod Dev. 1991;30(3):194-200. doi: 10.1002/mrd.1080300305.

Bai DP, Yang MM, Qu L, Chen YL. Generation of a transgenic cashmere goat using the piggyBac transposition system. Theriogenology. 2017;93:1-6. doi: 10.1016/j.theriogenology.2017.01.036.

Bauer DE, Canver MC, Orkin SH. Generation of genomic deletions in mammalian cell lines via CRISPR/Cas9. J Vis Exp. 2015;(95):e52118. doi: 10.3791/52118.

Bishop TF, Van Eenennaam AL. Genome editing approaches to augment livestock breeding programs. J Exp Biol. 2020;223(Pt Suppl 1):jeb207159. doi: 10.1242/jeb.207159.

Boel A, De Saffel H, Steyaert W, Callewaert B, De Paepe A, Coucke PJ, Willaert A. CRISPR/Cas9-mediated homology-directed repair by ssODNs in zebrafish induces complex mutational patterns resulting from genomic integration of repair-template fragments. Dis Model Mech. 2018;11(10):dmm035352. doi: 10.1242/dmm.035352.

Bollen, Y.; Post, J.; Koo, B.K.; Snippert, H.J.G. How to create state-of-the-art genetic model systems: strategies for optimal CRISPR-mediated genome editing. Nucleic Acids Res. 2018,46(13),6435-6454.

Bosch P, Forcato DO, Alustiza FE, Alessio AP, Fili AE, Olmos Nicotra MF, Liaudat AC, Rodríguez N, Talluri TR, Kues WA. Exogenous enzymes upgrade transgenesis and genetic engineering of farm animals. Cell Mol Life Sci. 2015;72(10):1907-29. doi: 10.1007/s00018-015-1842-1.

Burkard C, Lillico SG, Reid E, Jackson B, Mileham AJ, Ait-Ali T, Whitelaw CB, Archibald AL. Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. PLoS Pathog. 2017;13(2):e1006206. doi: 10.1371/journal.ppat.1006206.

Caplan AL, Parent B, Shen M, Plunkett C. No time to waste--the ethical challenges created by CRISPR: CRISPR/Cas, being an efficient, simple, and cheap technology to edit the genome of any organism, raises many ethical and regulatory issues beyond the use to manipulate human germ line cells. EMBO Rep. 2015;16(11):1421-6. doi: 10.15252/embr.201541337.

Carey K, Ryu J, Uh K, Lengi AJ, Clark-Deener S, Corl BA, Lee K. Frequency of off-targeting in genome edited pigs produced via direct injection of the CRISPR/Cas9 system into developing embryos. BMC Biotechnol. 2019;19(1):25. doi: 10.1186/s12896-019-0517-7.

Carlson DF, Garbe JR, Tan W, Martin MJ, Dobrinsky JR, Hackett PB, Clark KJ, Fahrenkrug SC. Strategies for selection marker-free swine transgenesis using the Sleeping Beauty transposon system. Transgenic Res. 2011;20(5):1125-37. doi: 10.1007/s11248-010-9481-7.

Chen F, Pruett-Miller SM, Davis GD. Gene editing using ssODNs with engineered endonucleases. Methods Mol Biol. 2015;1239:251-65. doi: 10.1007/978-1-4939-1862-1_14.

Chen F, Pruett-Miller SM, Huang Y, Gjoka M, Duda K, Taunton J, Collingwood TN, Frodin M, Davis GD. High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. Nat Methods. 2011;8(9):753-5. doi: 10.1038/nmeth.1653.

Chen J, Wang H, Bai J, Liu W, Liu X, Yu D, Feng T, Sun Z, Zhang L, Ma L, Hu Y, Zou Y, Tan T, Zhong J, Hu M, Bai X, Pan D, Xing Y, Zhao Y, Tian K, Hu X, Li N. Generation of Pigs Resistant to Highly Pathogenic-Porcine Reproductive and Respiratory Syndrome Virus through Gene Editing of CD163. Int J Biol Sci. 2019;15(2):481-492. doi: 10.7150/ijbs.25862.

Clark KJ, Carlson DF, Leaver MJ, Foster LK, Fahrenkrug SC. Passport, a native Tc1 transposon from flatfish, is functionally active in vertebrate cells. Nucleic Acids Res. 2009;37(4):1239-47. doi: 10.1093/nar/gkn1025.

Crispo M, Mulet AP, Tesson L, Barrera N, Cuadro F, dos Santos-Neto PC, Nguyen TH, Crénéguy A, Brusselle L, Anegón I, Menchaca A. Efficient Generation of Myostatin Knock-Out Sheep Using CRISPR/Cas9 Technology and Microinjection into Zygotes. PLoS One. 2015;10(8):e0136690. doi: 10.1371/journal.pone.0136690.

Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol. 2002;20(3):251-5. doi: 10.1038/nbt0302-251.

de Graeff N, Jongsma KR, Johnston J, Hartley S, Bredenoord AL. The ethics of genome editing in non-human animals: a systematic review of reasons reported in the academic literature. Philos Trans R Soc Lond B Biol Sci. 2019 374(1772):20180106. doi: 10.1098/rstb.2018.0106.

Deng S, Li G, Yu K, Tian X, Wang F, Li W, Jiang W, Ji P, Han H, Fu J, Zhang X, Zhang J, Liu Y, Lian Z, Liu G. RNAi combining Sleeping Beauty transposon system inhibits ex vivo expression of foot-and-mouth disease virus VP1 in transgenic sheep cells. Sci Rep. 2017;7(1):10065. doi: 10.1038/s41598-017-09302-1.

Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie WA, Rollo M, de Sousa P, Travers A, Wilmut I, Clark AJ. Deletion of the alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Nat Biotechnol. 2001;19(6):559-62. doi: 10.1038/89313.

Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R, Virgin HW, Listgarten J, Root DE. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol. 2016;34(2):184-191. doi: 10.1038/nbt.3437.

Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346(6213):1258096. doi: 10.1126/science.1258096.

Dua S, Bajwa KK, Prashar A, Bansal S, Beniwal M, Kumar P, Punetha M, Selokar NL, Yadav PS, Kumar D. Empowering of reproductive health of farm animals through genome editing technology. J Reprod Healthc Med. 2021; 2:4.

Eaton SL, Proudfoot C, Lillico SG, Skehel P, Kline RA, Hamer K, Rzechorzek NM, Clutton E, Gregson R, King T, O'Neill CA, Cooper JD, Thompson G, Whitelaw CB, Wishart TM. CRISPR/Cas9 mediated generation of an ovine model for infantile neuronal ceroid lipofuscinosis (CLN1 disease). Sci Rep. 2019;9(1):9891. doi: 10.1038/s41598-019-45859-9.

Eriksson S, Jonas E, Rydhmer L, Röcklinsberg H. Invited review: Breeding and ethical perspectives on genetically modified and genome edited cattle. J Dairy Sci. 2018;101(1):1-17. doi: 10.3168/jds.2017-12962.

FAO. World livestock: Transforming the livestock sector through the sustainable development goals. Rome. 2018; 222.

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 Biol. 2017;18(1):13. doi: 10.1186/s13059-016-1144-4.

Garrels W, Ivics Z, Kues WA. Precision genetic engineering in large mammals. Trends Biotechnol. 2012;30(7):386-93. doi: 10.1016/j.tibtech.2012.03.008.

Garrels W, Mátés L, Holler S, Dalda A, Taylor U, Petersen B, Niemann H, Izsvák Z, Ivics Z, Kues WA. Germline transgenic pigs by Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. PLoS One. 2011;6(8):e23573. doi: 10.1371/journal.pone.0023573.

Garrels W, Talluri TR, Apfelbaum R, Carratalá YP, Bosch P, Pötzsch K, Grueso E, Ivics Z, Kues WA. One-step Multiplex Transgenesis via Sleeping Beauty Transposition in Cattle. Sci Rep. 2016;6:21953. doi: 10.1038/srep21953.

Godber OF, Wall R. Livestock and food security: vulnerability to population growth and climate change. Glob Chang Biol. 2014;20(10):3092-102. doi: 10.1111/gcb.12589.

Gün G, Kues WA. Current progress of genetically engineered pig models for biomedical research. Biores Open Access. 2014;3(6):255-64. doi: 10.1089/biores.2014.0039.

Guo R, Wan Y, Xu D, Cui L, Deng M, Zhang G, Jia R, Zhou W, Wang Z, Deng K, Huang M, Wang F, Zhang Y. Generation and evaluation of Myostatin knock-out rabbits and goats using CRISPR/Cas9 system. Sci Rep. 2016;6:29855. doi: 10.1038/srep29855.

Hai T, Teng F, Guo R, Li W, Zhou Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res. 2014;24(3):372-5. doi: 10.1038/cr.2014.11. Epub 2014 Jan 31.

Hamernik DL. Farm animals are important biomedical models. Anim Front. 2019; 9(3):3-5. doi: 10.1093/af/vfz026.

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL. Production of transgenic rabbits, sheep and pigs by microinjection. Nature. 1985;315(6021):680-3. doi: 10.1038/315680a0.

Han K, Liang L, Li L, Ouyang Z, Zhao B, Wang Q, Liu Z, Zhao Y, Ren X, Jiang F, Lai C, Wang K, Yan S, Huang L, Guo L, Zeng K, Lai L, Fan N. Generation of Hoxc13 knockout pigs recapitulates human ectodermal dysplasia-9. Hum Mol Genet. 2017;26(1):184-191. doi: 10.1093/hmg/ddw378.

He Z, Zhang T, Jiang L, Zhou M, Wu D, Mei J, Cheng Y. Use of CRISPR/Cas9 technology efficiently targetted goat myostatin through zygotes microinjection resulting in double-muscled phenotype in goats. Biosci Rep. 2018;38(6):BSR20180742. doi: 10.1042/BSR20180742.

Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A. Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep. 2003;4(11):1054-60. doi: 10.1038/sj.embor.embor7400007.

Holm IE, Alstrup AK, Luo Y. Genetically modified pig models for neurodegenerative disorders. J Pathol. 2016;238(2):267-87. doi: 10.1002/path.4654.

Horan R, Powell R, McQuaid S, Gannon F, Houghton JA. Association of foreign DNA with porcine spermatozoa. Arch Androl. 1991;26(2):83-92. doi: 10.3109/01485019108987631.

Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014;157(6):1262-1278. doi: 10.1016/j.cell.2014.05.010.

Hu R, Fan ZY, Wang BY, Deng SL, Zhang XS, Zhang JL, Han HB, Lian ZX. Generation of FGF5 knockout sheep via the CRISPR/Cas9 system. J Anim Sci. 2017;95(5):2019-2024. doi: 10.2527/jas.2017.1503.

Huang L, Hua Z, Xiao H, Cheng Y, Xu K, Gao Q, Xia Y, Liu Y, Zhang X, Zheng X, Mu Y, Li K. CRISPR/Cas9-mediated ApoE-/- and LDLR-/- double gene knockout in pigs elevates serum LDL-C and TC levels. Oncotarget. 2017;8(23):37751-37760. doi: 10.18632/oncotarget.17154.

Hundleby PAC, Harwood WA. Impacts of the EU GMO regulatory framework for plant genome editing. Food Energy Secur. 2019;8(2):e00161. doi: 10.1002/fes3.161.

Ikeda M, Matsuyama S, Akagi S, Ohkoshi K, Nakamura S, Minabe S, Kimura K, Hosoe M. Correction of a Disease Mutation using CRISPR/Cas9-assisted Genome Editing in Japanese Black Cattle. Sci Rep. 2017 Dec 19;7(1):17827. doi: 10.1038/s41598-017-17968-w.

Ishii T. Genome-edited livestock: ethics and social acceptance. Anim Front 2017; 7: 24–32.

Ishino T, Hashimoto M, Amagasa M, Saito N, Dochi O, Kirisawa R, Kitamura H. Establishment of protocol for preparation of gene-edited bovine ear-derived fibroblasts for somatic cell nuclear transplantation. Biomed Res. 2018;39(2):95-104. doi: 10.2220/biomedres.39.95.

Ivancevic AM, Walsh AM, Kortschak RD, Adelson DL. Jumping the fine LINE between species: horizontal transfer of transposable elements in animals catalyses genome evolution. Bioessays. 2013;35(12):1071-82. doi: 10.1002/bies.201300072.

Ivics Z, Garrels W, Mátés L, Yau TY, Bashir S, Zidek V, Landa V, Geurts A, Pravenec M, Rülicke T, Kues WA, Izsvák Z. Germline transgenesis in pigs by cytoplasmic microinjection of Sleeping Beauty transposons. Nat Protoc. 2014;9(4):810-27. doi: 10.1038/nprot.2014.010.

Ivics Z, Li MA, Mátés L, Boeke JD, Nagy A, Bradley A, Izsvák Z. Transposon-mediated genome manipulation in vertebrates. Nat Methods. 2009;6(6):415-22. doi: 10.1038/nmeth.1332.

Jakobsen JE, Johansen MG, Schmidt M, Dagnæs-Hansen F, Dam K, Gunnarsson A, Liu Y, Kragh PM, Li R, Holm IE, Callesen H, Mikkelsen JG, Nielsen AL, Jørgensen AL. Generation of minipigs with targeted transgene insertion by recombinase-mediated cassette exchange (RMCE) and somatic cell nuclear transfer (SCNT). Transgenic Res. 2013;22(4):709-23. doi: 10.1007/s11248-012-9671-6.

Kalds P, Gao Y, Zhou S, Cai B, Huang X, Wang X, Chen Y. Redesigning small ruminant genomes with CRISPR toolkit: Overview and perspectives. Theriogenology. 2020;147:25-33. doi: 10.1016/j.theriogenology.2020.02.015.

Kalds P, Zhou S, Cai B, Liu J, Wang Y, Petersen B, Sonstegard T, Wang X, Chen Y. Sheep and Goat Genome Engineering: From Random Transgenesis to the CRISPR Era. Front Genet. 2019;10:750. doi: 10.3389/fgene.2019.00750.

Kambadur R, Sharma M, Smith TP, Bass JJ. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res. 1997;7(9):910-6. doi: 10.1101/gr.7.9.910.

Kawakami K, Shima A, Kawakami N. Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. Proc Natl Acad Sci U S A. 2000;97(21):11403-8. doi: 10.1073/pnas.97.21.11403.

Kawall K, Cotter J, Then C. Broadening the GMO risk assessment in the EU for genome editing technologies in agriculture. Environ. Sci. Eur. 2020; 32:106.

Komor AC, Badran AH, Liu DR. Editing the Genome Without Double-Stranded DNA Breaks. ACS Chem Biol. 2018;13(2):383-388. doi: 10.1021/acschembio.7b00710.

Kues WA, Kumar D, Selokar NL, Talluri TR. Applications of genome editing tools in stem cells towards regenerative medicine: An update. Curr Stem Cell Res Ther. 2022;17(3):267-279. doi: 10.2174/1574888X16666211124095527.

Kues WA, Niemann H. Advances in farm animal transgenesis. Prev Vet Med. 2011 102(2):146-56. doi: 10.1016/j.prevetmed.2011.04.009.

Kumar D, Kues WA. Application of genome editing in farm animals. In: genomics and biotechnological advances in veterinary, poultry, and fisheries. Academic Press 2020, 131-149.

Kumar D, Punetha M, Bansal S, Saini S, Kumar P, Sharma RK, Yadav PS. Double sperm cloning: Could improve the efficiency of animal cloning. Anim Reprod Update. 2022; 2(1):108-14. doi: 10.48165/aru.2022.2107.

Kumar D, Talluri TR, Anand T, Kues WA. Transposon-based reprogramming to induced pluripotency. Histol Histopathol. 2015;30(12):1397-409. doi: 10.14670/HH-11-656.

Kuroiwa Y, Kasinathan P, Choi YJ, Naeem R, Tomizuka K, Sullivan EJ, Knott JG, Duteau A, Goldsby RA, Osborne BA, Ishida I, Robl JM. Cloned transchromosomic calves producing human immunoglobulin. Nat Biotechnol. 2002;20(9):889-94. doi: 10.1038/nbt727.

Kuznetsov A, Kuznetsova I. The binding of exogenous DNA pRK31acZ by rabbit spermatozoa, its transfer to oocytes and expression in preimplantation embryos. Ontogenez, 1994; 26:300–309.

Lai L, Kang JX, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Starzl TE, Prather RS, Dai Y. Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol. 2006;24(4):435-6. doi: 10.1038/nbt1198.

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science. 2002;295(5557):1089-92. doi: 10.1126/science.1068228.

Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C. Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell. 1989;57(5):717-23. doi: 10.1016/0092-8674(89)90787-3.

Ledford H. CRISPR, the disruptor. Nature. 2015;522(7554):20-4. doi: 10.1038/522020a.

Lee K, Uh K, Farrell K. Current progress of genome editing in livestock. Theriogenology. 2020;150:229-235. doi: 10.1016/j.theriogenology.2020.01.036.

Li C, Zhou S, Li Y, Li G, Ding Y, Li L, Liu J, Qu L, Sonstegard T, Huang X, Jiang Y, Chen Y, Petersen B, Wang X. Trio-Based Deep Sequencing Reveals a Low Incidence of Off-Target Mutations in the Offspring of Genetically Edited Goats. Front Genet. 2018;9:449. doi: 10.3389/fgene.2018.00449.

Li Z, Duan X, An X, Feng T, Li P, Li L, Liu J, Wu P, Pan D, Du X, Wu S. Efficient RNA-guided base editing for disease modeling in pigs. Cell Discov. 2018;4:64. doi: 10.1038/s41421-018-0065-7.

Li Z, Yang HY, Wang Y, Zhang ML, Liu XR, Xiong Q, Zhang LN, Jin Y, Mou LS, Liu Y, Li RF, Rao Y, Dai YF. Generation of tryptophan hydroxylase 2 gene knockout pigs by CRISPR/Cas9-mediated gene targeting. J Biomed Res. 2017;31(5):445-452. doi: 10.7555/JBR.31.20170026.

Liu T, Dou H, Xiang X, Li L, Li Y, Lin L, Pang X, Zhang Y, Chen Y, Luan J, Xu Y, Yang Z, Yang W, Liu H, Li F, Wang H, Yang H, Bolund L, Vajta G, Du Y. Factors Determining the Efficiency of Porcine Somatic Cell Nuclear Transfer: Data Analysis with Over 200,000 Reconstructed Embryos. Cell Reprogram. 2015;17(6):463-71. doi: 10.1089/cell.2015.0037.

Liu X, Liu H, Wang M, Li R, Zeng J, Mo D, Cong P, Liu X, Chen Y, He Z. Disruption of the ZBED6 binding site in intron 3 of IGF2 by CRISPR/Cas9 leads to enhanced muscle development in Liang Guang Small Spotted pigs. Transgenic Res. 2019;28(1):141-150. doi: 10.1007/s11248-018-0107-9.

Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science. 2002;295(5556):868-72. doi: 10.1126/science.1067081.

Manesh SB, Samani RO, Manesh SB. Ethical issues of transplanting organs from transgenic animals into human beings. Cell J. 2014;16 (3): 353-60.

Mátés L, Chuah MK, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, Grzela DP, Schmitt A, Becker K, Matrai J, Ma L, Samara-Kuko E, Gysemans C, Pryputniewicz D, Miskey C, Fletcher B, VandenDriessche T, Ivics Z, Izsvák Z. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat Genet. 2009;41(6):753-61. doi: 10.1038/ng.343.

McFarlane GR, Salvesen HA, Sternberg A, Lillico SG. On-farm livestock genome editing using cutting edge reproductive technologies. Front Sustain Food Syst. 2019; 3:106.

Menchaca A, Dos Santos-Neto PC, Mulet AP, Crispo M. CRISPR in livestock: From editing to printing. Theriogenology. 2020;150:247-254. doi: 10.1016/j.theriogenology.2020.01.063.

Miskey C, Izsvák Z, Plasterk RH, Ivics Z. The Frog Prince: a reconstructed transposon from Rana pipiens with high transpositional activity in vertebrate cells. Nucleic Acids Res. 2003;31(23):6873-81. doi: 10.1093/nar/gkg910.

Mueller ML, Cole JB, Sonstegard TS, Van Eenennaam AL. Comparison of gene editing versus conventional breeding to introgress the POLLED allele into the US dairy cattle population. J Dairy Sci. 2019;102(5):4215-4226. doi: 10.3168/jds.2018-15892.

Muñoz-López M, García-Pérez JL. DNA transposons: nature and applications in genomics. Curr Genomics. 2010;11(2):115-28. doi: 10.2174/138920210790886871.

Nabarro D, Wannous C. The potential contribution of livestock to food and nutrition security: the application of the One Health approach in livestock policy and practice. Rev Sci Tech. 2014;33(2):475-85. doi: 10.20506/rst.33.2.2292.

Naeem M, Majeed S, Hoque MZ, Ahmad I. Latest Developed Strategies to Minimize the Off-Target Effects in CRISPR-Cas-Mediated Genome Editing. Cells. 2020;9(7):1608. doi: 10.3390/cells9071608.

Navarro-Serna S, Vilarino M, Park I, Gadea J, Ross PJ. Livestock Gene Editing by One-step Embryo Manipulation. J Equine Vet Sci. 2020;89:103025. doi: 10.1016/j.jevs.2020.103025.

Ni W, Qiao J, Hu S, Zhao X, Regouski M, Yang M, Polejaeva IA, Chen C. Efficient gene knockout in goats using CRISPR/Cas9 system. PLoS One. 2014;9(9):e106718. doi: 10.1371/journal.pone.0106718.

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W, Sha J. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell. 2014;156(4):836-43. doi: 10.1016/j.cell.2014.01.027.

Niu Y, Zhao X, Zhou J, Li Y, Huang Y, Cai B, Liu Y, Ding Q, Zhou S, Zhao J, Zhou G, Ma B, Huang X, Wang X, Chen Y. Efficient generation of goats with defined point mutation (I397V) in GDF9 through CRISPR/Cas9. Reprod Fertil Dev. 2018;30(2):307-312. doi: 10.1071/RD17068.

Park KE, Frey JF, Waters J, Simpson SG, Coutu C, Plummer S, Campbell M, Donovan DM, Telugu BP. One-Step Homology Mediated CRISPR-Cas Editing in Zygotes for Generating Genome Edited Cattle. CRISPR J. 2020;3(6):523-534. doi: 10.1089/crispr.2020.0047.

Park KE, Kaucher AV, Powell A, Waqas MS, Sandmaier SE, Oatley MJ, Park CH, Tibary A, Donovan DM, Blomberg LA, Lillico SG, Whitelaw CB, Mileham A, Telugu BP, Oatley JM. Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. Sci Rep. 2017;7:40176. doi: 10.1038/srep40176.

Park KW, Cheong HT, Lai L, Im GS, Kühholzer B, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim Biotechnol. 2001;12(2):173-81. doi: 10.1081/abio-100108344.

Peng J, Wang Y, Jiang J, Zhou X, Song L, Wang L, Ding C, Qin J, Liu L, Wang W, Liu J, Huang X, Wei H, Zhang P. Production of Human Albumin in Pigs Through CRISPR/Cas9-Mediated Knockin of Human cDNA into Swine Albumin Locus in the Zygotes. Sci Rep. 2015;5:16705. doi: 10.1038/srep16705.

Perisse IV, Fan Z, Singina GN, White KL, Polejaeva IA. Improvements in Gene Editing Technology Boost Its Applications in Livestock. Front Genet. 2021;11:614688. doi: 10.3389/fgene.2020.614688.

Perota A, Lagutina I, Duchi R, Zanfrini E, Lazzari G, Judor JP, Conchon S, Bach JM, Bottio T, Gerosa G, Costa C, Galiñanes M, Roussel JC, Padler-Karavani V, Cozzi E, Soulillou JP, Galli C. Generation of cattle knockout for galactose-α1,3-galactose and N-glycolylneuraminic acid antigens. Xenotransplantation. 2019;26(5):e12524. doi: 10.1111/xen.12524.

Petersen B. Basics of genome editing technology and its application in livestock species. Reprod Domest Anim. 2017;52 Suppl 3:4-13. doi: 10.1111/rda.13012.

Pfeifer A, Hofmann A. Lentiviral transgenesis. Methods Mol Biol. 2009;530:391-405. doi: 10.1007/978-1-59745-471-1_21.

Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci U S A. 2002;99(4):2140-5. doi: 10.1073/pnas.251682798.

Raman R. The impact of Genetically Modified (GM) crops in modern agriculture: A review. GM Crops Food. 2017;8(4):195-208. doi: 10.1080/21645698.2017.1413522.

Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol. 2016;34(3):339-44. doi: 10.1038/nbt.3481.

Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel

AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr, Zabner J, Prather RS, Welsh MJ. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science. 2008;321(5897):1837-41. doi: 10.1126/science.1163600.

Rottmann O, Antes R, Höfer P, Maierhofer G. Liposome mediated gene transfer via spermatozoa into avian egg cells. J Anim Breed Genet. 1992; 109: 64-70.

Ruan J, Li H, Xu K, Wu T, Wei J, Zhou R, Liu Z, Mu Y, Yang S, Ouyang H, Chen-Tsai RY, Li K. Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. Sci Rep. 2015;5:14253. doi: 10.1038/srep14253.

Schellander K, Peli J, Schmoll F, Brem G. Artificial insemination in cattle with DNA-treated sperm. Anim Biotechnol. 1995; 6: 41-50.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science. 1997;278(5346):2130-3. doi: 10.1126/science.278.5346.2130.

Schook LB, Rund L, Begnini KR, Remião MH, Seixas FK, Collares T. Emerging Technologies to Create Inducible and Genetically Defined Porcine Cancer Models. Front Genet. 2016;7:28. doi: 10.3389/fgene.2016.00028.

Schultz-Bergin MIs. CRISPR an ethical game changer. J Agric Environ Ethics. 2018;31:219.

Selokar NL, Kues WA. How farm animals are improving human health and welfare. Rev Sci Tech. 2018;37(1):83-96. doi: 10.20506/rst.37.1.2742.

Shinohara ET, Kaminski JM, Segal DJ, Pelczar P, Kolhe R, Ryan T, Coates CJ, Fraser MJ, Handler AM, Yanagimachi R, Moisyadi S. Active integration: new strategies for transgenesis. Transgenic Res. 2007;16(3):333-9. doi: 10.1007/s11248-007-9077-z.

Smith K, Spadafora C. Sperm-mediated gene transfer: applications and implications. Bioessays. 2005;27(5):551-62. doi: 10.1002/bies.20211.

Sperandio S, Lulli V, Bacci ML, Forni M, Maione B, Spadafora C, Lavitrano M. Sperm-mediated DNA transfer in bovine and swine species. Anim Biotechnol. 1996; 7(1): 59-77.

Tanihara F, Hirata M, Nguyen NT, Le QA, Hirano T, Takemoto T, Nakai M, Fuchimoto DI, Otoi T. Generation of a TP53-modified porcine cancer model by CRISPR/Cas9-mediated gene modification in porcine zygotes via electroporation. PLoS One. 2018;13(10):e0206360. doi: 10.1371/journal.pone.0206360.

Tanihara F, Takemoto T, Kitagawa E, Rao S, Do LT, Onishi A, Yamashita Y, Kosugi C, Suzuki H, Sembon S, Suzuki S, Nakai M, Hashimoto M, Yasue A, Matsuhisa M, Noji S, Fujimura T, Fuchimoto D, Otoi T. Somatic cell reprogramming-free generation of genetically modified pigs. Sci Adv. 2016;2(9):e1600803. doi: 10.1126/sciadv.1600803.

Tuggle CK, Waters WR. Tuberculosis-resistant transgenic cattle. Proc Natl Acad Sci U S A. 2015;112(13):3854-5. doi: 10.1073/pnas.1502972112.

Urnov FD. Genome Editing B.C. (Before CRISPR): Lasting Lessons from the "Old Testament". CRISPR J. 2018;1(1):34-46. doi: 10.1089/crispr.2018.29007.fyu.

Van Eenennaam AL. Application of genome editing in farm animals: cattle. Transgenic Res. 2019;28(Suppl 2):93-100. doi: 10.1007/s11248-019-00141-6.

Walsh AM, Kortschak RD, Gardner MG, Bertozzi T, Adelson DL. Widespread horizontal transfer of retrotransposons. Proc Natl Acad Sci U S A. 2013;110(3):1012-6. doi: 10.1073/pnas.1205856110.

Wang X, Cao C, Huang J, Yao J, Hai T, Zheng Q, Wang X, Zhang H, Qin G, Cheng J, Wang Y, Yuan Z, Zhou Q, Wang H, Zhao J. One-step generation of triple gene-targeted pigs using CRISPR/Cas9 system. Sci Rep. 2016;6:20620. doi: 10.1038/srep20620.

Wang X, Niu Y, Zhou J, Yu H, Kou Q, Lei A, Zhao X, Yan H, Cai B, Shen Q, Zhou S, Zhu H, Zhou G, Niu W, Hua J, Jiang Y, Huang X, Ma B, Chen Y. Multiplex gene editing via CRISPR/Cas9 exhibits desirable muscle hypertrophy without detectable off-target effects in sheep. Sci Rep. 2016;6:32271. doi: 10.1038/srep32271.

Wang Y, Du Y, Shen B, Zhou X, Li J, Liu Y, Wang J, Zhou J, Hu B, Kang N, Gao J, Yu L, Huang X, Wei H. Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Sci Rep. 2015;5:8256. doi: 10.1038/srep08256.

Wells KD, Bardot R, Whitworth KM, Trible BR, Fang Y, Mileham A, Kerrigan MA, Samuel MS, Prather RS, Rowland RRR. Replacement of Porcine CD163 Scavenger Receptor Cysteine-Rich Domain 5 with a CD163-Like Homolog Confers Resistance of Pigs to Genotype 1 but Not Genotype 2 Porcine Reproductive and Respiratory Syndrome Virus. J Virol. 2017;91(2):e01521-16. doi: 10.1128/JVI.01521-16.

Whelan AI, Lema MA. Regulatory framework for gene editing and other new breeding techniques (NBTs) in Argentina. GM Crops Food. 2015;6(4):253-65. doi: 10.1080/21645698.2015.1114698.

Whitelaw CB, Lillico SG, King T. Production of transgenic farm animals by viral vector-mediated gene transfer. Reprod

Domest Anim. 2008;43 Suppl 2:355-8. doi: 10.1111/j.1439-0531.2008.01184.x.

Whitelaw CB, Sheets TP, Lillico SG, Telugu BP. Engineering large animal models of human disease. J Pathol. 2016;238(2):247-56. doi: 10.1002/path.4648.

Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, Samuel MS, Mao J, O'Gorman C, Walters EM, Murphy CN, Driver J, Mileham A, McLaren D, Wells KD, Prather RS. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod. 2014;91(3):78. doi: 10.1095/biolreprod.114.121723.

Whitworth KM, Rowland RR, Ewen CL, Trible BR, Kerrigan MA, Cino-Ozuna AG, Samuel MS, Lightner JE, McLaren DG, Mileham AJ, Wells KD, Prather RS. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotechnol. 2016;34(1):20-2. doi: 10.1038/nbt.3434.

Whitworth KM, Rowland RRR, Petrovan V, Sheahan M, Cino-Ozuna AG, Fang Y, Hesse R, Mileham A, Samuel MS, Wells KD, Prather RS. Resistance to coronavirus infection in amino peptidase N-deficient pigs. Transgenic Res. 2019;28(1):21-32. doi: 10.1007/s11248-018-0100-3.

Williams DK, Pinzón C, Huggins S, Pryor JH, Falck A, Herman F, Oldeschulte J, Chavez MB, Foster BL, White SH, Westhusin ME, Suva LJ, Long CR, Gaddy D. Genetic engineering a large animal model of human hypophosphatasia in sheep. Sci Rep. 2018;8(1):16945. doi: 10.1038/s41598-018-35079-y.

Xie J, Ge W, Li N, Liu Q, Chen F, Yang X, Huang X, Ouyang Z, Zhang Q, Zhao Y, Liu Z, Gou S, Wu H, Lai C, Fan N, Jin Q, Shi H, Liang Y, Lan T, Quan L, Li X, Wang K, Lai L. Efficient base editing for multiple genes and loci in pigs using base editors. Nat Commun. 2019;10(1):2852. doi: 10.1038/s41467-019-10421-8.

Xie Z, Pang D, Yuan H, Jiao H, Lu C, Wang K, Yang Q, Li M, Chen X, Yu T, Chen X, Dai Z, Peng Y, Tang X, Li Z, Wang T, Guo H, Li L, Tu C, Lai L, Ouyang H. Genetically modified pigs are protected from classical swine fever virus. PLoS Pathog. 2018;14(12):e1007193. doi: 10.1371/journal.ppat.1007193.

Yan S, Tu Z, Liu Z, Fan N, Yang H, Yang S, Yang W, Zhao Y, Ouyang Z, Lai C, Yang H, Li L, Liu Q, Shi H, Xu G, Zhao H, Wei H, Pei Z, Li S, Lai L, Li XJ. A Huntingtin Knockin Pig Model Recapitulates Features of Selective Neurodegeneration in Huntington's Disease. Cell. 2018;173(4):989-1002.e13. doi: 10.1016/j.cell.2018.03.005.

Yang L, Guell M, Byrne S, Yang JL, De Los Angeles A, Mali P, Aach J, Kim-Kiselak C, Briggs AW, Rios X, Huang PY, Daley G, Church G. Optimization of scarless human stem cell genome editing. Nucleic Acids Res. 2013;41(19):9049-61. doi: 10.1093/nar/gkt555.

Yao J, Huang J, Hai T, Wang X, Qin G, Zhang H, Wu R, Cao C, Xi JJ, Yuan Z, Zhao J. Efficient bi-allelic gene knockout and site-specific knock-in mediated by TALENs in pigs. Sci Rep. 2014;4:6926. doi: 10.1038/srep06926.

Yao J, Huang J, Zhao J. Genome editing revolutionize the creation of genetically modified pigs for modeling human diseases. Hum Genet. 2016;135(9):1093-105. doi: 10.1007/s00439-016-1710-6.

Young AE, Mansour TA, McNabb BR, Owen JR, Trott JF, Brown CT, Van Eenennaam AL. Genomic and phenotypic analyses of six offspring of a genome-edited hornless bull. Nat Biotechnol. 2020;38(2):225-232. doi: 10.1038/s41587-019-0266-0.

Yu Y, Wang Y, Tong Q, Liu X, Su F, Quan F, Guo Z, Zhang Y. A site-specific recombinase-based method to produce antibiotic selectable marker free transgenic cattle. PLoS One. 2013;8(5):e62457. doi: 10.1371/journal.pone.0062457.

Yum SY, Lee SJ, Kim HM, Choi WJ, Park JH, Lee WW, Kim HS, Kim HJ, Bae SH, Lee JH, Moon JY, Lee JH, Lee CI, Son BJ, Song SH, Ji SM, Kim SJ, Jang G. Efficient generation of transgenic cattle using the DNA transposon and their analysis by next-generation sequencing. Sci Rep. 2016;6:27185. doi: 10.1038/srep27185.

Yum SY, Lee SJ, Park SG, Shin IG, Hahn SE, Choi WJ, Kim HS, Kim HJ, Bae SH, Lee JH, Moon JY, Lee WS, Lee JH, Lee CI, Kim SJ, Jang G. Long-term health and germline transmission in transgenic cattle following transposon-mediated gene transfer. BMC Genomics. 2018;19(1):387. doi: 10.1186/s12864-018-4760-4. P

Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci U S A. 2011;108(4):1531-6. doi: 10.1073/pnas.1008322108.

Zayed H, Izsvák Z, Walisko O, Ivics Z. Development of hyperactive sleeping beauty transposon vectors by mutational analysis. Mol Ther. 2004;9(2):292-304. doi: 10.1016/j.ymthe.2003.11.024.

Zhang J, Cui ML, Nie YW, Dai B, Li FR, Liu DJ, Liang H, Cang M. CRISPR/Cas9-mediated specific integration of fat-1 at the goat MSTN locus. FEBS J. 2018;285(15):2828-2839. doi: 10.1111/febs.14520.

Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. Mol Ther Nucleic Acids. 2015;4(11):e264. doi: 10.1038/mtna.2015.37.

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. J Cell Biochem. 2018. doi: 10.1002/jcb.27474.

Zhao J, Lai L, Ji W, Zhou Q. Genome editing in large animals: current status and future prospects. Natl Sci Rev. 2019;6(3):402-420. doi: 10.1093/nsr/nwz013.

Zheng Q, Lin J, Huang J, Zhang H, Zhang R, Zhang X, Cao C, Hambly C, Qin G, Yao J, Song R, Jia Q, Wang X, Li Y, Zhang N, Piao Z, Ye R, Speakman JR, Wang H, Zhou Q, Wang Y, Jin W, Zhao J. Reconstitution of UCP1 using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and improves thermogenic capacity. Proc Natl Acad Sci U S A. 2017;114(45):E9474-E9482. doi: 10.1073/pnas.1707853114.

Zhou X, Xin J, Fan N, Zou Q, Huang J, Ouyang Z, Zhao Y, Zhao B, Liu Z, Lai S, Yi X, Guo L, Esteban MA, Zeng Y, Yang H, Lai L. Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer. Cell Mol Life Sci. 2015;72(6):1175-84. doi: 10.1007/s00018-014-1744-7.