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CRISPR/Cas9 technology: Current status and future scenario in livestock

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Abstract
Genome editing especially CRISPR/Cas9 is a suite of state-of-the-art reproductive technologies for on farm genetic improvement to sustain germline of economically important livestock species**.** As a consequence of **s**pecified straightforwardness and extent in functioning of CRISPR-Cas tool, it can be anticipated that a considerable number of genome-edited livestock will dominate over the next decade for the perfection not only in human beings but also in animal population. Animal breeders can now selectively and proficiently modify animal DNA by adopting this influential skill. This tool has aimed for maintaining the present beneficial potential in chief genetic characteristics of the herd and to bring in more desirable traits such as polled, thermo-resilient and disease tolerant animals with clear-cut genetic modification to eliminate harmful recessive lethal genetic mutations. Conventional breeding and selection methods for genetic modification are limited by available genetic design in terms of linkage and variant within the variety. In livestock, the CRISPR/Cas system has capability to generate single step alteration in pleotrophic and polymorphic traits with multiple genes and directly amend genetic mutations in target tissues and cells to assist conventional management. Genome editing permit animal breeders to bring in diverse polymorphisms in the gene pool of elite stock by conquering all spatiotemporal biological barriers to direct increased profits in animal based food products.

Keywords: CRISPR, livestock, genetic improvement, agro-economy, designer animals

Introduction
As per the future forecast of 2050 for the purpose of global demand of food security animal-based food products need to be increased by 70% in proportion with gradually increasing 9.8 Million (world) human populations (FAO, 2009) [15]. This have to be achieved but with minimal impact on the environment with implementation of advanced technologies. Genome editing is a tool that allows livestock breeders to improve animal welfare, performance and efficiency to achieve more sustainable future for livestock and agriculture with cutting-edge reproductive technologies (McFariane *et al.*, 2019). Genome editing using biomedical research has been recent refurbishment in the research and development field. In order to study the mechanism of human disease, drug development and organ transplantation, it is essential to construct an appropriate animal model with the growth of germline genome editing for scientific requirements. At present, this field has undoubtedly updated with the use of CRISPR system. Wellbeing and ethical issues can also be notified with the great prospective of editing tools for medical and agricultural purposes. Auxiliary studies to craft more genome edited animals can be helpful at this verge of competitive universal trades to resolve off-target possessions and possible jeopardy for host genome. This suite of state-of-the-art reproductive technologies is technically sound which applies genome editing in agricultural milieu to rapidly picking up productivity, fertility, sustainability, and animal safety with negligible infrastructure and modest fiscal assistance. The means to open ways to these benefits is currently in the hands of supervisory body which validate these researches to disseminate desired superior germplam to rural farmer's community.
This review article focused to enlighten the present and future scenario for editing strategy especially in livestock genome based on CRISPR/Cas9 and comparing results with ZFNs and TALENs.

Genetic engineering can be defined as "The deliberate modification of the characteristics of an organism by manipulating its genetic material." This is widely used in various fields such as research, medicine (protein/enzyme production), agriculture (crops) and industrial

biotechnology. Genome Editing is one technique of genetic engineering for targeted genetic modifications, enabling the knockout and knocking in of specific DNA fragments. Combining with reproductive technology this can be used for biomedical research, clinics, agriculture, disease research viz. constructing appropriate animal models and gene therapy.

For genome editing four major varieties of nucleases are mostly used such as Meganucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeat-associated nuclease Cas9 (CRISPR-Cas9). Among these variants of genome editor toolbox last three except meganucleases were applied for livestock species such as Pigs, Cattle, Sheep, Goats and Chicken for various purposes. Principle behind these editor tools is more or less similar to fabricate Double Strand DNA Breaks (DSBs) with specific binding and nuclease domains by any one way either NHEJ or HDR. First is Non-homologous End Joining (NHEJ), which is simple but Error Prone because of Indels (insertion and deletion) Nucleotide for achieving genetic modification by knocking out the cut sequences. Second is Homologous DNA Repair Template (HDRT) via the Homology-directed Repair (HDR) pathway (Fernandez *et al.*, 2017) [14]. Later one is more complex but can be used for knocking in the desired sequences at cut site.

Important varieties of genetically modified animals

The spectrum for Genetically Modified livestock can be enhanced through this paradigm shift from conventional breeding and selection methods to advance tools of gene editing via artificial selection (indexing, REML, BLUP, Marker Assisted and Genomic Selection) with human interventions to reduces the costs and increase the potential of preferred mutant animals *viz.* improved Thermoregulatory responses, enhanced meat quality, disease resistance and superior germplasm with economical production (Chen *et al.*, 2007) [7]. Till now these Genome Editing tool in Livestock were used for various modification such as

1. **Milk alteration:** Reduces allergenic potential of β-Lactoglobulin (BLG), knocking in the human lactoferrin (hLF) gene and high expression of human serum albumin variation attained by using ZFNs and TALENs (Yu *et al.*, 2011; Wei *et al.*, 2018; Cui *et al.*, 2015; Luo *et al.*, 2016) [61, 52, 10, 34].
2. **Meat production, composition and quality improvement:** Increases muscle mass and decrease fat accumulation via implementing MSTN (myostatin)-mutation in cattle, sheep, goat, swine, dogs and humans with ZFNs editor (Kambadur *et al.*, 1997; Qian *et al.*, 2015; Wang *et al.*, 2018; Zhang *et al.*, 2018a) [28, 43, 49, 62].
3. **UCP1-knockin pigs:** Maintain temperature in acute cold, increased lean meat and decreased fat deposition. Decreased fecal nitrogen, phosphorus outputs, increased growth and feed conversion rates. This progress was accomplished by CRISPR/Cas9 to include mouse adiponectin-UCP1 (Zheng *et al.*, 2017; Zhang *et al.*, 2018b) [65, 63].
4. **Disease resistance:** Porcine reproductive and respiratory syndrome (PRRS) with single-gene i.e. CD163 deletion by CRISPR/Cas9 editor, Foot-and-mouth disease virus (FMDV) with tiny interfering RNAs by small hairpin RNAs (shRNAs), Bovine tuberculosis with inclusion of the mouse SP110 gene by TALEN to produce resistant swine and cattle respectively (Whitworth *et al.*, 2016; Wells *et al.*, 2017; Burkard *et al.*, 2017; Hu *et al.*, 2015; Grange 2001; Gao *et al.*, 2017; Wu *et al.*, 2015) [54, 53, 4, 54, 19, 17, 56].
5. **Animal welfare:** Introduction of candidate 'polled' allele to avoid losses due to unintentional fight into dairy cattle with TALEN-mediated genome amendment and reproductive cloning (Carlson *et al.*, 2016) [8].
6. **Bioreactors:** Transgenic piglets as knocking in human serum albumin (HSA) by the means of CRISPR/Cas9 (Peng *et al.*, 2015) [42].

Up to date

1. Xenotransplantation

Recently University of Maryland School of Medicine (10th Jan 2022) publicized about revolutionary achievement in xenotransplantation, highlighted as a patient received a heart from a genetically altered pig in USA. The pig had 10 genetic modifications. Four genes were knocked out, or inactivated, including one that encodes a molecule that causes an aggressive human rejection response. Six human genes were inserted into the genome of the donor pig modifications designed to make the porcine organs more tolerable to the human immune system.

2. FNCAS9 Editor-limited Uniform Detection Assay (FELUDA test)

An accurate and low-cost paper-based test strip used for the detection of genes specific to sars-cov-2 virus (Gulati *et al.*, 2021). Give accurate result in 30-45 min., takes short time interval. Credit goes to collaborative research of CSIR and TATA group done by scientist team lead by Debojyoti Chakraborty and Souvik Maiti. Test is very much reliable as it has 96% sensitivity and 98% specificity. Test procedure is simple and can be followed by reading manual at home by patient or nearby family members.

Comparison of various nucleases used for genome editing

Different engineered nucleases can be compared on the ground of recognition location, targeting restrictions, specificity in terms of mismatching sequences, difficulties of engineering and difficulties of *in vivo* delivery with the aid of various vectors (Li *et al.*, 2019; table 1) [32]. CRISPR/Cas9 is RNA based editing tool while other tools are protein based editors. Re-designing and re-engineering of new set of proteins hamper broad adoption of protein based tools (Zhao *et al.*, 2019) [66]. These technical barriers are not seen in CRISPR system and it is a flexible and robust method with high editing efficiency. Success and promise of CRISPR/Cas are due to its virtues of having simple, elegant, customizable, modular and evolutionary tool for multiple targeting, minimize and/or eradicate off-target modification which makes this tool far better than others with its precise cutting property.

Table 1: Comparative Analyses of Different Engineered Nucleases

Platforms	ZFNs	TALENs	Cas9	Meganuclease
Recognition sequences	9–18 bp per monomer, 18–36 bp per pair	14–20 bp per monomer, 28–40 bp per pair	20 bp guide sequence + PAM sequence	Between 14 and 40 bp
Restriction target	Difficult for non-G-rich sites	5′ targeted base must be a T	Targeted site should precede a PAM sequence	Low efficiency for targeting novel sites
Specificity	Tolerating few positional mismatches	Tolerating few positional mismatches	Tolerating positional and multiple consecutive mismatches	Tolerating few positional mismatches
Difficulties of engineering	Requiring substantial protein engineering	Requiring complex molecular cloning methods	Using easy cloning methods and oligo synthesis	Requiring substantial protein engineering
Difficulties vector mediated *in vivo* processing	Relatively easy as small size of expression elements suitable for varieties of viral vectors	Difficult due to the large size of functional components	Commonly used SpCas9 with large size may cause packaging problems for viral vectors such as AAV	Relatively easy as small size of expression elements suitable for varieties of viral vectors

Historical background of CRISPR tool

From its inception as 1st report on CRISPR (Ishino *et all.*, 1987) [26] till now with receiving 2020 Nobel Prize by Jennifer Doudna and Emmanuelle Charpentier for Chemistry this has emerged very rapidly with wide application from prokaryotes to eukaryotes such as laboratory animals and now non-human primates amplified from 2008 (Yang *et al.*, 2008) [59] to 2013 and still on revolutionary path (Kornegay, 2017) [32].

Mechanism of action

This is a part of adaptive immune system of bacterial cell to combat viral infection also present in some archea, initially discovered in E coli cells (Barrangou *et al.*, 2007; Horvath and Barrangou, 2010) [3, 23]. There are two sequences in this system one is spacer sequences which are complimentary sequences to viral genes transcribed into tracrRNA another is CRISPR sequences which are small repetitive palindromic sequences which transcribed into guided RNA (Wiedenheft *et al.*, 2012) [57]. There are three phases in functioning of CRISPR system: adaptation, expression and interference. In adaptation phase short piece of invading forign DNA is captured and integrated into spacer element to be transcribed into precrRNA and finally to crRNA which form effector complex with Cas9 protein system. Cas9 protein has both helicase and nucleases activity helped by recognition protospacer motif (PAM) sequences for nickase activity and two nuclease domains HNH and RuvC perform complementary (target strand of DNA) and non-complementary (non-target DNA strand) cleavage respectively (Gasiunas *et al.*, 2012; Cong *et al.*, 2013; Jinek *et al.*, 2012) [16, 9, 18].

CRISPR and genetic gain in livestock

CRISPR/Cas9 Technology has a wide applications in molecular and cytogenetic research such as base editing, gene repression, gene activation, chromatin topography, epigenome editing, chromatin imaging (Sun *et al.*, 2021) [44]. With the use of CRISPR/Cas9 system various researchers promoted a precise form of repair (homology-directed repair; HDR) to construct indels or knockouts by providing a matching template DNA sequence to insert (knock in) into the break in a cell. For this some of the appliance comprises as alteration of a promoter sequence or gene, inclusion of an exogenous reporter (*viz.* a fluorescent protein), or manufacturing a clincally pertinent SNP for a disease model. Another application is by cutting two replicas with the aid of the Cas9/sgRNA complex in which knocking in acted upon to repair one replica of the gene/sequence via crafting a knockout/indel at the second replica using the non-homologous end joining (NHEJ) pathway. However the efficiency of knocking in is generally lower than knocking out contrast to this knocking in is frequently attempted then knocking out. Till now various changes with these two approaches either knocking in or knocking out through CRISPR/Cas9 editor (table 2) were accomplished for various purposes mostly dominated as use of animal for disease model studies to safeguard human beings.

Table 2: Genetically modified animals with CRISPR/Cas9

Species		Gene	Applications (Disease Model)	References
Cynomolgus KO		PPARγ /RAG1 p53 DAX1	Metabolic Diseases and Immunodeficiency Tumorigenesis AHC-HH	Niu *et al.*, 2014 [40]; Chen *et al.*, 2015 [8] Wan *et al.*, 2015 [46] Kang *et al.*, 2015 [29]
Rhesus (KO)		Dystrophin	DMD	Chen *et al.*, 2015 [8]
Pig	KO	ApoE/LDLR, Npc1l1 MITF TPH2 TYR Hoxc13G GTA1/CMAH/β4GalNT2 vWF TP53/PTEN/APC/BRCA1/BRCA2/KRAS Parkin/DJ-1/PARK2/PINK1 PERV CD163	Cardiovascular and Metabolic Diseases Waardenburg Syndrome 5-HT Deficiency Induced Behavior Abnormality Albinism ED-9 Xenotransplantation vWD lung cancer PD Xenotransplantation Disease Resistance to PRRSV	Huang *et al.*, 2017 [25] Wang *et al.*, 2015 [46] Li *et al.*, 2017 [33] Zhou *et al.*, 2015 [66] Han *et al.*, 2017 [22] Butler *et al.*, 2016 [5] Hai *et al.*, 2014 [21] Wang *et al.*, 2017 [48] Wang *et al.*, 2016 [50] Yang *et al.*, 2015 [58] Niu *et al.*, 2017 [39] Whitworth *et al.*, 2016 [54] Burkard *et al.*, 2017 [4]
	KI	CD163 (SRCR 5 domain, hCD163L1 SRCR	Disease Resistance to PRRSV	Wells *et al.*, 2017 [53]

		domain 8 homolog) UCP1 Human albumin Huntingtin	Meat Production, Composition and Quality Bioreactor HD	Zheng *et al.*, 2017 [65] Peng *et al.*, 2015 [42] Yan *et al.*, 2018 [57]
Dog (KO)		MSTN ApoE Dystrophin	Improve Muscle Growth Cardiovascular Disease DMD Gene Therapy	Zou *et al.*, 2015 Feng *et al.*, 2018 [13] Amoasii *et al.*, 2018 [1]
Goat		MSTN (KO) MSTN (fat-1) (KI)	Meat Production, Composition and Quality	Wang *et al.*, 2018 [49] Zhang *et al.*, 2018 [63]
Cattle (KI)		NRAMP1	Disease Resistance to Tuberculosis	Gao *et al.*, 2017 [17]

KO: Knocking Out; KI: Knocking In; MSTN: Myostatin

Malpotra *et al.*, 2017 [37] had exploited knocking out property of the CRISPR/Cas9 method to distinguish its efficiency while working on Rig-I gene (retinoic acid-inducible gene-1) in Goat primary fibroblasts by using a NHEJ pathway. Rig-I a cytoplasmic sensor is an innate immune response regulator which we can be an asset for the management of viral diseases, immune disorders, cancer and other conditions in mammalian species. Cell screening of thirty colonies revealed inactivation of the Rig-I gene by deletion with two positive clones by simple and cost-effective CRISPR/Cas9 technique in primary fibroblast cell culture. Dumne, 2020 [14] studied on molecular cloning and characterization of *cox-2* gene using CRISPR/Cas9 method in buffalo. PTGS2 (Prostaglandin-endoperoxide synthase 2) gene is responsible for predetermination of Cyclooxygenase-2 or COX-2. This is of great concerned in inflammatory response as an important precursor of prostacyclin for mediating the conversion of arachidonic acid to prostaglandin H2.

On-farm improvement by genome editing can be helpful for enhancing genetic gain and sustainable future for livestock in dam as well as in sire. Zygote electroporation (Laible, 2018; Miao *et al.*, 2019; Namula *et al.*, 2019) [31, 37, 38] or zygote transductions of recombinant adeno-associated viruses (rAAV) (Yoon *et al.*, 2018; Bak and Porteus, 2017) [60, 2] are method of choice for dam's selection and dissemination of superior animals. Oocytes are collected from donor females using ovum pick up. Collected oocytes are matured and fertilized *in vitro*. Validated genome editing reagents are introduced into the zygote using electroporation or transduction. Embryos are cultured *in vitro* to blastocyst stage. A biopsy is taken from each blastocyst, DNA is extracted and sequenced on-farm using a portable DNA sequencer. Embryos with the desired edits are transferred into recipient females, who give birth to genome edited offspring. Animals with confirmed genotypes are added into the breeding program to disseminate their superior genetics. Surrogate sire technology (SST) (Giasetti *et al.*, 2019; Park *et al.*, 2017; Wang *et al.*, 2017) [18, 41, 50] is method of choice for sire selection. Spermatogonial stem cells (SSCs) can be collected with needle testicular biopsy from a donor male with suitable genetic merit. After confirming fertility of surrogate sires cultured cells will then be introduced into the breeding program to disseminate the superior germline genetics to the farmer. These methods have yet to be used on livestock for wide application into breeding herd. Electroporation is a well-known method introduced into mammalian cells, although it was only recently optimized for use with zygotes. Till now applied for pigs and cattle because of its ease ever more frequent in profit-making for stock animals. With handy movable equipment and minimal training, conjugate electroporation can be included into established embryo transfer programs with little or no trouble. Virus mediated manipulation method zygotic transduction was experimented only for mice and still awaited to be used in domestic animals. This editing procedure in genome of cultured zygotes using rAAV transduction is a quite uncomplicated practice and no prerequisite for supplementary equipment contrast to standard ET plans. The cost effective operation and level of expertise can direct this technology to be extensively used on farms in progressive era. Third SST was applied for pigs and mice hitherto valuable to pertain for various livestock breeds. Tropical countries viz. India, due to having vast and polymorphic potential of resilient dams and sires from local breeds can be managed properly. To produce beneficial production traits with surrogate sires produced from circulated genome-edited sperm permit livestock farmers to attain their goals in fewer time intervals (McFariane *et al.*, 2019). Authenticated Genome prophecy will bring rapidity to genetic gain by reducing the generation gaps for each animal to cut the time of sexual maturity and reflect desired changes into recipient mature males from by transplanting altered young germline through elite SSCs genomes.

Thus far rapid advancement with editing into the genome of large animals has come up with useful impact for agro-economy and human as disease models, gene therapy and xenotransplantation. Additionally, to hasten the expansion of existing genome edited and modified organs, tissues and animals new accurate tools need to be designed for genetic modification in the field of agricultural including livestock sector, regenerative medicine and remedial appliances. Revolutionary scientific tool, CRISPR/Cas will be more suitable in its specificity and precision for on farms further improvements, with a significantly less frequent for of off-target menace due to naturally occurring events such as spontaneous mutations in animal genomes.

Ethical predicament

There is several ethical considerations *viz.* lacking the proper watch to ensure several issues as explained, proof for desired alteration; accuracy to do only guided modification; consent and law enforcement with some uniform rules and regulations; and disastrous consequences such as eugenics and racism, weapon of mass destruction, bio-terrorism, for the use of these types of editing tools. As every technique has its own pros and cons CRISPR/Cas9 also has its merits and demerits. Designer individuals are debatable point resulted from these types of genome editing techniques. Due to ethical and legal implications these techniques are facing a hold to be used in near future because they result to make changes in germline of living organisms.

Conclusion

Today we are talking about designer eggs with some dietary modification for laying hens and designer human babies to cure some serious health ailments. Designer animals can also

be the area to be entranced with these types of advance biotechnology tools for various domestic animals for better performance in economically important traits. CRISPR/Cas9 is a valuable tool for enhancing the animal breeding environment combining with genome editing and progressive reproductive technologies to improve the genetically heritable potential not only to the presented generation but also to next upcoming generations.

Declaration of Competing Interest
The authors declare that they have no conflict of interest.

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Author contributions
NY made the review effort and finalized the manuscript; UP helped in manuscript drafting, visualization and manuscript preparation; KY conceptualized the study; SM and DPR helped in understanding the deeper aspect of subject by their previous practical experience and knowledge; KB helped in formatting the draft. All authors approved the manuscript.

References

1. Amoasii L, Hildyard JC, Li H, Sanchez-Ortiz E, Mireault A, Caballero D, *et al*. Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. Science. 2018;362(6410):86-91.
2. Bak RO, Porteus MH. CRISPR-mediated integration of large gene cassettes using AAV donor vectors. Cell reports. 2017;20(3):750-756.
3. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, *et al*. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315(5819):1709-1712.
4. Burkard C, Lillico SG, Reid E, Jackson B, Mileham AJ, Ait-Ali T, *et 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 pathogens. 2017;13(2):e1006206.
5. Butler JR, Paris LL, Blankenship RL, Sidner RA, Martens GR, Ladowski JM, *et al*. Silencing porcine CMAH and GGTA1 genes significantly reduces xenogeneic consumption of human platelets by porcine livers. Transplantation. 2016;100(3):571.
6. Carlson DF, Lancto CA, Zang B, Kim ES, Walton M, Oldeschulte D, Fahrenkrug SC. Production of hornless dairy cattle from genome-edited cell lines. Nature biotechnology. 2016;34(5):479-481.
7. Chen K, Baxter T, Muir WM, Groenen MA, Schook LB. Genetic resources, genome mapping and evolutionary genomics of the pig (*Sus scrofa*). International Journal of Biological Sciences. 2007;3(3):153.
8. Chen Y, Cui Y, Shen B, Niu Y, Zhao X, Wang L, *et al*. Germline acquisition of Cas9/RNA-mediated gene modifications in monkeys. Cell research. 2015;25(2):262-265.
9. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, *et al*. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-823.
10. Cui C, Song Y, Liu J, Ge H, Li Q, Huang H, *et al*. Gene targeting by TALEN-induced homologous recombination in goats directs production of β-lactoglobulin-free, high-human lactoferrin milk. Scientific reports. 2015:5(1):1-11.
11. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346(6213).
12. Dumne PR. Molecular cloning and characterizationof *cox-2* gene and its crispr guide RNA in buffalo. M.V.Sc. Thesis, ICAR-NDRI, Karnal, Haryana, India, 2020.
13. Feng C, Wang X, Shi H, Yan Q, Zheng M, Li J, *et al*. Generation of ApoE deficient dogs via combination of embryo injection of CRISPR/Cas9 with somatic cell nuclear transfer. Journal of genetics and genomics= Yi chuan xue bao. 2018;45(1):47-50.
14. Fernandez A, Josa S, Montoliu L. A history of genome editing in mammals. Mamm. Genome. 2017;28:6299.
15. Food and Agriculture Organization of the United Nations (FAO). How to Feed the World 2050. High-Level Expert Forum, 2009.
16. Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proceedings of the National Academy of Sciences. 2012;109(39):E2579-E2586.
17. Gao Y, Wu H, Wang Y, Liu X, Chen L, Li Q, *et al*. Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. Genome biology. 2017;18(1):1-15.
18. Giassetti MI, Ciccarelli M, Oatley JM. Spermatogonial stem cell transplantation: insights and outlook for domestic animals. Annual review of animal biosciences. 2019;7:385-401.
19. Grange JM. Mycobacterium bovis infection in human beings. Tuberculosis. 2001;81(1-2):71-77.
20. Gulati S, Maiti S. Chakraborty D. Low-cost CRISPR diagnostics for resource-limited settings. Trends in Genetics. 2021;37(9):776-779.
21. Hai T, Teng F, Guo R, Li W, Zhou Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell research. 2014;24(3):372-375.
22. Han K, Liang L, Li L, Ouyang Z, Zhao B, Wang Q, *et al*. Generation of Hoxc13 knockout pigs recapitulates human ectodermal dysplasia–9. Human molecular genetics. 2017;26(1):184-191.
23. Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. Science. 2010;327(5962):167-170.
24. Hu S, Qiao J, Fu Q, Chen C, Ni W, Wujiafu S, *et al*. Transgenic shRNA pigs reduce susceptibility to foot and mouth disease virus infection. Elife. 2015;4:e06951.
25. Huang L, Hua Z, Xiao H, Cheng Y, Xu K, Gao Q, *et al*. CRISPR/Cas9-mediated ApoE-/-and LDLR-/-double gene knockout in pigs elevates serum LDL-C and TC levels. Oncotarget. 2017;8(23):37751.
26. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. Journal of bacteriology. 1987;169(12):5429-5433.
27. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-821.
28. Kambadur R, Sharma M, Smith TP, Bass JJ. Mutations in

myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome research. 1997;7(9):910-915.
29. Kang Y, Zheng B, Shen B, Chen Y, Wang L, Wang J, *et al*. CRISPR/Cas9-mediated Dax1 knockout in the monkey recapitulates human AHC-HH. Human molecular genetics. 2015;24(25):7255-7264.
30. Kornegay JN. The golden retriever model of Duchenne muscular dystrophy. Skeletal muscle. 2017;7(1):1-21.
31. Laible G. Production of transgenic livestock: overview of transgenic technologies. Animal Biotechnology. 2018;2:95-121.
32. Li Q, Qin Z, Wang Q, Xu T, Yang Y, He Z. Applications of genome editing technology in animal disease modeling and gene therapy. Computational and structural biotechnology journal. 2019;17:689-698.
33. Li Z, Yang HY, Wang Y, Zhang ML, Liu XR, Xiong Q, *et al*. Generation of tryptophan hydroxylase 2 gene knockout pigs by CRISPR/Cas9-mediated gene targeting. Journal of biomedical research. 2017;31(5):445.
34. Luo Y, Wang Y, Liu J, Cui C, Wu Y, Lan H, *et al*. Generation of TALE nickase-mediated gene-targeted cows expressing human serum albumin in mammary glands. Scientific reports. 2016;6(1):1-11.
35. Malpotra S, Vats A, Kumar S, Gautam D, De S. Generation of genomic deletions (of Rig-I GENE) in goat primary cell culture using CRISPR/CAS9 method. Animal biotechnology. 2017;29(2):142-52.
36. McFarlane GR, Salvesen HA, Sternberg A, Lillico SG. On-farm livestock genome editing using cutting edge reproductive technologies. Frontiers in Sustainable Food Systems. 2019;3:106.
37. Miao D, Giassetti MI, Ciccarelli M, Lopez-Biladeau B, Oatley JM. Simplified pipelines for genetic engineering of mammalian embryos by CRISPR-Cas9 electroporation. Biology of reproduction. 2019;101(1):177-187.
38. Namula Z, Wittayarat M, Hirata M, Hirano T, Nguyen NT, Le QA, *et al*. Genome mutation after the introduction of the gene editing by electroporation of Cas9 protein (GEEP) system into bovine putative zygotes. *in vitro* Cellular & Developmental Biology-Animal. 2019;55(8):598-603.
39. Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, *et al*. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science. 2017;357(6357):1303-1307.
40. Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, *et al*. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell. 2014;156(4):836-843.
41. Park KE, Kaucher AV, Powell A, Waqas MS, Sandmaier SE, Oatley MJ, *et al*. Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. Scientific Reports. 2017;7(1):1-9.
42. Peng J, Wang Y, Jiang J, Zhou X, Song L, Wang L, *et al*. Production of human albumin in pigs through CRISPR/Cas9-mediated knockin of human cDNA into swine albumin locus in the zygotes. Scientific reports. 2015;5(1):1-6.
43. Qian L, Tang M, Yang J, Wang Q, Cai C, Jiang S, *et al*. Targeted mutations in myostatin by zinc-finger nucleases result in double-muscled phenotype in Meishan pigs. Scientific reports. 2015;5(1):1-13.
44. Sun B, Chen H, Gao X. Versatile modification of the CRISPR/Cas9 ribonucleoprotein system to facilitate *in vivo* application. Journal of Controlled Release. 2021;337:698-717.
45. University of Maryland School of Medicine. Successful transplant of porcine heart into adult human with end-stage heart disease: First-of-its-kind transplant was patient's only option for survival after being deemed ineligible for traditional transplant. Science Daily. (2022, January 10).
46. Wan H, Feng C, Teng F, Yang S, Hu B, Niu Y, *et al*. One-step generation of p53 gene biallelic mutant Cynomolgus monkey via the CRISPR/Cas system. Cell research. 2015;25(2):258-261.
47. Wang K, Jin Q, Ruan D, Yang Y, Liu Q, Wu H, *et al*. Cre-dependent Cas9-expressing pigs enable efficient *in vivo* genome editing. Genome research. 2017;27(12):2061-2071.
48. Wang X, Cao C, Huang J, Yao J, Hai T, Zheng Q, *et al*. One-step generation of triple gene-targeted pigs using CRISPR/Cas9 system. Scientific reports. 2016;6(1):1-7.
49. Wang X, Niu Y, Zhou J, Zhu H, Ma B, Yu H, *et al*. CRISPR/Cas9-mediated MSTN disruption and heritable mutagenesis in goats causes increased body mass. Animal genetics. 2018;49(1):43-51.
50. Wang Y, Ding Y, Li J. CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. In RNAi and Small Regulatory RNAs in Stem Cells. Humana Press, New York, NY. 2017;293-305.
51. Wang Y, Du Y, Shen B, Zhou X, Li J, Liu Y, *et al*. Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Scientific reports. 2015;5(1):1-9.
52. Wei J, Wagner S, Maclean P, Brophy B, Cole S, Smolenski G, *et al*. Cattle with a precise, zygote-mediated deletion safely eliminate the major milk allergen beta-lactoglobulin. Scientific reports. 2018;8(1):1-13.
53. Wells KD, Bardot R, Whitworth KM, Trible BR, Fang Y, Mileham A, *et al*. 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. Journal of virology. 2017;91(2):e01521-16.
54. Whitworth KM, Rowland RR, Ewen CL, Trible BR, Kerrigan MA, Cino-Ozuna AG, *et al*. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nature biotechnology. 2016;34(1):20-22.
55. Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. Nature. 2012;482(7385):331-338.
56. Wu H, Wang Y, Zhang Y, Yang M, Lv J, Liu J, *et al*. TALE nickase-mediated SP110 knockin endows cattle with increased resistance to tuberculosis. Proceedings of the National Academy of Sciences. 2015;112(13):E1530-E1539.
57. Yan S, Tu Z, Liu Z, Fan N, Yang H, Yang S, *et al*. A huntingtin knockin pig model recapitulates features of selective neurodegeneration in Huntington's disease. Cell. 2018;173(4):989-1002.
58. Yang L, Güell M, Niu D, George H, Lesha E, Grishin D, *et al*. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science. 2015;350(6264):1101-

1104.
59. Yang SH, Cheng PH, Banta H, Piotrowska-Nitsche K, Yang JJ, Cheng EC, *et al*. Towards a transgenic model of Huntington's disease in a non-human primate. Nature. 2008;453(7197):921-924.
60. Yoon Y, Wang D, Tai PW, Riley J, Gao G, Rivera-Pérez JA. Streamlined ex vivo and *in vivo* genome editing in mouse embryos using recombinant adeno-associated viruses. Nature communications. 2018;9(1):1-12.
61. Yu S, Luo J, Song Z, Ding F, Dai Y, Li N. Highly efficient modification of beta-lactoglobulin (BLG) gene via zinc-finger nucleases in cattle. Cell research. 2011;21(11):1638-1640.
62. Zhang J, Cui ML, Nie YW, Dai B, Li FR, Liu DJ, *et al*. CRISPR/Cas9-mediated specific integration of fat-1 at the goat MSTN locus. The FEBS journal. 2018;285(15):2828-2839.
63. Zhang X, Li Z, Yang H, Liu D, Cai G, Li G, *et al*. Novel transgenic pigs with enhanced growth and reduced environmental impact. Elife. 2018;7:e34286.
64. Zhao J, Lai L, Ji W, Zhou Q. Genome editing in large animals: current status and future prospects. National Science Review. 2019;6(3):402-420.
65. Zheng Q, Lin J, Huang J, Zhang H, Zhang R, Zhang X, *et al*. Reconstitution of UCP1 using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and improves thermogenic capacity. Proceedings of the National Academy of Sciences. 2017;114(45):E9474-E9482.
66. Zhou X, Xin J, Fan N, Zou Q, Huang J, Ouyang Z, *et al*. Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer. Cellular and molecular life sciences. 2015;72(6):1175-1184.