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CRISPR in livestock: From editing to printing

A. Menchaca [a, *], P.C. dos Santos-Neto [a], A.P. Mulet [b], M. Crispo [b]

[a] Instituto de Reproducción Animal Uruguay, Fundación IRAUy, Cruz del Sur 2350, Montevideo, Uruguay
[b] Unidad de Animales Transgénicos y de Experimentación (UATE), Institut Pasteur de Montevideo, Mataojo, 2020, Montevideo, Uruguay

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ABSTRACT

Precise genome editing of large animals applied to livestock and biomedicine is nowadays possible since the CRISPR revolution. This review summarizes the latest advances and the main technical issues that determine the success of this technology. The pathway from editing to printing, from engineering the genome to achieving the desired animals, does not always imply an easy, fast and safe journey. When applied in large animals, CRISPR involves time- and cost-consuming projects, and it is mandatory not only to choose the best approach for genome editing, but also for embryo production, zygote microinjection or electroporation, cryopreservation and embryo transfer. The main technical refinements and most frequent questions to improve this disruptive biotechnology in large animals are presented. In addition, we discuss some CRISPR applications to enhance livestock production in the context of a growing global demand of food, in terms of increasing efficiency, reducing the impact of farming on the environment, enhancing pest control, animal welfare and health. The challenge is no longer technical. Controversies and consensus, opportunities and threats, benefits and risks, ethics and science should be reconsidered to enter into the CRISPR era.

1. Introduction

Genome editing in large animals may be applied with different purposes, including biotechnology to improve food production, animal health and pest control, and the generation of animal models for biomedicine and basic research. The main question for an innovative technology is why it should be used. In livestock, the global context of food demand and production, as well as new concerns in terms of environmental sustainability and animal welfare, may explain the potential usefulness of CRISPR.

Increasing demand for food required by the world's growing population is one of the biggest challenges for the future of the human species. According to FAO, UN and WB, this consumption level is not only increased by the global population growth, exceeding 9 billion by 2050 [1], but also by a higher *per capita* consumption of animal protein over vegetable carbohydrates. Extreme poverty rates have significantly decreased over the past years and people have more access to better quality food [2]. This growing demand has already pushed livestock production and this phenomenon will increase in the coming years [3]. Our challenge will be to achieve a balance to attain greater food production, ensuring global sustainability, preserving climate change and deforestation, respecting biological diversity and animal well-being, and guaranteeing equity to global food access.

Classical approaches to enhance productivity based on the improvement of animal health, nutrition, genetics, reproduction and management, will make an important contribution. However, this will not be enough to ensure the productive change required. This scenario needs novel ideas and disruptive technologies, and CRISPR appears as a powerful tool to contribute to global livestock transformation. How would the future of farm animals be with novel production traits, with resistance to transmissible diseases, or even with the eradication of pests that have harmed livestock throughout the history? With CRISPR we are closer to make these things happen.

2. Bases of the CRISPR-Cas system

CRISPR-Cas system is present in nature as a component of the bacteria and archaea immune system, protecting them from invading DNA contaminants [4]. In CRISPR-Cas system type II used in genetic engineering, the specificity of the cut is given by the CRISPR RNA (crRNA). This molecule needs to interact with a trans-activating crRNA (tracrRNA) to form a crRNA_tracrRNA duplex,

* Corresponding author.
E-mail address: menchaca.alejo@gmail.com (A. Menchaca).

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directing the CRISPR-associated (Cas) nuclease to specific sites (spCas9 in the case of the *Streptococcus pyogenes* type II CRISPR system). The crRNA and the tracrRNA sequences can be combined into a single guide RNA (sgRNA), directing Cas9 to the desired site and catalyzing the DNA cleavage. Once these components are introduced within the cell (or into the zygote), they will guide the Cas9 to the complementary locus in the genome and will create a double-strand break (DSB) [5].

The generated DSBs will be repaired through two mechanisms, non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Fig. 1). The NHEJ pathway frequently leads to small insertions or deletions (indels), or chromosomal rearrangements. These often disrupt open reading frames, effectively creating gene knockouts. This mechanism led to the generation of the first successful disruption of endogenous genes by CRISPR-Cas system in livestock (sheep [6,7], goats [8,9], cows [10] and pigs [11–14]). On the other hand, the HDR pathway employs a homologous repair template to fix the DSB. This mechanism allows the creation of specific changes in the DNA, which is mediated by the addition of the adequate repair template containing the desired insertion or modification. Genome editing conducted by this mechanism can be used to insert a predefined single nucleotide or sequence, or even change or delete it in existing genes. After the first knockouts births reported by NHEJ, successful generation of knock-in large animals by HDR were achieved in sheep [15,16], goats [17] and pigs [18].

Amazing advancement of this technology does not cease to surprise us. Recently, fusion of an inactive form of Cas9 with a cytidine deaminase enzyme permitted to develop a new CRISPR tool called base editors that perform C→T substitutions [19]. Furthermore, a new promising CRISPR variant consisting of a

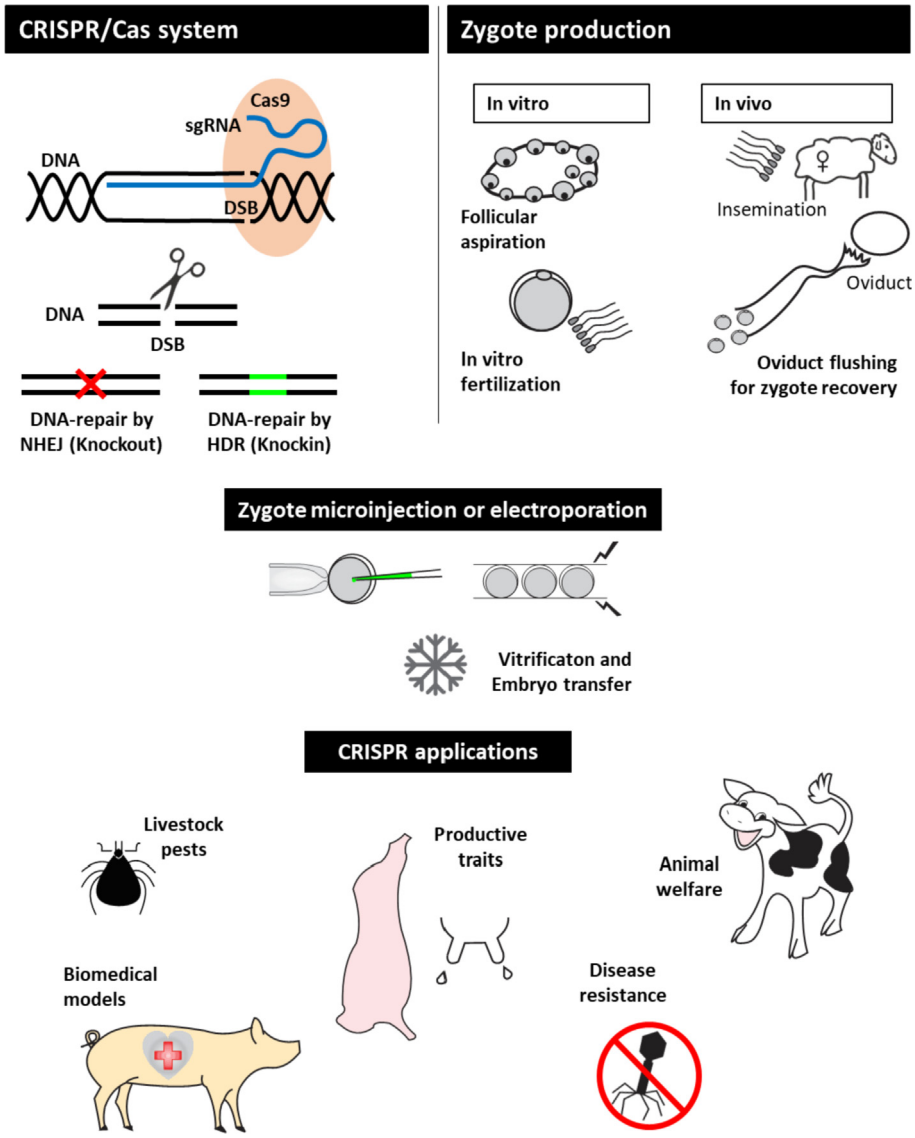

Fig. 1. The CRISPR pipeline to transform livestock: From editing to printing. Different approaches for CRISPR-Cas system, consisting of a single guide RNA (sgRNA) designed to direct Cas9 to the desired DNA site and induce double strand breaks (DSB). DNA cleavage results in different gene repair mechanisms as nonhomologous end joining (NHEJ) or homology-directed repair (HDR, *i.e.*, in the presence of a DNA donor template). Zygotes are obtained by *in vitro* embryo production (*in vitro* maturation and fertilization), or derived from *in vivo* production (insemination and oviduct flushing). Delivery of CRISPR-Cas components into the zygote is performed by direct microinjection into the cytoplasm, or alternatively, by electroporation with no need for embryo micromanipulation. Embryo transfer is carried out either with fresh or cryopreserved embryos by vitrification with minimum volume method (*e.g.*, Cryotop). The wide range of CRISPR applications in large animals include improving productive traits, enhancing animal welfare through adaptation and resilience, conferring resistance to infectious and transmissible diseases, generating animal models for biomedical research, and suppressing other species considered as pests for livestock.

catalytically impaired Cas9 endonuclease fused to a reverse transcriptase allows performing precise targeted insertions, deletions and point mutations without requiring DSBs or donor DNA templates. This approach, known as prime editing, presents lower off-targets activity and fewer byproducts than previous alternatives [20].

3. The design of the CRISPR components

3.1. The effector nucleases

When designing an experiment or project, one of the main things to determine is which nuclease to use. The spCas9 nuclease is widely used but there are other options that should be considered depending on the purpose of the project. Nucleases such as Cas12a or CasX are also available and have unique structural and functional features, providing new opportunities for genome editing applications. Cas proteins can be introduced as DNA expression plasmid, *in vitro* transcript, or as a recombinant protein bound to the sgRNA in a ribonucleoprotein particle (RNP). Despite being the easiest system, plasmid DNA can be randomly integrated into the genome and thus integration need to be carefully assessed. RNA reagents are easy to generate and can be cloning-free, however, Cas9 time-course experiments revealed that the RNP complex triggers mutations faster than Cas9 mRNA + sgRNA [21]. Moreover, the longer they last inside the cell, the bigger the probability to generate complex modifications and mosaics as well as more off-target effects.

3.2. The sgRNAs

The second element for genome editing by CRISPR involves target site selection and sgRNA design. Any DNA locus containing the sequence NGG, named as protospacer adjacent motif (PAM), represents a potential target site for CRISPR/Cas9 system. Nevertheless, not all the sequences induce cuts with the same efficiency and specificity. sgRNAs sequences should be as unique as possible, to limit the possibility of unintended DSBs in partially complementary sequences. It is preferable to select crRNAs whose seed sequences (first 12 nucleotides adjacent to the PAM) are unique, since those are essential for the specificity on a 20 nucleotides crRNA.

In livestock, where gestation length is long and high-cost projects are required, failure to achieve edited offspring or inefficiencies in the system due to the editing design or reagents quality have frustrating impact and should be minimized. In this kind of projects, a careful design and previous validation of the sgRNAs becomes essential. For *in silico* design, many web tools and software packages allow predicting the activity and specificity of the sgRNAs. After selecting these sequences, it is highly recommended to validate them before generating the animals. This analysis can be done *in vitro* in different cell-free systems, in *in vitro* cultured cells of the same species, or directly in embryos. Since the chromatin context influences the cutting efficiency of the CRISPR-Cas system, more reliable results will be obtained if the sgRNAs are analyzed in embryos. Embryo sequencing may be conducted in the whole embryo and then, if efficiency is acceptable, proceed to embryo production sessions for embryo transfer. Although it is feasible to perform a biopsy in the same blastocysts that will be transferred, pre-implantation analysis of biopsied blastomeres is not always representative of the genotype of the born animals due to the occurrence of mosaicism [22,23].

3.3. The repair template

The incidence of HDR for DSB repair is low compared to NHEJ, thus designing optimal DNA donor templates can increase HDR frequencies. ssDNA donor templates have shown greater recombination efficiency and require shorter homology arms than dsDNA. When searching for specific mutations or small sequences insertions, the most used templates are single-stranded donor oligonucleotides (ssODNs). Homology arms for ssODN donor should be approximately 60–70 bp each. Although some reports suggest that asymmetric arms improve efficiency, there is no consensus in the convenience of this strategy in the template design [24,25]. Larger insertions require dsDNA templates with homology arms of 1–3 kb at either side of DSB sites or long ssDNAs. The insertion efficiency of plasmid-mediated HDR with dsDNA is much lower than with ssDNA [26]. Nucleases may re-cut the target locus after the knock-in sequence is inserted. To avoid recurrent DSBs, a silent mutation should be inserted at the PAM site or the target site. If an incompatible PAM or target site sequence is introduced into the genome via the knock-in, the sgRNA-Cas9 complex can no longer bind to the target site, and no further edits can be made. The use of ssODN to induce knock-in or to improve knockout efficiency has been reported recently in ruminant species (sheep: [15,16]; goats: [17]; cows [27]).

4. Embryo manipulation

Advanced reproductive technologies play a critical role in the generation and propagation of edited animals. Novel molecular strategies are available for the generation of genome engineered animals, but mainly in livestock, the success always requires mastering several reproductive technologies. *In vitro* embryo production (IVEP) is the preferable method of choice to obtain enough quantity of excellent quality zygotes available to be microinjected with the CRISPR components (Fig. 1). Oocytes are collected after follicular aspiration from live females, or moreover from ovaries of a nearby slaughterhouse. Of notice, although zygotes may be collected from the oviduct, *in vivo* zygote production involves several issues [28] that were overcome with the development of IVEP technology. The success of *in vitro* production implies that the IVEP laboratory should be capable to obtain good fertilization and cleavage rates (~80–90%) and should have optimum culture conditions to achieve acceptable *in vitro* development rates (~30–40% blastocysts). For further revision of the procedure for IVEP conducted in our laboratory, see previous review [29].

Standard microinjection protocols originally developed to obtain genome-edited mice have been adapted to livestock zygotes, since CRISPR system can be easily injected into the dark cytoplasm of livestock species with no need to visualize the pronucleus. The possibility of cytoplasmic injection (instead of pronuclear injection), represents another great advantage of CRISPR technology, avoiding the centrifugation of zygotes required in farm animals for pronuclear injection. Thus, this technical advantage allows an easier, faster, more efficient and more innocuous microinjection in terms of embryo survival. For this reason, CRISPR microinjection into the cytoplasm of zygotes appears as the preferable method in mammals [28]. On the other hand, as an alternative to direct microinjection into the zygotes, CRISPR cell transfection for somatic cell nuclear transfer (SCNT) is also used in livestock species. The generation of a live edited animal resulting from SCNT embryos is not an easy task, adding complexity to the whole procedure of genome editing. Although CRISPR success in donor cells is acceptable, SCNT procedure has low efficiency and a low proportion of transferred embryos results in live offspring. Embryos produced by cloning have low development rates, are susceptible to

developmental and epigenetic reprogramming anomalies, thus inducing pregnancy failure, stillborn or low newborn survival rates. When SCNT is mastered by the laboratory, the low efficient rate will be compensated with most of the healthy born animals carrying the desired mutation. Genome editing mediated by SCNT is applied by some laboratories in some kind of projects (*e.g.*, multiplex editing), but the high efficiency of CRISPR after direct microinjection into zygotes has allowed an easier approach (sheep: [6,7,15,16]; goats: [9,17]; pigs: [11,13]). All together, these reports showed that with CRISPR it is possible to perform direct microinjection into the zygote of different species, with minimum effects on developmental competence and pregnancy rates, high newborn survival rate, and high editing rate with acceptable homozygous proportion.

For zygote microinjection, the mixture containing the CRISPR components is prepared in the laboratory. Cas9, sgRNA and ssODNs purity, quality and concentrations are critical to achieve a good balance between embryo survival and editing rate. As no absolute rule exists, each laboratory should test their reagents and concentrations before launching projects, since getting negative results in the offspring will be discouraging and expensive. Although reagents concentration is not a major problem and may vary among laboratories, as a reference we usually use Cas9 RNP at 50–500 ng/μl, sgRNAs at 5–200 ng/μl, and ssODNs at 200 ng/μl. With these concentrations, acceptable editing rates of 40–50% were achieved in lambs, with 50–60% of them being knock-in (summarized from 95 lambs born [7], and unpublished data).

Microinjection is usually performed under an inverted microscope connected with two micromanipulators. Up to 500 presumptive zygotes can be microinjected one by one into the cytoplasm (2–5 pl of injection mix) in each session. On the other hand, electroporation has recently been described as an alternative approach to deliver small CRISPR reagents into mouse and rat zygotes [30,31] and it is currently used in our laboratory [28,32]. This procedure avoids the technically demanding microinjection technique allowing a high throughput scheme in the laboratory. Microinjection requires investment in expensive equipment for embryo micromanipulation, long-term expertise and technical skills. Electroporation also requires equipment, but not as expensive as the microscope and micromanipulators, and it may be already available at the bench for other purposes. The procedure is much easier, the learning curve for the technicians is much faster, and many zygotes may be electroporated in few minutes. Although this method has clear advantages compared with microinjection, little information is reported in livestock to date [33–36]. Proper adaptation from mice and rats protocols to large animals' species would allow the replacement of microinjection by electroporation, as is happening in mice.

After microinjection or electroporation, zygotes can be transferred immediately into the oviduct, or cultured *in vitro* until the blastocyst stage to be transferred into the uterine horn, or cryopreserved in different stages to be stored until embryo transfer.

One of the greatest challenges in *in vitro* produced embryos in farm animals is to overcome the low cryotolerance compared with *in vivo* derived embryos. In addition, for genome editing the embryos are subjected to microinjection, and thus survival rate and cryotolerance may be further affected. For this reason, in most of the projects involving microinjection, embryos are transferred fresh. However, in long-term programs with hundreds of embryos produced every week, a large and continuous supply of synchronized recipients ready to use are required. In this context, current improvements on cryopreservation of *in vitro* produced embryos deserve to be considered.

Novel vitrification systems named as minimal volume methods have been proposed for *in vitro* produced embryos [37]. We have been conducted a series of experiments to evaluate these minimum volume methods for different stage ovine embryos [32,38–40], allowing to standardize the method for cryopreservation in genome editing projects. The outcomes on 1207 embryos microinjected with CRISPR that were transferred in different developmental stages, associated or not to vitrification, are presented in Table 1. Vitrification by minimum volume methods (Crytop and Spatula MVD) are used as a routine in our laboratory with acceptable outcomes, both in livestock and murine embryos after microinjection for genome modification [32,41]. This practice simplifies embryo transfer programs to produce genome-edited animals, mainly in large-scale projects involving livestock.

5. Gene editing analysis

Once presumptive genome-edited animals are born, an exhaustive genotypic analysis in addition to the phenotypic one is necessary. Those animals in which CRISPR/Cas system was effective, will carry a different combination of mutated alleles, which in some cases may be in the form of mosaic. The available methods to analyze CRISPR editing efficiency vary depending on the type of intended change. As NHEJ generates random and heterogeneous indels in the animals, the sgRNA can be designed in such a way that the cut intervenes in a restriction site to facilitate subsequent genotyping, however, this approach limits the number of available loci. In addition, there are some methods that are based on the detection of heteroduplex formed when a wild type and a mutant PCR amplicon (or amplicons that carry two different mutations) bind together. These techniques allow genotyping mutations in any locus. Methods based on nucleases include Surveyor nuclease and T7 Endonuclease I assay (T7EI assay). Both nucleases target and digest mismatched heteroduplex double-stranded DNA, and as a result produces two or more smaller fragments in an enzymatic reaction. The digested DNA fragments can thus be resolved and visualized by agarose gel electrophoresis. Heteroduplex mobility assay (HMA) is based on the differential migration of DNA molecules with and without mismatches in a non-denaturing PAGE and will show a retarded electrophoresis migration.

None of the above assays can exactly reveal the nature of the mutation introduced. In the end, it is always necessary to sequence the region to determine the mutation. Sanger sequencing of amplicons from founding animals can be problematic, mainly in the presence of mosaics, since it results in asynchronous and overlapping chromatograms. One way to overcome this problem is to clone PCR products into plasmids and sequence a sufficient number of independent clones to obtain a representative sample of the present alleles in the animal (usually between 10 and 20 clones).

Editing involving HDR, which introduces new DNA sequences, can be assessed by a number of methods such as the restriction enzyme digest if the mutation results in a loss or gain of a restriction enzyme site. An alternative is the use of deep sequencing or digital PCR of the area of interest. This system allows detecting alleles that are in low abundance, and in addition, it can be simultaneously used to identify off-target sites. Although off-target sites should always be analyzed mainly in those projects involving livestock for food production, the frequency and number of off-targets produced by the Cas9 nuclease is low in live animals compared to *in vitro* cultured cells [42,43]. In addition, the possible off-targets can be removed with the subsequent selected breeding of animals. As a rule, a careful selection of target sequences to avoid predicted off-target sites is mandatory, avoiding guides with possible off-targets sites on the same chromosome.

6. Printing the edited: CRISPR applications

Some of the prospective applications of CRISPR include

Table 1
Pregnancy outcomes after zygote microinjection with CRISPR/Cas system of *in vitro* produced embryos transferred fresh or subjected to vitrification in sheep (unpublished data).

Stage of embryos	Fresh or Vitrified	Embryo transfer	No. of recipients	No. of transferred embryos	Pregnant/transferred recipientes (%)	Birth/pregnant embryos (%) *
Early stages						
Day 2 (2–8 cells; trial #1)	Fresh	Oviduct	50	262	24.0% (12/50)[a]	72.2% (13/18)[a]
		Uterine horn	52	276	25.0% (13/52)[a]	100% (17/17)[a]
Day 2 (2–8 cells; trial #2)	Fresh	Uterine horn	24	120	54.2% (13/24)	88.2% (15/17)
Late stages						
Day 6 (Blastocysts; trial #3)	Fresh	Uterine horn	25	75	48.0% (12/25)[a]	75.0% (12/16)[a]
	Vitrified	Uterine horn	159	474	30.8% (49/159)[b]	85.7% (60/70)[a]
Overall results			310	1207	32.0% (99/310)	84.8% (117/138)

For different trial # (three trials), a vs. b, $P < 0.05$ (General Linear Mixed Models). *Some births were twins.

improving productive and fitness traits in large animals, conferring resistance to infectious and transmissible diseases, enhancing animal welfare through improving adaptation and resilience in animals, and suppressing other species considered as pests for livestock. These uses for CRISPR have been either reported as a proof of concept, for research, or proposed for commercial use. Some CRISPR edited animals are summarized below, illustrating the main or first reported ideas to give an overview of how this technology can contribute to the livestock transformation (Fig. 1). A wider list of reports is summarized in previous reviews [28,44,45].

6.1. Improving productive traits

The first animals edited by CRISPR in our laboratory were born in 2014 [7]. In that project Superfine Merino breed was used as genetic background to introduce a *MSTN* mutation that improves meat production. Superfine Merino is recognized as the best breed to produce the finest and highest quality wool, but the problem for farmers is the lower growing rate and smaller size of the lambs in comparison with other breeds. On the other hand, several breeds have been improved through the years by classic genetics (*i.e.*, selective breeding) to produce meat, which was in detriment of wool quality. A good example is Texel, the most popular breed in the Netherlands and distributed worldwide. The impressive growth rate and meat production of this breed is associated with a spontaneous mutation at the *MSTN* gene encoding for myostatin (or GDF8) that is involved in muscle cell grow and differentiation. By suppressing the function of *MSTN*, the muscle mass increases. In our study, Merino embryos were edited with CRISPR to disrupt the *MSTN* gene. As a result, double muscle lambs were obtained, thus achieving greater growing rate and heavier body weight than Superfine Merino lamb counterparts. The knockout lambs were 25% heavier [7] and maintained the same wool quality traits than Merino lambs (unpublished data). Although this proof of concept did not include the study of the offspring, in following studies germline transmission of *MSTN* mutation have been reported by its presence in the gonads of founders (in sheep [46] and goats [47]) and offspring [47]. Amazingly, these early studies suggested that what farmers have pursued for centuries (*i.e.*, animals producing both high-quality wool and more meat), might be achieved by CRISPR in few months.

Other similar models to improve productive or fitness traits have been reported in other species. In China, Cashmere goats, which are known primarily for their high-quality hair, were simultaneously edited by CRISPR at the *MSTN* and *FGF5* gene, in order to improve both meat and hair production [9]. In pigs also, after the first report conducted to disrupt *MSTN* using CRISPR in this species [48], several studies have been conducted to improve carcass traits through this strategy. In addition, novel ideas have been proposed to use CRISPR for altering male:female ratio in livestock, particularly when the desired product is provided by only one sex (*e.g.*, meat from males or milk from females). Only male offspring strategy has been proposed in beef cattle [49], since males grow faster and are bigger than females, and only male production may improve efficiency and avoid female culling by farmers. Another approach has been proposed in pigs, in this case to suppress testis development in the fetal gonadal ridges resulting in a female phenotype and avoiding the undesirable male-specific boar taint [50]. Both strategies still needs to be proven and require further investigation. These and other improvements have been proposed in different species and serve as examples of the opportunities of CRISPR to enhance livestock efficiency. Disruption of single genes that have significant effects on traits of economic relevance appears as an interesting approach to improve farm animal genetics.

6.2. Disease-resistant animals

Diseases affecting livestock can have a devastating impact on production, industry and trade of live animals and derived products, and even on zoonosis and public health. Not only limited to the region or country in which the disease appears, some pandemics have even global effects as is now happening with African swine fever in Asia, disturbing the meat and crop market and producing global consequences. Animal welfare should be also taken into account, as the first affected subject are the proper animals suffering the disease and death. According to OIE publications [51], due to the intensification of livestock production among other factors, the world is facing an unprecedented increase of emerging and re-emerging animal diseases and zoonosis. Thus, decreasing disease susceptibility in livestock has become an interesting focus of research.

With CRISPR, the generation of disease-resistant animals may be achieved. Some examples have been reported recently, and one of the clearest models was the generation of resistant pigs to Porcine Respiratory and Reproductive Syndrome virus (PRRSV). This disease causes the most important economic losses in the pig industry, with more than 2.5 billion dollars per year only in US and Europe. Vaccines have not been able to control the disease, there is no effective treatment, and due to the high level of infection and expansion, the only effective method to eradicate the virus during a PRRSV outbreak is depopulation of the herd, sterilization, and repopulation. The macrophage surface protein CD163, which belongs to the scavenger receptor cysteine rich family, mediates the entry of PRRSV into the host cell. Based on this information, genome-edited pigs with a disruption in the *CD163* gene were produced by CRISPR, conferring resistance to PRRSV infection. *CD163* edited piglets, reported in two different projects in US and Scotland, were completely resistant to North American and European PRRSV strains [52,53]. The authors reported that the animals

showed no symptoms or suffered no infection to the viral *in vivo* challenge, demonstrating the effectiveness of this strategy.

African swine fever, a transboundary animal disease for which there are no approved vaccines and euthanasia is required, is currently producing massive losses in pig industry in Asia and represents a risk for other regions. Although the virus affect domestic and wild pigs, the infection is generally asymptomatic in warthog (a wild pig of sub-Saharan Africa). This type of resistance to African swine fever virus in warthog was associated with a variation in the gene encoding RELA [54]. Researchers from the Roslin Institute have proposed to edit the domestic pig inducing this variation in *RELA* with the aim to confer disease resilience to this virus [55,56]. In another study, CRISPR has also been used to produce Coronavirus resistant pigs, by editing a putative receptor of the transmissible gastroenteritis virus [57]. In China, edited cows with increased resistance to tuberculosis were produced through CRISPR [10]. These examples show the potential of CRISPR as a novel strategy to control infectious diseases in livestock.

6.3. Improving animal welfare

Attempts to move towards more compassion and respect for animals have been encouraged in the last years, seeking to avoid unnecessary suffering in livestock. Most of the strategies consisted in the improvement of the animal practices or the environment, but adaptation (or edition) of the animals to the productive systems had not been addressed. Gene editing can also make a contribution in this regard. The concept of welfare-enhanced animals is a novel strategy to avoid animal suffering, designing genetic adaptation and resilience [58]. Many routine procedures used to mitigate some of the consequences of intensive livestock, such as calf dehorning, male castration, tail-docking in dairy cattle, mulesing and tail-docking in sheep, abortion or offspring culling of not desired gender, often results in both immediate or chronic pain. Some of these practices may be avoided with the application of genome editing.

Horn removal is a routine practice in calves, however, pain and stress for the animals is an inevitable consequence, increasingly questioned by consumers. Several beef breeds like Angus, are naturally horn-free due to a dominant trait referred to as polled, with two allelic variants on the bovine chromosome. Genetics improvement programs by selective breeding have not been effective to introduce this polled trait into Holstein cattle. Using TALENs, Carlson et al. [59] introgressed the causative celtic mutation (Pc) into the Holstein cattle genome resulting in a polled phenotype of the offspring. This example represents a potential approach for reducing physical dehorning in dairy cattle without loss of productivity. As mentioned, the use of CRISPR to produce germline ablated male pigs has been proposed [50], and even thought this remains to be proven, it would offer new opportunities to finish with surgical castration in pigs [60,61]. Avoiding these practices in animal husbandry may encourage public support of genome-edited animals for food chain production.

6.4. Large animals models in research and biomedicine

Although the regulatory system of CRISPR is still unclear for agricultural applications in some countries, genome editing will continue to advance biomedicine and basic research. Large animal models can now answer basic and applied questions using novel approaches not available before (*e.g.*, knockouts and single-base changes). In biomedicine, many human diseases including cancer, diabetes, heart diseases, and various neurological conditions, are caused by numerous variants in genes. Generation of animal models that replicates human mutations are a well-established tool in mice since homologous recombination in embryonic stem cells was achieved in this species several years ago. On the contrary, this was not technically feasible in large animals until the genome-editing era arrives. Since then, using farm animals as relevant preclinical models for human therapies is gaining worldwide interest. Although the pig is the most used animal species in biomedicine and xenotransplantation, sheep, goats and cattle are also interesting models to be studied.

Recently, CRISPR-Cas system was used to produce a sheep knockout model (assisted by ssODN) for human deafness by editing the *OTOF* gene (Menchaca et al., unpublished data). The absence of otoferlin, encoded by this gene, is involved in hearing impairment in humans, and a large animal model that mimics this disorder will be useful to test diverse therapies that could reverse the hearing disability. From 73 lambs born, 13 showed indel mutations (17.8%), and eight of them (61.5%) carried knock-in mutations by HDR (unpublished results). Also in sheep, researchers from Utah developed a knockout model for cystic fibrosis, a genetic disease with progressive lung affectation [62]. They combined CRISPR with SCNT to obtain $CFTR^{-/-}$ lambs with a similar phenotype as in humans, providing an interesting model to advance the development of new therapies. Another sheep model of a rare human bone disease, hypophosphatasia, was recently reported using CRISPR/Cas9 to introduce a single point mutation in the tissue nonspecific alkaline phosphatase (*TNSALP*) gene, which induced the same disorder as in humans [15]. In addition, an ovine model for infantile neuronal ceroid lipofuscinosis (CLN1 disease), a devastating neurodegenerative pediatric disorder with no cure, was achieved more recently using CRISPR/Cas9 system to introduce the same human mutation [16]. The availability of CRISPR to accurately replicate the clinical phenotype of human diseases in large animals is an invaluable tool for the understanding of disease progression and the development of more effective therapeutics.

Several biomedical models have been reported in pigs, but one of the main contributions of this species is in xenotransplantation. Animal organs and tissues are considered to be a promising solution to overcome the global shortage due to the growing demand for human transplantation. The CRISPR system may be used to produce edited pigs to intend preventing hyperacute rejection, acute vascular rejection mechanisms, and potentially promote tolerance in pig-to-human xenotransplantation. Interesting strategies have been reported mainly including knockouts models [63]. Although the use of these pigs as donors in preclinical nonhuman primate models has been limited up to date, *in vitro* analysis of their cells has provided invaluable information. An interesting approach to inactivate porcine endogenous retrovirus (PERV) was reported in CRISPR edited pigs [64]. Endogenous retrovirus is viewed as a potential infectious risk in xenotransplantation of pig organs, and this strategy opens new opportunities for research in this field. The increasing availability of CRISPR large animal models for human diseases and xenotransplantation will help to develop global therapies and personalized medicine to improve human health.

6.5. No pests in livestock: the challenge of gene drive

If genetic engineering in livestock has been technically amazing and ethically controversial, its application in wild population is even much more challenging. Gene drive enabled by CRISPR has brought an unprecedented possibility to propagate genetics through populations in wild species. Since 2015, CRISPR was fine-tuned to develop a genetic system with the ability to 'drive' themselves and nearby genes through populations, in a greater frequency than predicted by Mendelian inheritance. In sexual reproduction, offspring inherit two versions of every gene, one from each parent with the same opportunity to be transmitted

(50:50). On the opposite, gene drive ensures that the genetic edition will almost always be transmitted, allowing the variant to rapidly spread through a given population. This application differs from classic gene editing by associating a sequence that expresses a CRISPR endonuclease to the RNA guide into the target site, cutting the sister allele in the homologous chromosome. DNA repair occurs by HDR, and CRISPR and any additional sequence included in the cassette is copied onto the homologous chromosome. The system ensures homozygosity for the edited segment. This kind of 'super-Mendelian' inheritance makes possible to drive any edition through the desired population, and if the edition compromises essential traits, the entire population is suppressed. Thus, when applied in pests affecting livestock, the environment (or fauna) where large animals will be raised can be engineered. After releasing relatively few edited insects in wildlife, a great impact in a given target population is expected, including eradication or extinction. For this reason, gene drive is as promising as controversial.

A pest is any animal or plant detrimental for humans, affecting directly public health, livestock, agriculture or wildlife. In livestock, these species usually compete for the same resources than humans, and they can generate losses either by a direct effect on the host or by acting as a vector of other diseases. Approximately 6 million metric tons of pesticides are sprayed onto the global landscape each year [65]. Because chemical-based farming contributes negatively to sustainability, the exploration of different approaches is encouraged. Gene drive holds the potential to control pests without the use of chemicals or pesticides [66]. As preliminary advances, gene drive system has been successfully reported in proof-of-concept studies in insects [67,68], mainly with the focus on controlling vector-borne diseases. Interesting ongoing projects and novel ideas are being explored nowadays, mainly in human vector-borne diseases; in our case we are involved in a collaborative project to suppress screwworm fly population (*Cochliomyia hominivorax*) that produce important loses in livestock in South America. After the insects, targeting invasive vertebrate pests affecting livestock, agriculture and wildlife will be for sure the next step. Application of this technology will require a global discussion and a case by case study; who decides to use gene drive and when, is a big challenge for the coming years.

7. Concluding remarks and perspectives

The development of CRISPR for genome editing has led to a range of novel ideas addressing challenges associated with modern livestock, including productive and fitness traits, animal health and welfare, environmental preservation, and impacts on human health. This technology allows moulding the animal kingdom and the environment as never achieved before, in order to pursue human purposes to enhance global well-being. Once the technical challenge has been overcome, the focus will become political. Who decides, and when and how it should be regulated, are questions under current debate. Any decision that could affect the global population must be collectively discussed. In the end, probably sooner rather than later, this technology will be applied as part of the globalization we all live in. Restrictive regulations in some countries will become opportunities for others; those who excessively restrict biotechnology today will pay-per-use tomorrow. We should decide on which side the future will find us.

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