GENOME EDITING IN CATTLE: RECENT DEVELOPMENTS AND PROSPECTS

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TAKE HOME MESSAGES

- Genome editing refers to use of site-directed nucleases (e.g. Zinc finger nuclease, TALENS, CRISPR/Cas9) to introduce targeted alterations into genomic DNA sequences
- Genome editing offers a way to repair genetic defects, inactivate or knock-out undesired genes, or move beneficial alleles and haplotypes between breeds in the absence of linkage drag
- Genome editing synergistically complements, not replaces, traditional breeding programs
- Genome editing has been employed to introduce useful genetic variants impacting disease resistance, product quality, adaptability, and welfare (e.g., polled or hornlessness) traits into cattle breeding programs
- Regulatory oversight of genome editing in animals varies by country; the U.S. FDA proposes to treat each "intentional genomic alteration" introduced by genome editing as a new animal drug

INTRODUCTION

Genome editing involves using a nuclease (e.g., Zinc finger nuclease, TALENS, CRISPR/Cas9) targeted to a specific sequence in the genome to introduce a double-stranded break in the DNA double helix at that target site. One method that cells use to repair double-stranded breaks is non-homologous end joining (**NHEJ**) where the two broken ends are brought back together and the phosphodiester bonds reformed. This method is error-prone and often results in small insertions and deletions at the target cleavage site because of mistakes in the repair process. These alter the nuclease target site and prevent further cleavage events. An alternative repair mechanism is homology-directed repair (**HDR**) using homologous DNA as a repair template. A DNA repair template can be added with desired modifications between regions of homology to either side of the DSB. This template can be used to introduce a range of genome edits from point mutations to whole-gene insertions (Figure 1).

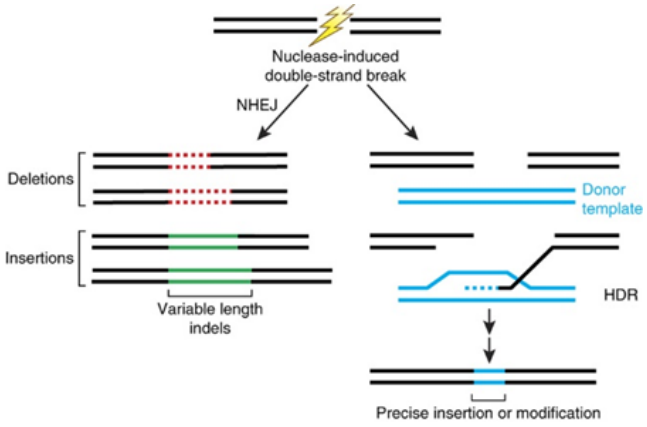

Figure 1. Nuclease-induced double-strand breaks can be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. Imprecise non-homologous end joining-mediated repair can produce variable-length insertion and deletion mutations at the site of the double-strand break. Homology-directed repair-mediated repair can introduce precise point mutations or insertions from a single-stranded or double-stranded DNA donor template (Sander and Joung, 2014).

The previous generation of genetic engineering tools, resulting in the first transgenic livestock more than 30 years ago (Hammer et al., 1985), was limited to the insertion of foreign DNA into the genome. This DNA was generally in the form of a recombinant DNA (**rDNA**) construct comprised of a promoter and a protein coding region (protein upregulation), or an inhibitory RNA encoding region (protein downregulation). As integration was random, there was no way of predicting all the possible effects that introducing the transgene would have on the animal as the epigenetic environment varies among different regions of the genome. It also meant that each genetically engineered founder animal had the gene inserted into a different location in the genome. There is only one single approved genetically engineered animal for food purposes globally, the fast-growing AquAdvantage Atlantic salmon.

Genome editing presents an approach to introduce targeted modifications into existing genes and regulatory elements within a breed or species, without necessarily the introduction of foreign DNA, potentially avoiding concerns regarding transgenesis. It offers a new opportunity to accelerate the rate of genetic gain in livestock by precisely introducing useful extant genetic variants into structured livestock breeding programs. These variants may repair genetic defects, inactivate or knock-out undesired genes, or involve the movement of beneficial alleles and haplotypes between breeds in the absence of linkage drag.

Genome editing research in cattle to date has focused primarily on monogenic (single gene) traits like disease resistance (e.g., tuberculosis), production (e.g., myostatin knockout), generation of single gender (all-male) offspring (Owen (2019), A.L. Van Eenennaam laboratory, unpublished data), elimination of allergens (e.g., beta-lactoglobulin knockout), and welfare traits (e.g., polled or hornlessness; Table 1).

Table 1. Examples of proposed and potential targets for genome editing in cattle. Updated from Van Eenennaam (2017).

Target	**Targeted Trait/Goal**	**Reference**
Intraspecies *POLLED* allele substitution	No horns/welfare trait	Carlson et al. (2016)
Intraspecies *SLICK* allele substitution	Heat tolerance	Porto-Neto et al. (2018) (not yet published)
Myostatin (MSTN) gene knockout	Increased lean muscle yield	Proudfoot et al. (2015)
Beta-lactoglobulin gene knockout	Elimination of milk allergen	Yu et al. (2011) Wei et al. (2018)
Prion protein (PRNP) knockout	Elimination of prion protein	Bevacqua et al. (2016)
Intraspecies *CALPAIN* & *CAPASTATIN* allele substitution	Improved meat tenderness	Casas et al. (2006) (not yet reduced to practice)
Insertion of lysostaphin/lysozyme transgene	Resistance to mastitis	Liu et al. (2013) Liu et al. (2014)
CD18 gene edit	Resistance to BRD (bovine respiratory disease)	Shanthalingam et al. (2016) (not yet reduced to practice)
Insertion of SP110, NRAMP1	Resistance to tuberculosis	Wu et al. (2015) Gao et al. (2017)
Intraspecies SRY translocation onto X chromosome	All male offspring	Owen (2019) (not yet published)
NANOS gene knockout	Infertility (for gonial cell transfer i.e. surrogate sires/dams)	Ideta et al. (2016)

Data coming out of some of the large-scale genomic and sequencing projects are revealing situations in which the sequence of one naturally occurring allele results in superior performance to that observed when animals inherit an alternative allele of that gene. It is envisioned that it might be possible to edit an animal's genome to the superior allele, and to do that at several genomic locations, or for several different genes. Genome editing could be used to introduce useful alleles (e.g., heat tolerance, disease resistance) at precise genomic locations, and other useful haplotypes into native locally adapted cattle breeds, thereby helping to improve their productivity while retaining their adaptive traits. Simultaneous targeting of different genes has allowed bi-allelic modification of up to three genes at the same time. The advantage of gene editing over conventional selection to move these naturally occurring alleles from one animal to another is that favorable alleles rarely all occur in one single individual. Editing offers the opportunity to increase the frequency of desirable alleles in an individual or a breed more rapidly than could be achieved through conventional breeding, and in the absence of undesirable linkage drag.

One could potentially envision editing several alleles for different traits – such as known fertility impairing haplotypes (VanRaden et al., 2011), polled, and to correct known Mendelian genetic defects that affect cattle (Casas and Kehrli, 2016) all while using conventional selection methods to keep making genetic progress towards a given selection objective. Although monogenic traits present good targets for genome editing and can have tangible animal health, environmental and economic outcomes, nearly all economically important livestock traits are complex polygenic traits (Georges et al., 2019). These traits include milk yield and composition, carcass yield, composition and quality, feed conversion, feed efficiency, growth rate, wool yield and quality, fertility, egg yield, and disease resistance.

Gene editing conceptually offers an approach to translate the thousands of SNP markers discovered through livestock sequencing projects, the information obtained from numerous genome wide association studies, and the discovery of causative SNPs (Quantitative Trait Nucleotides; **QTNs**) into useful genetic variation for use in animal breeding programs (Hickey, 2013). One modeling study reported that combining gene editing with traditional genomic selection could improve the response to selection four-fold after 20 generations (Jenko et al., 2015). It is worth noting, however, that this study modeled editing a quantitative trait that had 10,000 known QTN. In reality, animal breeders do not currently have a comprehensive understanding of which edits would be impactful on quantitative traits (i.e., those controlled by many genes).

It is neither likely that all the genes affecting such traits are known, nor is it typically evident which edits might be the most desirable for these genes (i.e., what is the sequence of the desirable allele). It is likely that, at least in the short term, editing will focus on large effect loci and known targets to correct genetic defects or decrease disease susceptibility, and conventional selection will continue to make progress in selecting for all of the many small effect loci that influence the complex traits that contribute to the breeding objective. In other words, editing will complement, not replace, conventional breeding programs

Polled (Hornlessness)

Dehorning is painful but protects animals and humans from injury. Much interest has been generated by use of editing to introduce the polled allele into dairy breeds. Breeding for polled (hornlessness) has typically not been adopted because of the low genetic merit and scarcity of polled dairy sires (Figure 2).

Alternatively, gene editing to produce high genetic merit polled sires has been proposed. Horns are inherited as an autosomal recessive trait. Two candidate POLLED mutations have been predicted in cattle. One is an 80,128 base pair (bp) duplication of Friesian origin ($\mathbf{P_F}$). The second is a simple allele of Celtic origin ($\mathbf{P_C}$) corresponding to a duplication of 212 bp in place of a 10 bp deletion. This dominant P_C Celtic POLLED allele was introduced into the genome of a crossbred dairy bull using genome editing and HDR of a donor template (Carlson et al., 2016).

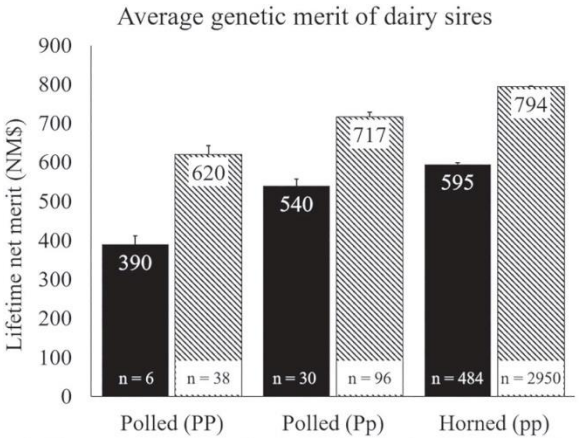

Figure 2. Average lifetime net merit (NM$) of the top 50% polled and horned Holstein (black and white hatched bars) and Jersey (solid black bars) sires and the total number of each genotype and breed registered with the National Association of Animal Breeders under active, foreign, genomic, or limited status in March 2018. Error bars represent SEM. (Mueller et al., 2019)

This genome-edited polled dairy bull (P_CP_C) was crossed with horned cows (pp), resulting in six heterozygous (P_Cp) calves. The calves had no horns and were otherwise healthy and phenotypically unremarkable. It was recently reported that whole genome sequencing of these animals revealed the bull was a compound heterozygote, carrying one naturally occurring P_C Celtic POLLED allele, and an allele containing an additional introgression of the HDR donor plasmid along with the P_C Celtic allele. These alleles segregated in the offspring of this bull with two obtaining only the naturally occurring P_C Celtic POLLED allele, although interestingly inheritance of either allele produced polled calves. No other unintended genomic alterations were observed (Young et al., 2019).

Segregating out exogenous sequence from the editing cassettes is a routine occurrence in plant breeding where conventional genome editing typically involves the delivery and integration into the host genome of rDNA cassettes encoding editing components. Final products are typically null-segregants containing the intended genomic alteration but none of the exogenous DNA. This finding reinforces the need to screen for plasmid sequence when genome editing involves a plasmid containing the HDR repair template, as has been done previously (Wei et al., 2018). Such an outcome would not be an issue when knocking out a gene using editing reagents but no donor template (i.e., NHEJ), or when using a single-stranded oligodeoxynucleotide or DNA donor template, rather than a donor plasmid.

Modeling the Introgression of Polled in U.S. Dairy Populations

Mueller et al. (2019) simulated the introgression of the POLLED allele into the U.S. dairy cattle population via conventional breeding or gene editing for 20 yr (Figure 3). Gene editing decreased the frequency of the HORNED allele to <0.1 after 20 yr, which was as fast, or faster, than conventional breeding for both breeds. In a mating scenario that required use of only the existing homozygous polled sires, inbreeding increased to 17% (Holstein) and 14% (Jersey), compared with less than 7% in the baseline scenarios. In contrast, gene editing in the same mating scheme resulted in significantly less inbreeding, 9% (Holstein) and 8% (Jersey). In addition, gene editing resulted in significantly greater NM$ after 20 yr compared with conventional breeding with polled sires for both breeds. Moreover, the gene editing scenarios of both breeds used a significantly greater number of unique sires compared with either the conventional breeding or baseline scenarios. Taken as a whole, these simulations showed that given the current genetic merit of horned and polled dairy sires, use of conventional breeding methods to decrease the frequency of the HORNED allele would increase inbreeding and slow genetic improvement. Gene editing only the top 1% of elite artificial insemination sires per year, resulted in a better outcome with the POLLED allele being rapidly disseminated while maintaining genetic diversity and genetic progress.

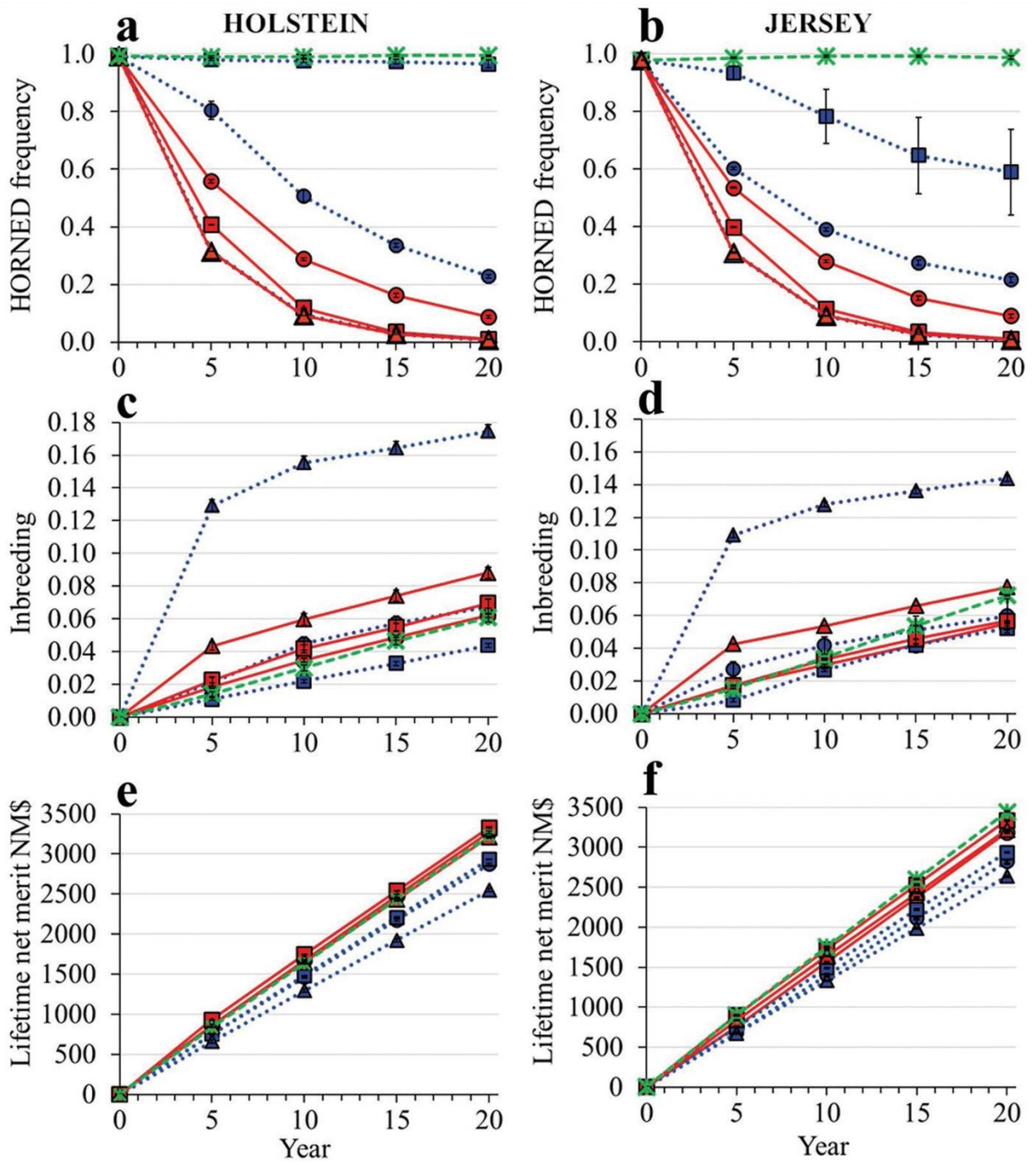

Figure 3. Effect of editing for polled (solid red lines), breeding for polled (dotted blue lines), or not considering polled (dashed green line) in selection scenarios on (a, b) *HORNED* allele frequency, (c, d) inbreeding, and (e, f) genetic merit for Holstein (a, c, e) and Jersey (b, d, f). Error bars represent SEM (Mueller et al., 2019).

Surrogate Gonads

Of particular interest to the livestock industry is the possibility of generating germ cells from high merit donor animals exogenously in the gonads of otherwise sterile host animals, thereby expanding the availability of gametes from genetically desirable dams and sires (Gottardo et al., 2019). This idea has been termed surrogate sires/dams (Figure 4). This could be achieved by using genome editing to knockout the germ cell lineage in surrogate sires/dams, and then filling that developmental niche with the germline of superior animals. There have been reports of spermatogenesis following the transplantation of exogenous germ cells into the testes of sterile mice and rats. It has been proposed that sterile testes from NANOS2-/- boars (Park et al., 2017) could harbor donor-derived spermatogenesis following transplantation of wild-type spermatogonial stem cells (Giassetti et al., 2019). Exogenous germ cells have also been generated in the sterile ovaries of NANOS3 knock-out cow (Ideta et al., 2016).

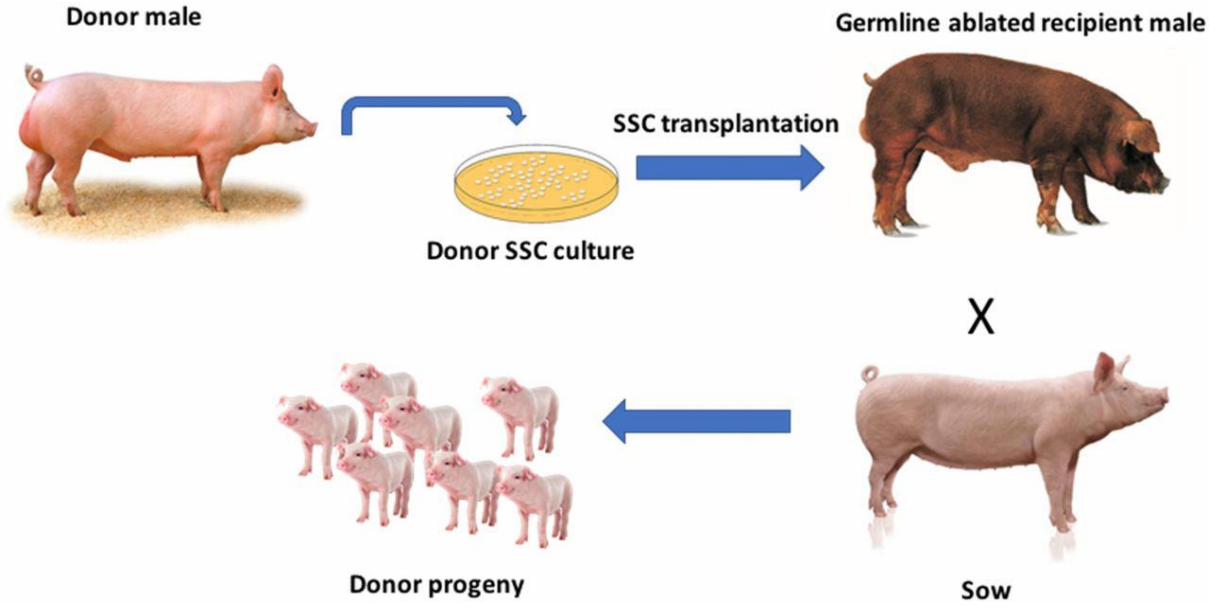

Figure 4. Schematic depicting the possible application of surrogate sire technology where a boar of one breed is producing sperm derived from a donor male of another breed (Gottardo et al., 2019)

Intersection with Conventional Breeding

To become an important driver of genetic change, genome-editing methods must seamlessly integrate with conventional animal breeding programs. That means that they must reliably function to germline-edit animals that are selected to be the next generation of parents. Edits can be introduced through gene editing of somatic cells followed by somatic cell nuclear transfer (**SCNT**) cloning, or injection of the gene editing reagents into the cytoplasm (**CPI**) of early-stage zygotes of the next generation of selection candidates.

To date, SCNT has been the primary method to deliver nuclease-mediated genetic changes into livestock (Tan et al., 2016). The advantage of SCNT is that the gene-edited cell line can be genotyped and/or screened before transfer into the enucleated oocyte to ensure that the desired edits, and no donor template integrations, have occurred. The disadvantage is that there are well-documented drawbacks and inefficiencies associated with cloning including early embryonic losses, and birth defects (Keefer, 2015).

Direct editing of zygotes is advantageous because it is modifying the next generation, but

the disadvantage is that not all embryos will have the desired edit, and often embryos are mosaic–meaning some cells are edited and some are not (Tan et al., 2013). On average, however, fewer embryos are required to gene edit a pig, for example, using this approach compared with SCNT because of the inefficiencies associated with cloning (Tan et al., 2016). Knockouts using NHEJ have been achieved through CPI of zygotes from several livestock species, and can be obtained with relatively high frequency, with some reports of 100% efficiency. Targeted gene knock-ins have proven more challenging. Entire interspecies allele substitutions have been successfully knocked-in using CPI of zygotes in pigs (Peng et al., 2015, Lillico et al., 2016). To date no group has reported bovine knock-in calves resulting from CPI of zygotes. Owen et al. (2019) reported some success at obtaining targeted gene knock-ins in developing bovine embryos. Using CPI of a modified donor vector, in addition to editing reagents into mature oocytes followed by fertilization resulted in a 34% knock-in efficiency rate. Moreover, approximately a quarter of those knock-in embryos were non-mosaic, meaning the edit took place before the first cell division.

Regulations

As with earlier genetic engineering approaches, whether breeders will be able to employ genome editing in cattle genetic improvement programs will very much depend upon global decisions around the regulatory framework and governance of genome editing for food animals. The United States Department of Agriculture (**USDA**) has announced that genome edited plants containing genomic alterations that could have been achieved using conventional breeding methods, are not going to be treated differently from a regulatory perspective to crop varieties developed using conventional breeding.

In contrast, the United States Food and Drug Administration (**FDA**) came out in 2017 with a draft guidance on the regulation of genome edited animals entitled, "Regulation of Intentionally Altered Genomic DNA in Animals" (Food and Drug Administration, 2017). This guidance states that "intentional genomic alterations" produced using modern molecular technologies including genome editing are going to be regulated as "new animal drugs". It proposes that the presence of any "intentionally altered genomic DNA" would trigger mandatory, premarket new animal drug evaluation, irrespective of product risk or novelty of the genomic alteration. The draft guidance suggests the need for genotypic and phenotypic durability studies over multiple generations, including, where feasible, data on inheritance from at least two generations, preferably more, and recommends that at least two of the sampling points be from non-contiguous generations (e.g., F1 and F3).

One procedural problem with the proposed guidance is differentiating between "intentional genomic alterations", off-target genome editing alterations, and *de novo* mutations (Van Eenennaam, 2018). In one analysis of whole-genome sequence data from 234 taurine cattle representing three breeds, more that 28 million variants were observed, comprising insertions, deletions, and single-nucleotide variants (Daetwyler et al., 2014). Another recent study found that on average every new animal will have approximately 65 de novo mutations, of which approximately five will be small insertion/deletions and the remaining 60 will be single-nucleotide substitutions (Harland et al., 2017). These naturally occurring variants are the basis for all selection programs, and evolution, and are not regulated anywhere in the world.

Furthermore, the draft guidance recommends that all investigational animals, including offspring of genome edited animals, and their biological products be disposed of by incineration, burial, or composting. Multigenerational studies with large food animals like cattle take years and are beyond the resources of most academic laboratories, especially if the investigational animals must be incinerated rather than sold for food purposes. While these requirements might make some sense in the context of animals expressing a pharmaceutical protein (i.e., an actual drug), they make little sense in the context of a DNA variant or a naturally occurring allele in food. How can the absence of a small piece of DNA, or a SNP, rationally be considered a drug? Several industry and research groups have argued that the FDA's proposed new animal drug regulatory approach

for genome editing in animals is not a suitable regulatory approach (Van Eenennaam et al., 2019).

In contrast, Argentina's regulatory approach is to treat plants and animals being genome edited for food purposes similarly. They ask two questions of the final product (i.e., food entering commerce): "Is there a new combination of genetic material in the final product?", and "Is the final product free of transgenes?" If the answer to both of these questions is no, then that product does not trigger the genetic engineering regulatory approval process. The "GMO" regulations pertain to plants and animals containing exogenous rDNA constructs containing new combinations of DNA that could potentially present a hazard in the form of a new food allergen or toxin (Whelan and Lema, 2015).

From a risk perspective, it does not make a lot of sense to have a different set of rules for a genome edited calf carrying a naturally-occurring allele, free of transgenes, differently from a calf that inherited that same naturally-occurring allele from its parent (Carroll et al., 2016). In several countries such a calf would trigger regulatory scrutiny as a "GMO", or as a new animal drug in the United States. Figure 5 reveals the disharmonious state of proposed regulations regarding genome editing in animals globally.

Country	Additional Regulations?	Basis of trigger/regulation?
Argentina	No	Novel (foreign) DNA sequence/transgene
Australia	Yes	Use of "long" template
Brazil	No	Novel (foreign) DNA sequence/transgene
Canada	No	Trait novelty (i.e. novel product risk)
European Union	Yes	Is a GMO if used a mutagenesis technique not in existence before 2001
Japan	No	Presence of foreign genes
New Zealand	Yes	Using of in vitro technique that modifies the genes/genetic material
United States	Yes	Intentional genomic alteration from useof modern molecular techniques

Figure 5. Current regulatory thinking as to whether genome edited livestock carrying a naturally occurring allele introduced using genome editing and an HDR donor template would be treated differently to conventional breeding and subjected to additional regulatory requirements.

CONCLUSIONS

Significant improvements in the efficiency of milk and beef production have historically been accomplished through conventional breeding of superior individuals with an eye towards specific breeding objectives. Genome editing is a tool that is well suited for modifying qualitative, single-gene traits at comparatively rapid rates in the absence of linkage drag and could be used in conjunction with conventional selection approaches to address issues such as disease resistance and improved welfare traits. Animal breeders need regulatory certainty regarding genome editing. If editing is used to introduce alterations that are no different from those that could have been obtained using conventional breeding, it should not trigger additional layers of regulatory scrutiny and expense. Regulations

should be proportionate to any novel risks inherent in the product, and not the process used to produce that product. At the current time, the arbitrary trigger for regulation of genome-edited livestock in the United States is the presence of "intentional genomic alterations", introduced using modern molecular techniques. This new animal drug regulatory paradigm will put the United States at a competitive disadvantage when it comes to incorporating genome editing into animals breeding program, relative to other countries (e.g., Argentina, Canada) where novel product risk-based regulatory approaches have been implemented.

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