Gene editing: Today and in the Future—Alison Van Eenennaam, PhD

Take Home Messages

- Gene editing refers to the use of site directed nucleases (e.g. Zinc finger nucleases, TALENS, CRISPR/Cas9) to introduce targeted alterations into genomic DNA sequence.
- It offers a way to correct genetic defects, inactivate or knock-out undesirable genes, and/or move beneficial alleles and haplotypes between breeds in the absence of linkage drag.
- Gene editing would synergistically complement, not replace, traditional breeding programs.
- It has been used to introduce useful genetic variants impacting disease resistance, product quality, adaptability, and welfare (e.g. polled) traits in research settings.
- It could also be used to alter the sex ratio of offspring, and enable novel breeding schemes to accelerate the rate of genetic gain or reduce genetic lag in beef cattle breeding programs.
- The regulatory oversight of gene editing in animals varies by country; in 2017 the US Food and Drug Administration (FDA) released a regulatory guidance stating that it plans to treat "all intentional alterations" introduced into the genome of animals as new animal drugs.
- The USDA challenged this in 2021 by releasing a notice of proposed rule making claiming regulatory authority of certain livestock species, including cattle, developed using modern biotechnology that are intended for agricultural purposes such as human food and fiber.
- The FDA is opposed to sharing regulatory oversight of genetically engineered and genome edited animals with USDA, and as of May, 2021 it is unclear how this regulatory turf battle will play out; the outcome will likely determine whether it will be feasible to incorporate gene editing into US livestock genetic improvement programs.

Introduction

Gene editing involves using a site-specific nuclease (e.g. Zinc finger nuclease, TALENS, CRISPR/Cas9) to cut DNA and introduce a double-stranded break (DSB) at a targeted, specific sequence in the genomic DNA double helix. It is effectively a sophisticated pair of molecular scissors. The DSBs are then repaired by machinery in the cells using one of two mechanisms. One method is non-homologous end joining (NHEJ) where the two broken ends are brought alongside each other and are glued together. This method is error-prone and often results in small insertions and deletions (indels) at the target cleavage site due to inevitable mistakes in the repair process. These errors 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 that match up either side of the DSB. This can be used to introduce a range of genome edits, from point mutations to whole-gene insertions.

Gene editing presents an approach to introduce targeted modifications into existing genes and regulatory elements within a breed or species, without necessarily introducing 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 (genes introduced along with the beneficial gene during backcrossing.)

Introduction of Editing Components Into the Genome

Gene editing reagents can be delivered into target cells via physical methods or through the employment of vectors (viral or non-viral). Gene edited mammalian livestock have predominantly been produced using physical methods which include electroporation of somatic cells (typically fetal fibroblasts) and microinjection, or more recently electroporation, of zygotes (one-cell embryo). Electroporation uses high-voltage pulses to induce transient pore formation in the cell membrane. These pores allow the flow of gene editing components from the suspension liquid into the cell cytoplasm (Lin and Van Eenennaam, 2021). Although electroporation has traditionally been used to edit cultured cell lines, it is also effective on zygotes (Chen et al., 2016).

For a long time, cytoplasmic microinjection (CPI) has been the go-to technique for delivering gene editing components directly into livestock zygotes. Electroporation has only recently begun to show its potential for this purpose with effective introduction of indel mutations into zygotes of cattle (Wei et al., 2018a, Miao et al., 2019, Namula et al., 2019,

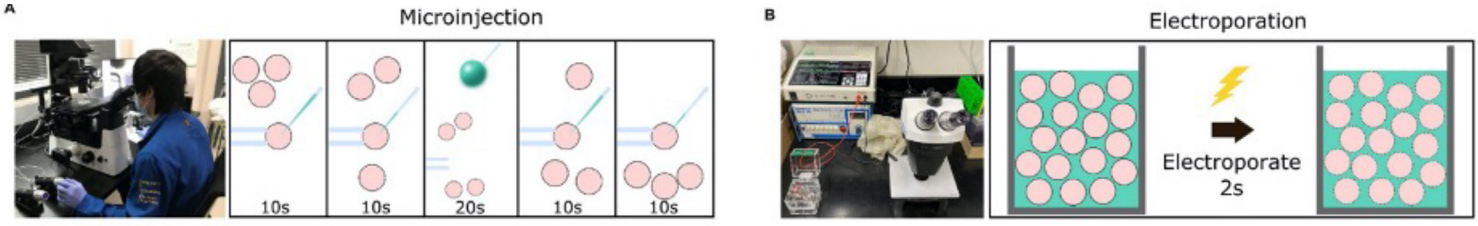

Figure 1. Graphical schematic of a comparison between setup and time necessary for the microinjection vs. electroporation of embryos. (A) The equipment necessary for the microinjection of embryos and the workflow involved to introduce editing reagents (green) into four presumptive zygotes (pink) using a holding needle (left) to stabilize the zygote before introducing the injection needle (right). (B) The equipment necessary for the electroporation of embryos and the workflow involved to introduce editing reagents into 30–100 presumptive zygotes via a cuvette. Image from Lin and Van Eenennaam (2021).

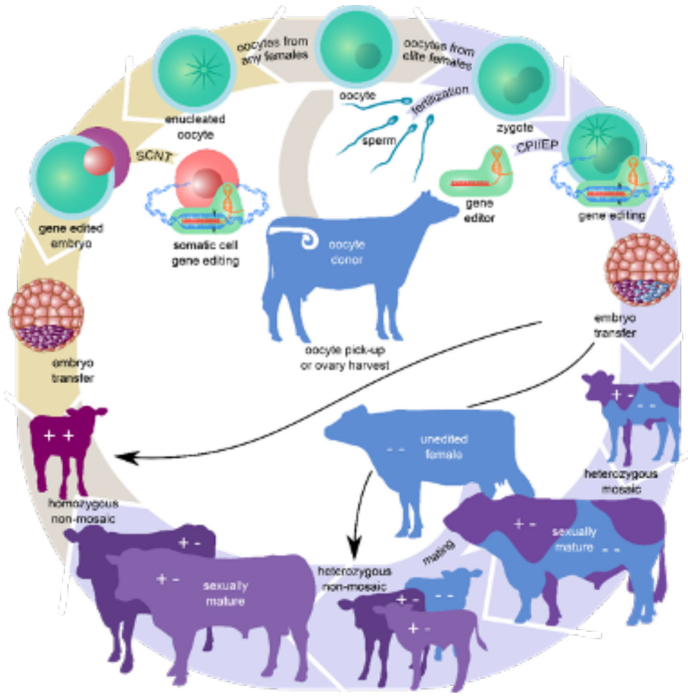

Figure 2. Steps for producing genome-edited livestock through somatic cell nuclear transfer (SCNT) or zygote editing. Schematic showing the steps involved to produce homozygous, non-mosaic livestock by either SCNT cloning of gene-edited and screened somatic cells (yellow arrows) or cytoplasmic injection (CPI)/electroporation (EP) of zygotes (purple arrows) with gene editing components. Image from Bishop and Van Eenennaam (2020).

Camargo et al., 2020). Unlike CPI, where a needle is used to deliver gene editing reagents into zygotes individually, electroporation allows the manipulation of zygotes en masse, reducing the time and expertise required (Figure 1).

Introducing gene editing reagents directly into zygotes using both methods has been a successful approach to achieve targeted knock-outs in embryos. However, issues still exist. Firstly mosaicism, meaning 2 or more genetically different sets of cells in an animal, is a common problem that can reduce the efficiency of producing a line of knock-out animals if the germ line (i.e. sperm and eggs) is derived from a subset of cells that were not gene edited. Second, inserting new genes is much more difficult than targeted knock-outs. Targeted whole-gene insertions relies on using the HDR pathway of repair which tends to only be active in dividing cells. As such it is difficult to achieve gene knock-ins in zygotes.

Gene Editing in Cattle Genetic Improvement

In animal breeding programs, germline transmission is the ultimate goal because edits must be passed on to the next generation to achieve genetic improvement. In mammalian livestock species, gene editing can be performed either in somatic cells and the edited cell line subsequently cloned by somatic cell nuclear transfer (SCNT), or in developing zygotes. Most targeted gene knock-outs in mammalian livestock, and a few targeted gene insertions, have been achieved by editing in cell culture, followed by SCNT (Tan et al., 2016). The use of SCNT to derive embryos from edited cells greatly reduces the efficiency of the method due to the low rate of birth of healthy cloned animals, particularly in cattle (Akagi et al., 2013, Keefer, 2015).

Delivery of gene editing components into the zygote avoids the shortcomings of SCNT, but has the drawback of significant rates of mosaicism when the editing event occurs at a multinuclear/multicellular stage, and unknown editing success prior to the birth of the calf, unless the embryo is biopsied prior to transfer. For mosaic animals, a breeding strategy must be employed to obtain homozygous, non-mosaic animals (Figure 2). Gene editing of zygotes also has the advantage of producing a diversity of foundation animals as each zygote will produce a genetically distinct animal, as opposed to animals derived from a clonal cell line.

Table 1. Publications using gene editing in cattle for agricultural applications. Modified from Mueller (2021).

Trait category	Goal	Genome target and function	Reference
Animal health/ welfare	Prevent horn growth	Horn/Poll	Tan et al. (2013); Carlson et al. (2016)
	Disease resistance: mastitis	CSN2 (Beta-casein): milk protein gene	Liu et al. (2013) Liu et al. (2014)
	Disease resistance: tuberculosis	Intergenic region between SFTPA1 and MAT1A	Wu et al. (2015)
	Intergenic region between FSCN1 and ACTB	Disease resistance: bovine respiratory disease (BRD) ITGB2 (integrin subunit beta 2): encodes the leukocyte signal peptide CD18	Shanthalingam et al. (2016)
	Disease resistance: bovine spongiform encephalopathy (BSE)	PRNP (prion protein): susceptibility to BSE	Bevacqua et al. (2016)
	Repair mutation: IARS syndrome	Isoleucyl-tRNA synthetase (IARS)	Ikeda et al. (2017); Ishino et al. (2018)
	Thermotolerance	PMEL (premelanosomal protein gene): coat color	Laible et al. (2020)
		PRLR (prolactin receptor): hair coat length	Rodriguez-Villamil et al. (2021)
Product yield or quality	Eliminate a milk allergen	PAEP (Beta lactoglobulin): whey protein gene	Yu et al. (2011) Wei et al. (2015) Wei et al. (2018b)
	Increase lean muscle yield	CSN2 (Beta-casein): milk protein gene	Su et al. (2018)
Reproduction and novel breeding schemes	Generate host for germ cell transfer	NANOS2 (Nanos C2HC-Type Zinc Finger 2): necessary for male germline development	Miao et al. (2019), Ciccarelli et al. (2020)
	All male offspring	Safe harbor loci, H11	Owen et al. (2021)

Gene editing research in cattle to date has focused primarily on monogenic (single gene) traits for animal health and welfare, or product yield and quality. There are also some applications that focus on reproduction and novel breeding schemes that may be of relevance to beef cattle breeding programs (Table 1).

It should be emphasized that many of the processes involved in gene editing livestock are time consuming, and at present inefficient. There are a large number of procedural steps and unpredictable biological variables including gamete collection and maturation, introduction of the editing reagents, cloning and transfer of embryos into synchronized surrogate dams, all of which have their own limitations and constraints. Microinjection of zygotes that result in mosaic offspring, and then subsequently breeding to produce heterozygous and homozygous edited offspring is both time consuming and expensive when performed in large food animals. Many gene editing applications require homozygous modifications to ensure inheritance of one copy in the F1 generation, or for alleles with a recessive mode of inheritance. The complexity and inefficiencies associated with many of these processes makes the gene editing of livestock far from routine at the current time (Figure 3).

It is perhaps not obvious to those outside of this field, but a source of bovine oocytes for in vitro maturation and fertilization has to be readily available to perform zygote editing, often obtained from ovaries collected at a local slaughter facility, unless specific female genetics is required, in which case ovum pick-up may be used. To produce viable mammalian offspring, it is also necessary to have a ready supply of synchronized recipient or surrogate cows. This is not an inexpensive undertaking in the case of large livestock species, and due to seasonal breeding and other climatic factors, it is almost impossible to conduct this work during certain times of the year.

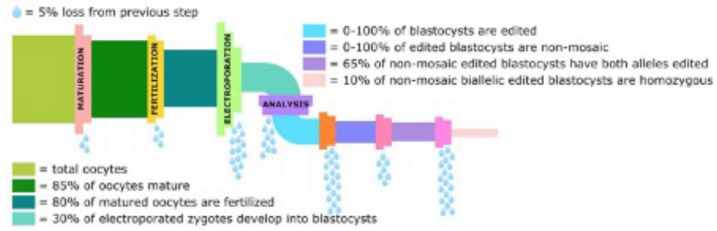

Figure 3. Graphical representation of the losses in the gene editing pipeline from collection of oocytes to the percentage of blastocysts that are non-mosaic homozygotes for the intended edit. Image from Lin and Van Eenennaam (2021).

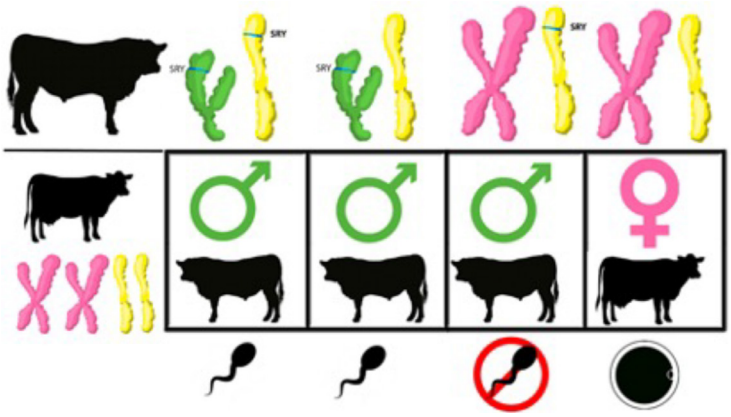

Figure 4. Cosmo will produce sperm carrying either an X (pink) or a Y (green) sex chromosome, and one copy of Chromosome 17. All Y-bearing sperm will produce a male calf, whereas only half of the X-bearing sperm will produce a female. The other half carrying the SRY gene on Chromosome 17 (yellow) are expected to produce a male-appearing XX individual. However, this animal would not be expected to be produce fertile sperm.

Future Applications of Gene Editing

Skewing of sex ratios

In mammals, sex determination is typically dependent on the inheritance of the sex chromosomes, X and Y. Individuals with two X chromosomes are genetically female and individuals with one X chromosome and one Y chromosome are genetically male. Dairy farmers often use "X-sorted" semen in artificial insemination as it contains only sperm carrying an X chromosome and will result in all female calves.

It is actually only a single gene on the Y chromosome that determines whether an embryo develops as a male or female. This gene is known as the sex-determining region of the Y chromosome or "SRY" for short. SRY expresses a protein in early embryogenesis that initiates male sexual differentiation by triggering a cascade of factors necessary for male gonadal development and shutting down formation of the female gonad.

In 2020 we generated a gene edited calf, Cosmo, who carries an extra copy of SRY on one of his non-sex chromosomes (Owen et al., 2021). Cosmo is expected to produce 75% male offspring: 50% of which will be XY males; 25% of which will be XX females; and 25% of which are expected to be XX individuals that appear male due to the inheritance of the chromosome 17 carrying the SRY gene. These XX males are not expected to produce viable sperm as that requires the expression products of additional genes located on the Y chromosome (Figure 4).

Cosmo turned one year of age in April 2021, and he will be bred to study if inheriting the SRY gene on Chromosome 17 is sufficient to trigger the male developmental pathway in XX embryos. Such bulls could produce a higher proportion of male market calves. However, at this time the project is still in the research stage and is highly regulated by the Food and Drug Administration, meaning Cosmo and his offspring are not allowed to be marketed, enter the food supply, or even be rendered.

Bulls and cows carrying gametes belonging to a different animal

There is a lag in the genetic improvement between the elite nucleus seedstock sector and commercial animals. One way to decrease this lag would be to make germline copies of elite animals. This aim could be achieved through the use of surrogate sires (Gottardo et al., 2019) which involves replacing the germline of inferior males (e.g. herd sires) with the germline of genetically elite males (e.g. AI sires) by introducing germ cells derived from the elite sires into the testes of the herd sires.

Recently, gene editing has been used to knock out genes necessary for an animal's own germ cell production (Ideta et al., 2016, Park et al., 2017, Taylor et al., 2017). These germline knock-out animals make ideal hosts for elite donor-derived germ cell production. In germline knock-out mice, pigs and goats, transplantation of donor spermatogonial stem cells (Ciccarelli et al., 2020), or embryonic stem cells (Miura et al., 2021) resulted in donor-derived sperm production in the otherwise sterile testes. Additionally, donor-derived oocytes have been generated in sterile ovaries of germline knock-out heifers (Ideta et al., 2016).

In vitro breeding: New advances in vitro with germ cell and gamete development from mouse ESCs have led to recent interest in the potential for in vitro breeding in livestock (Goszczynski et al., 2018). The advantage of this proposed method would be that it could effectively remove the wait required for animals to reach sexual maturity prior to meiosis and conception. This has the potential to dramatically decrease the generation interval component of the breeders' equation. If both in vitro gametogenesis and fertilization could be successfully accomplished in a petri dish, this offers the possibility of maintaining an entire breeding population of large animals in a laboratory (Figure 6). Gene editing could be included at the ESC stage to introduce useful genetic variation in the selected cell line.

Figure 6. In vitro breeding (IVB). Diagram of the strategy, estimated times, and possible alternatives for its implementation in animal production systems. NT: nuclear transfer. IVF: In vitro fertilization. ESCs: Embryonic Stem Cells. Image from Goszczynski et al. (2018).

Regulations

As with earlier genetic engineering approaches, whether breeders will be able to employ gene editing in cattle genetic improvement programs will very much depend upon global decisions around regulatory frameworks and governance of gene editing for food animals. Argentina was the first country to publish its proposed regulatory approach for gene editing and other new breeding techniques (Whelan and Lema, 2015). The Argentine approach is that if there is no "new combination of genetic material", and if the final product is free of "transgenes", then that product will not be subject to regulation as a genetically modified organism (GMO). In this system, no distinction is drawn between gene edited plants and animals. In 2018, a gene edited line of tilapia, which did not contain any foreign DNA or a new combination of genetic material, received regulatory exemption by Argentina's National Advisory Commission on Agricultural Biotechnology. Similarly, Brazil ruled that the intraspecies polled allele substitution that results in hornless cattle would not be regulated as a GMO.

In 2020, the United States Department of Agriculture (USDA) published its SECURE (Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient) rule which confirmed that the "USDA does not regulate or have any plans to regulate plants that could otherwise have been produced through traditional breeding techniques."

However, the United States Food and Drug Administration (FDA) has taken a very different approach for edited animals, and in a 2017 draft guidance announced that "all intentional alterations" in the genome of animals would be regulated as new animal drugs (FDA, 2017; Maxmen, 2017). The guidance elaborates that each alteration would need to go through a mandatory premarket multigenerational safety and efficacy review, irrespective of whether that alteration already exists in the target species or could have been achieved using conventional breeding. It should be noted that only two genetically engineered animals for agricultural purposes (fast-growing AquAdvantage salmon, and the GalSafe pig) have ever been approved using this regulatory approach, whereas numerous genetically engineered crops, and even a couple of gene edited crop varieties are commercially available.

Unapproved animal drugs are not allowed to enter the food or rendering chain, requiring incineration or burial following euthanasia of experimental gene-edited food animals. This added expense is inhibitory for gene editing research into food animal species. Typically, the income derived from marketing surplus animals, and the milk, meat and eggs produced by both university and USDA (e.g. MARC) herds and flocks, used in both research and teaching, is an integral offset to the sizable costs associated with large animal research. Categorizing all gene edits as drugs, irrespective of novelty, eliminates saleable products from edited livestock, and increases the costs associated with this research considerably. It also dramatically increases the developmental costs associated with commercializing gene edited livestock. A US 2019 petition calling for regulations that are proportionate to unique product risks, and the harmonization of regulations for gene edited plants and animals was supported by hundreds of scientists (Van Eenennaam et al., 2019)

On January 19, 2021, the USDA announced the finalization of a Memorandum of Understanding (MOU) with the US Department of Health and Human Services outlining regulatory responsibilities over certain animals developed using genetic engineering that are intended for agricultural purposes (such as human food, fiber, and labor). However, the FDA is opposed to losing their regulatory oversight of genetically engineered and gene edited livestock for food

purposes. A public comment period on the USDA proposal closed in May 2021, and as of writing this paper it is unclear how this regulatory turf battle will play out.

Meanwhile in Europe, the Court of Justice of the European Union (ECJ) ruled in 2018 that gene-edited crops should be subject to the same stringent regulations as conventional GMOs (Callaway, 2018). This will likely hinder both the use of gene editing by both plant and animal researchers in the EU, and the adoption of this technology in European agriculture.

Conclusions

Gene editing is a tool that is well-suited for modifying qualitative, single-gene traits at comparatively rapid rates and which could be used in conjunction with conventional selection approaches to address issues such as disease resistance, improved product yield or quality, and animal welfare traits. It could also be used to introduce traits that skew the sex ratio of offspring, and enable novel breeding schemes to accelerate the rate of genetic gain. The availability of this technology for use by industry likely hinges on the regulatory framework imposed, which varies dramatically by country. From a risk-based perspective, it makes little sense to regulate gene edited animals carrying the same allelic DNA at the targeted locus as conventionally bred animals differently, solely because the former was produced using gene editing. Regulations should be fit-for-purpose, proportionate to novel product risks, if any, and agnostic to method, rather than being triggered and predicated on the use of an arbitrarily defined subset of breeding methods.

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