Gene Editing as a Tool for genetic Improvement of Beef Cattle—Maci Mueller, University of California-Davis

INTRODUCTION

Gene editing is a suite of molecular tools that allow livestock breeders to precisely add, delete, or replace letters in the genetic code in order to influence a specific trait of interest (e.g., disease resistance), in as little as one generation. Several studies have produced gene edited livestock embryos and live animals, including cattle, for a multitude of traits. However, gene editing has not yet been applied on a commercial scale in livestock, so studies related to incorporating gene editing into livestock breeding programs have been limited. Moreover, the beef industry, which primarily raises animals in extensive grazing systems has several unique considerations compared to other more intensively managed industries (e.g., dairy). Therefore, this review aims to summarize gene editing research related to beef cattle improvement and to discuss strategies for disseminating traits improved via gene editing in extensive beef cattle grazing systems.

REVIEW OF LITERATURE

What is gene editing?

Genome or gene editing refers to the use of site-directed nucleases (i.e., nucleic acid cleaving enzymes) to precisely introduce double-stranded breaks (DSB) at predetermined locations in the genome [1]. Cells have evolved two primary pathways to repair DSBs: non-homologous end joining (NHEJ) and homology-directed repair (HDR). The underlying principle of both pathways is that the cell's endogenous repair factors will identify and congregate at the site of the DSB to repair the DNA in an efficient manner.

When using the NHEJ pathway, the cell attempts to fuse the broken DNA ends back together through blunt-end ligation. NHEJ is referred to as "non-homologous" because the ligation occurs without the use of a homologous template (e.g., sister chromatid) [2]. Consequently, this pathway is error-prone and often introduces variable-length insertion and deletion mutations (indels) at the DSB site [3]. In other words, the NHEJ pathway allows for the efficient disruption or knockout of a gene by targeting breaks to the coding region of the gene, where indels can result in frameshift or nonsense mutations.

On the other hand, the cell can use the HDR pathway if a nucleic acid template is provided. HDR templates can be designed to include desired modifications between regions of homology to either side of the DSB and templates are generally provided to the cell in the form of single-stranded or double-stranded DNA. The cell's repair enzymes can use the template as a model for precise repair by homologous recombination. The HDR pathway can be used to introduce, or knock-in, a range of gene edits, from point mutations to allelic substitutions, to entire transgenes [3].

There are currently three primary site-directed nucleases used for gene editing in livestock: 1) zinc finger nucleases (ZFN); 2) transcription activator-like effector nucleases (TALENs); and 3) clustered regularly interspersed short palindromic repeats (CRISPR)-associated protein 9 (Cas9) (Table 1). Since 2012, all three editing systems have been used to perform both gene knockouts and knock-ins in livestock cells and zygotes [4-6].

How can gene editing be applied for genetic improvement of beef cattle?

Regardless of the gene editing system used, the experiments in cattle have primarily focused on three main areas of improvement 1) animal health and welfare, 2) product yield or quality, and 3) reproduction or novel breeding schemes (Table 1). All three of these areas are highly aligned with the goals of conventional breeding programs [4, 7, 8].

In particular, a highly anticipated application of gene editing in livestock is to enable breeders to tackle specific animal health and welfare issues at a genetic level that through conventional breeding alone would either not be possible or likely result in decreased production efficiency. For example, gene editing enabled Wu et al. [9] and Gao et al. [10] to precisely insert genes from other species (mouse SP110 and human NRAMP1, respectively) into an intergenic region of the bovine genome to decrease susceptibility to tuberculosis. This scientific feat would not have been possible through conventional breeding methods alone. Gene editing has also enabled researchers to replicate a beneficial mutation in the prolactin receptor (PRLR) gene, first found in Senepol cattle and hypothesized to result in a SLICK phenotype (i.e., short, sleek hair coat), in Angus cattle to increase thermotolerance [11]. Although the Senepol PRLR mutation could be introgressed into another breed, such as Angus, through conventional breeding methods alone, the process would require multiple generations of backcrossing to restore genetic merit to pre-introgression levels, due to linkage drag [12]. In a species like cattle, with a long generation interval, backcrossing is a time-consuming and expensive process [13, 14]. Additionally, it is important to note that genetic solutions for animal health and welfare issues are often more sustainable and less work for livestock producers than chemical or mechanical methods [15, 16].

Overall, the potential for gene editing to improve livestock sustainability is evident. For instance, the 2018 National Academies of Sciences, Engineering, and Medicine (NASEM) study, Science Breakthroughs 2030: A Strategy for Food and Agricultural Research, identified "the ability to carry out routine gene editing of agriculturally important organisms," as one of the five most promising scientific breakthroughs that are possible to achieve in the next decade to increase the U.S. food and agriculture system's sustainability, competitiveness, and resilience

[17]. However, strategies for effectively incorporating gene editing into existing animal breeding programs, especially for species with long-generation intervals, such as cattle, are less obvious.

How can gene editing be integrated into beef cattle breeding programs?

In order for gene editing to be an important factor for genetic change, it must integrate smoothly into conventional cattle breeding programs and reliably edit the germline of breeding stock [6]. Therefore, the potential of gene editing cannot fully be realized without being used in conjunction with genomic selection (GS) and assisted reproductive technologies (ART) to accelerate genetic gain by simultaneously altering components of the breeder's equation [7, 18, 19].

GS, which has been advanced by the development of high-throughput genotyping of single nucleotide polymorphisms (SNPs), is used to predict the genetic merit of an animal based on its DNA data [20]. In livestock, GS has been used to improve the accuracy of selection and to provide useful information on traits that would otherwise be difficult to measure [21-23]. Concurrently, ART, such as artificial insemination (AI), multiple ovulation embryo transfer (MOET), and more recently ovum-pick up with in vitro embryo production (OPU-IVP), have been incorporated into cattle breeding schemes to increase selection intensity. Moreover, the benefits of each of these tools (i.e., GS and ART) can be maximized when used synergistically to accurately select young animals, which can drastically reduce the generation interval and ultimately accelerate genetic gain [24].

For example, GS can be used to accurately select high-genetic-merit young donor females for MOET or OPU and bulls for semen collection. Therefore, embryos produced from these matings will also have high genetic merit [24]. However, due to Mendelian sampling variance, not all full-sibling embryos will have the same genetic merit and there is a large cost and natural resource drain in gestating embryo transfer (ET) calves of unknown genetic merit to later cull [25].

An additional strategy is genomic screening of embryos (GSE), sometimes referred to as embryo genotyping, which is the genotyping of cells biopsied from preimplantation embryos (i.e., before ET into a recipient female). GSE can be used to predict an embryo's genetic merit so that only the embryos with the highest genetic merit are used for ET. Moreover, since a larger number of embryos can be in vitro produced (IVP) compared to live-born animals, GSE can be used to select a small number of animals from a large pool of candidates (in their embryo stage), which will further increase the selection intensity [24, 26, 27]. Although GSE holds great potential, there are currently several technical limitations to overcome.

There is an inverse relationship between the viability of a biopsied embryo and the ability to obtain enough DNA sufficient for genotyping [28]. DNA extracted from embryo biopsies can be used for genetic diagnosis (i.e., genotyping of a few specific loci via polymerase chain reaction (PCR)), for GS, or a combination of both). DNA from one to several biopsied cells has been used successfully for genetic diagnosis (primarily, sex identification) of preimplantation bovine embryos [28-31]. Moreover, de Sousa et al. [29] took biopsies of a limited number of cells (10-20 blastomeres) from the trophectoderm of both in vivo derived and IVP bovine embryos on day 7 of development and demonstrated that the biopsies were sufficient for embryo sexing via PCR and that there was no significant ($P > 0.05$) difference on day 60 pregnancy rates of fresh transfer, biopsied embryos compared to control, non-biopsied embryos. It is important to note that this study did not investigate pregnancy rates of biopsied and cryopreserved embryos. Due to the limited amount of time between being able to biopsy an embryo and needing to transfer the fresh embryo (i.e., both on day 7 of in vitro culture), the ability to cryopreserve biopsied embryos will likely be a critical process for applying GSE on a commercial scale.

While embryo biopsies for sex determination have been routinely used in ET programs [28, 32, 33], GS of embryos has been limited since a much larger number of cells (minimum of 30-40 cells) must be biopsied and genotyped to make accurate selection decisions [27, 28]. Although taking a biopsy of more than ~20 cells will drastically decrease embryo viability, alternatives to generate a sufficient amount of DNA for GS from only a small number of biopsied cells have been investigated, such as growing biopsied cells in culture [34, 35], and using whole genome amplification of biopsied cells in combination with imputation from known parental and population genotypes [35-37].

An adaption to traditional GSE was developed by Kasinathan et al. (2015) to genomically screen unborn bovine fetuses rather than embryos. Their strategy utilized multiple ET's and subsequent embryo flushing (21-26 day fetuses) to generate fetal fibroblast lines. DNA was extracted from the fibroblast lines for GS and the resulting genomic breeding values (U.S. dairy, Lifetime Net Merit index (NM$)) were used to select the line with the highest genetic merit. Cells from the selected elite fibroblast line were used as donor cells for somatic cell nuclear transfer (SCNT) cloning. Following ET of the cloned embryos, five healthy calves with elite dairy genetics were born [38]. This scheme does overcome the challenges of taking embryo biopsies for GS but still relies on the inefficient process of SCNT cloning to produce live offspring.

Similar challenges also exist for producing live, homozygous gene edited offspring. Currently, there are two primary methods to generate gene edited bovine embryos

and each has associated tradeoffs (Figure 1). One option is to introduce the gene editing reagents (e.g., CRISPR/Cas9) into a somatic cell line and subsequently clone the cell line by SCNT to produce embryos. To date, this has been the primary method for gene edited livestock production because the clonal colony growth of cell lines provides large amounts of DNA that can be genomically sequenced to confirm and isolate cells with the desired edit in order to only produce animals with intended edits. However, due to faulty or incomplete epigenetic reprogramming of the donor cell genome, SCNT cloning often results in high rates of pregnancy loss and can also negatively affect the viability of live-born calves [39, 40]. Additionally, unless a scheme similar to Kasinathan et al. [38] is used, adult somatic cloning increases the generation interval by one generation (equivalent to two years in cattle), compared to ET of in vivo derived or IVP embryos.

Alternatively, gene editing reagents can be introduced directly into the cytoplasm of an IVP zygote (i.e., single-cell embryo), typically via microinjection (Figure 2) or more recently, via electroporation. Gene editing of zygotes is an attractive option because it avoids the inefficiencies associated with SCNT cloning, allows for the production of a diversity of foundation animals as each zygote will produce a genetically distinct animal, as opposed to animals derived from a clonal cell line, and does not increase the generation interval because the editing process is occurring in the next generation of animals. However, characterizing gene edited zygotes is difficult due to the challenges of GSE discussed above. Specifically, a major challenge associated with gene editing of zygotes is the production of mosaic animals [6, 19, 41]. Mosaicism arises from mutations that occur after DNA replication [42], resulting in one individual having two or more different genotypes. It is important to keep in mind that many livestock gene editing applications require homozygous modifications (i.e., two copies) to ensure inheritance of one copy in the F1 generation [6]. Therefore, mosaic gene edited animals will require subsequent breeding to produce homozygous edited offspring (Figure 1). Regardless of the method used to generate gene edited bovine embryos, ET into synchronized recipient females is a crucial step in producing live gene edited offspring (Figure 1).

Due to the fact that gene editing has not yet been applied on a commercial scale in livestock, strategies for incorporating gene editing into livestock breeding programs have primarily been modeled via computer simulation. One of the first simulation studies to explore the potential of combining gene editing with GS in a livestock breeding program was by Jenko et al. [18]. They modeled a breeding scheme called promotion of alleles by genome editing (PAGE) to improve quantitative traits, by selecting and gene editing the best animals based on their breeding values and then compared this scheme to GS alone. Jenko et al. [18] found that when gene editing was combined with GS the rate of genetic gain could be doubled as compared with GS alone. It is important to note that this simulation assumed a quantitative trait that had 10,000 known quantitative trait nucleotides (QTN), but identifying such QTN is not a trivial exercise and to date relatively few QTN with large effects on quantitative traits have been identified [43].

Bastiaansen et al. [44] modeled gene editing of a monogenic trait at the zygote stage in a generic livestock population combined with GS for a polygenic trait (i.e., index-based selection). In this simulation, zygotes from either 0, 10, or 100% of matings from genomically-selected elite parents were gene edited for the desired monogenic trait. Additionally, due to the low efficiencies of gene editing reported in the literature (Tan et al., 2016), they modeled various gene editing success and embryo survival rates. When they modeled 100% gene editing efficiency and embryo survival, they observed a strong favorable impact of gene editing on decreasing the time to fixation for the desired allele (four-fold faster), compared to GS alone. However, when they modeled a 4% gene editing efficiency, this had a major impact on the number of editing procedures needed (increased by 72%) and increased by eight-fold the loss in selection response for the polygenic trait, compared to the 100% efficiency model [44]. As discussed above, gene editing of zygotes is typically not 100% and mosaic animals are common [19, 41]. Therefore, in a commercial setting gene edited embryos will likely need to be biopsied to confirm the desired change before ET and avoid transferring non-edited embryos. Moreover, the current technical limitations of taking embryo biopsies for GS will need to be overcome to not only identify embryos with the intended edit(s) but also to select embryos with superior genetic merit in order to improve selection intensity.

Van Eenennaam [7] proposed a scheme where gene editing could be incorporated as an added step to the Kasinathan et al. [38] elite cattle production system (Figure 3). This approach was modeled to introduce a beneficial, monogenic, dominant allele (i.e., POLLED) into the U.S. dairy cattle [45] and northern Australian beef cattle populations [46]. In these simulations, fetal tissue from the next generation of yet-to-be-born bulls was genomically screened and selected, gene edited, and then successfully cloned such that this production system added 3–5 months to produce a homozygous gene edited, bull (Figure 3).

In the U.S. dairy population, Mueller et al. [45] found that the use of gene editing was the most effective way to increase the frequency of the desired allele while minimizing detrimental effects on inbreeding and genetic merit based on an economic selection index (i.e., NM$). The addition of gene editing only the top 1% of genetic merit bull calves per year to mating schemes that placed moderate selection pressure on polled was sufficient to maintain the same or better rate of genetic gain compared

to conventional selection on genetic merit alone, while significantly increasing POLLED allele frequency to greater than 90% [45]. Additionally, both Bastiaansen et al. [44] and Mueller et al. [45] found that gene editing reduced long-term inbreeding levels in scenarios that placed moderate to strong selection emphasis on the monogenic trait of interest (e.g., polled) compared to conventional breeding alone. Importantly, Mueller et al. [45] modeled breeding to represent the widespread use of AI in the U.S. dairy population (i.e., maximum of 5,000 (5%) matings/bull/year) [23, 47-49], so a single dairy sire was able to have an immense impact on the whole population. Therefore, only a small number of elite dairy sires needed to be gene edited to see population-level results [45].

In contrast, AI is not widely used in northern Australian breeding herds [50], thus Mueller et al. [46] modeled all matings via natural service (i.e., maximum of 35 matings/bull/year). The natural mating limits prevented individual gene edited beef bulls from having an extensive impact on the whole population. Consequently, gene editing only the top 1% of seedstock beef bull calves per year in mating schemes that placed moderate to strong selection on polled resulted in significantly slower rates of genetic gain compared to conventional selection on genetic merit alone. However, they did find that if the proportion of gene edited animals was increased to the top 10% of seedstock beef bull calves per year in similar polled mating schemes then similar rates of genetic gain could be achieved compared to conventional selection on genetic merit alone. In all scenarios, regardless of if gene editing was applied, the population inbreeding level never exceeded 1%, which is well below the acceptable level [51]. This simulation study modeled solely natural mating because currently reproductive tools are scarcely used in this population [50]. However, the authors explain that, "this is unlikely to be the situation with valuable gene edited bulls. It is more probable that a high-genetic-merit homozygous polled sire would be used for AI or in vitro embryo production followed by ET, in the seedstock sector. This system would amplify the reach of each gene edited bull using well-proven advanced reproductive technologies and enable these bulls to produce hundreds or even thousands of progeny, and thus have a greater impact on the whole population."

Although Mueller et al. [46] modeled a northern Australian beef cattle population, many findings are also applicable to the U.S. beef industry [52]. Presently, only 12% of U.S. beef producers use AI, and even fewer (7%) use estrus synchronization. In 2017, this resulted in less than 10% of all females being bred via AI. A larger portion of heifers (19%) were bred via AI compared to only 7% of cows. Additionally, the majority of females bred via AI were also exposed to a clean-up bull (>80%). Interestingly, more operations in the U.S. Central region (22%) reported using AI compared to either the East or West regions (~8% each). Overall, AI is not currently widely practiced on U.S. beef operations largely due to the logistical challenges and additional labor required to identify females in estrus and constrain them to perform AI [52]. Therefore, a large number of gene edited natural service bulls will be needed to broadly disseminate gene edited traits in the U.S. beef industry.

A potential alternative to AI is the use of surrogate sires. Surrogate sires are host bulls that carry germ cells from more genetically elite donor sires, and they will be able to pass on these desirable donor genetics through natural mating to improve beef production efficiency [53]. Additionally, surrogate sire technology could potentially provide an efficient means for the distribution of traits that have been improved through gene editing [54].

It is anticipated that surrogate sire technology can be realized through germline complementation, which consists of using donor cells from one genetic background to complement or replace the germline of an otherwise sterile host of a different genetic background [55, 56]. Germline complementation requires two components: 1) hosts that lack their own germlines, but otherwise have normal gonadal development (e.g., intact seminiferous tubules and somatic support cell populations), and 2) donor cells that are capable of becoming gametes (Figure 4).

One method to generate germline-deficient hosts is via treatment with chemotoxic drugs (e.g., busulfan) or local irradiation, but these methods are not efficient in livestock because they either fail to completely eliminate the endogenous germline, or the treatment has undesirable side effects on animal health [55]. A promising alternative is to use gene editing to knockout a gene (e.g., NANOS2 or DAZL) in a zygote that is necessary for an animal's own germ cell production [57-61].

Donor cells can be blastomeres (i.e., embryo cells) or stem cells, as reviewed by Bishop and Van Eenennaam [6] and McLean et al. [19]. Stem cells provide several advantages over blastomeres. An embryo has a limited number of blastomeres and therefore a limited amount of genomic screening and multiplication potential [19]. In contrast, stem cells are self-replicating so they can provide a potentially unlimited supply of donor cells. Additionally, stem cells could be gene edited in culture, possibly multiple times sequentially, and then DNA could be extracted without harming the viability of the remaining stem cells to both confirm the intended gene edit was made and use GS to determine the genetic merit of each line. This scheme would be especially useful when applied to embryonic stem cells (ESCs) to overcome the current challenges associated with GSE and to avoid the mosaicism issues currently associated with zygote gene editing.

One source of germline competent stem cells is spermatogonial stem cells (SSCs), which can be isolated from mature or juvenile testes [55, 59]. Another potential source of donor cells is ESCs, which are derived from the inner cell mass (i.e., the tight cluster of cells inside a 7-day

old embryo that will eventually give rise to the definitive structures of the fetus) of a preimplantation embryo [62]. Alternatively, induced pluripotent stem cells (iPSC) can be derived from somatic cells. Additionally, ESCs or iPSCs can be induced in culture to become primordial germ cell-like cells (PGCLCs) and subsequently induced to form sperm [63].

The process of germline complementation (i.e., combing donor cells with a host) can occur at different stages of a host animal's development, depending on the donor cell source (Figure 4). If the donor cells are SSCs or PGCLCs then they can be injected into a juvenile or adult host's germline-deficient gonad (Figure 4A). SSCs transfer has been demonstrated in pigs and goats and represents germline cloning of the current generation of sires [57, 59]. On the other hand, PGCLCs derived from ESCs would represent germline cloning of the next generation since the donor cells originated from an unborn 7-day old embryo. Alternatively, donor blastomeres or ESCs, which both represent the next generation could be combined with the host at the developing embryo stage (Figure 4B) [19, 64].

Irrespective of the production method, surrogate sires could unlock an opportunity to both accelerate genetic improvement of beef cattle and widely distribute traits improved via gene editing. The selection of only elite males for donor cells would increase selection intensity. Additionally, since the use of surrogate sires will not require any additional labor for commercial producers, there could be widespread adoption of this technology, which would dramatically reduce the lag in genetic merit that typically exists between the seedstock sector and the commercial sector. Gottardo et al. [53] performed simulations to develop and test a strategy for exploiting surrogate sire technology in pig breeding programs. Their model projected that using surrogate sire technology would significantly increase the genetic merit of commercial sires, by as much as 6.5 to 9.2 years' worth of genetic gain as compared to a conventional breeding program. An important question that should be addressed in future research is how to best accommodate both surrogate sires and their progeny and gene edited animals and their products into genetic evaluations.

Conclusions and Implications to Genetic Improvement of Beef Cattle

The ability of gene editing to inactivate targeted gene function (i.e., knockout genes), knock-in genes from other species, and/or achieve intraspecies allele introgression in the absence of undesired linkage drag, offers promising opportunities to introduce useful genetic variation into livestock breeding programs. Specifically, gene editing is well-suited for modifying qualitative, single-gene traits, at a much more rapid pace than conventional selection alone. Moreover, if gene editing is synergistically combined with GS and ART, genetic gain can be accelerated by simultaneously altering multiple components of the breeder's equation. It also offers the opportunity to improve currently elusive traits, such as disease resistance and improved animal welfare. Although the potential for gene editing to improve livestock sustainability is evident, strategies for effectively incorporating gene editing into existing animal breeding programs are less apparent. Several gene editing schemes have been modeled for livestock populations, and the most efficient schemes have relied heavily on widespread adoption of ART, especially commercial sector use of AI. Considering the currently limited adoption of AI in the U.S. commercial beef industry, novel breeding schemes, such as gene editing applied to surrogate sire production (i.e., host bulls that carry germ cells from more genetically elite donor sires), will be required to widely disseminate desired traits improved via gene editing. Furthermore, this system could have the added benefit of reducing the genetic lag that typically exists between the seedstock sector and the commercial sector in beef cattle breeding programs.