DNA-based Biotechnologies

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Animal breeders have made incredible genetic progress by selecting animals with desirable traits as parents of the next generation. Remarkably, this selective breeding, or artificial selection, was historically accomplished based solely on outward appearances (phenotypes) and then later with genetic prediction estimates, without understanding which genes influence particular characteristics. Advances in the field of genetics enabled breeders to make more rapid progress toward their explicit breeding objectives, with modern approaches combining genomics and statistics to rank individuals based on their genetic merit (Georges et al., 2019).

During the past century, several new technologies have been incorporated into programs aimed at accelerating the rate of the genetic improvement of livestock by providing tools for breeders to maximize the genetic contributions of highly productive animals. These include artificial insemination (AI), the use of hormones to control the female reproductive cycle to allow for synchronization and superovulation, and embryo transfer. Prior to their eventual widespread adoption, some of these new technologies (e.g. AI) were initially controversial and their introduction met with some resistance. In the past decade, applied DNA-based technologies have become available as a tool that livestock producers can use to aid in making their selection decisions.

What Is Biotechnology?

Biotechnology is defined as technology based on biology. From this definition, it is obvious that animal breeders have been using biotechnology for many years. For example, traditional selection techniques involve using observations on the physical attributes and biological characteristics of animals to select the parents of the next generation. One only needs to look at the amazing variety of dog breeds to realize the influence that breeders can have on the appearance and characteristics of animals from a single species. Genetic improvement through selection has been an important contributor to the dramatic advances in agricultural productivity that have been achieved in the past century.

Genetic improvement is an important component of sustainability. U.S. farmers and ranchers produced 12.725 million metric tons of beef in 2019 with approximately 95 million head of cattle (Figure 1), approximately 40 million fewer cattle than would have been required to produce that same amount of beef using 1975 genetics and technologies. Looked at another way, in 2018 the U.S. produced 18% of the world's beef with only 6% of the global cattle population.

In the past two decades, applied DNA-based technologies have become available as a tool that livestock producers can use to aid in making their selection decisions. The intent of this chapter is to provide the necessary background to create an understanding of DNA-based technologies and to discuss some of the recent developments and future applications in cattle production systems.

What Is DNA?

Living organisms are made up of cells, and located inside each cell is deoxyribonucleic acid, or **DNA** for short. DNA is made up of pairs of four nucleotides abbreviated as "A," "C," "G," and "T" (Figure 2). The entire genetic makeup, or **genome**, of an organism is stored in one or more chromosomes located inside each cell. DNA has two important functions; first, it transmits genetic information between generations during reproduction, and second, it continually

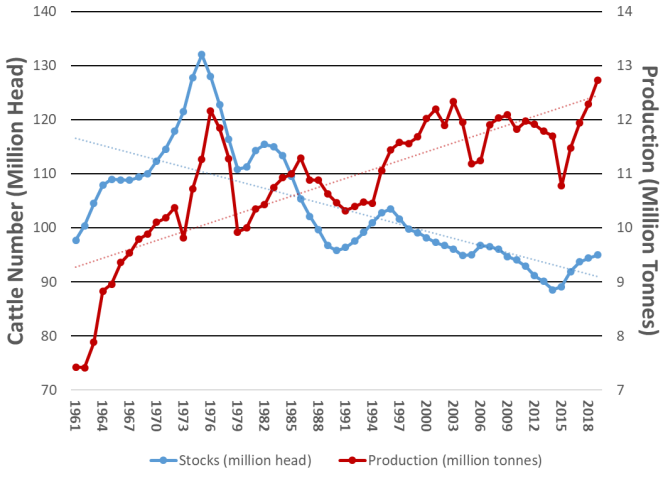

Figure 1. U.S. cattle inventory 1961-2019 (blue line; million head, left axis) and beef production (red line; million tonne, right axis). Data from USDA FAS Beef and Veal production statistics. Data derived from USDA FAS https://apps.fas.usda.gov/psdonline/app/index.html#/app/downloads.

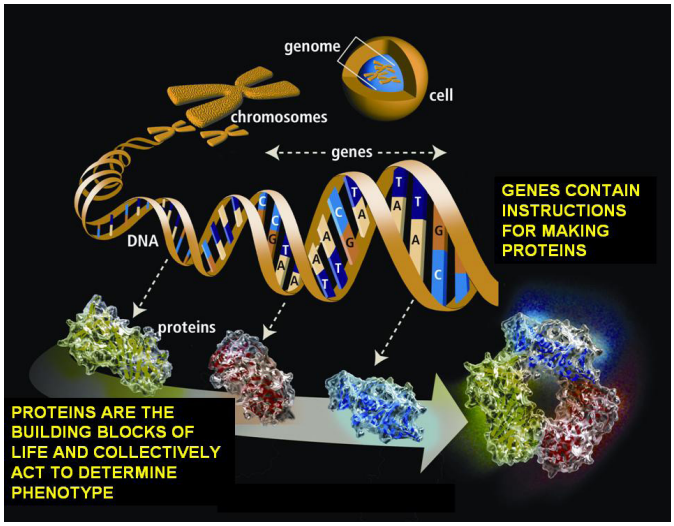

Figure 2. DNA (deoxyribonucleic acid) contains the instructions for making proteins. Differences in the nucleotide sequence of a gene's DNA can influence the type or amount of protein that is made, and this can have an effect on the observed performance of an animal. Original graphic obtained from the U.S. Department of Energy Human Genome Program, http://www.doegenomes.org

spells out the identity and the rate of assembly of proteins. **Proteins** are essential to the structure and function of plants and animals. A **gene** is a distinct sequence of DNA that contains all of the instructions for making a protein. It is possible for the DNA sequence that makes up a gene or "**locus**" to differ between individuals. A single nucleotide polymorphism (**SNP**), pronounced "snip," is a variation at a single position in the sequence of DNA among individuals.

These alternative DNA variants or forms of a gene are called **alleles**, and they can result in differences in the amount or type of protein being produced by that gene among different individual animals. This can affect the performance or appearance of animals that carry different alleles. Alleles can be **recessive**, meaning that an animal must inherit the same allele (i.e. the same sequence) from both parents before there is an effect, **additive** meaning that the effect is proportional to the number of an allelic variants inherited by the animal (i.e. carrying two copies of a particular allele produces double the effect of carrying one copy), or **dominant** meaning that the presence of one allele is sufficient to result in an effect on the trait or attribute of interest. Coat color is a well-known example of a simple trait where the presence of the dominant black allele dictates black over the recessive red alleles.

Scientists have started to identify regions in chromosomal sequence of DNA that influence production traits. They have used the techniques of molecular biology and quantitative genetics to find differences in the DNA sequence in these regions. Tests have been developed to identify these subtle sequence differences and so identify whether an animal is carrying a segment of DNA that is positively or negatively associated with a trait of interest.

Genotyping refers to the process of using laboratory methods to determine which DNA-marker alleles an individual animal carries, usually at particular genes or locations (loci) in the genome. The genotype identifies the marker alleles an animal carries. Because an animal gets one allele of each gene from its sire, and one allele of each gene from its dam, it can only carry two alleles of any given marker locus or gene. If an animal gets the same marker allele from each parent it is referred to as homozygous, or it may inherit different alleles from each parent in which case it is referred to as heterozygous. DNA testing can be used to distinguish between animals carrying different marker alleles and this information can also be used for tracking parentage.

Most of the economically relevant traits for cattle production (calving ease, weaning weight, growth, reproduction, milk production, carcass quality, etc.) are **complex traits** controlled by the protein products of many genes and also influenced by the production environment. The protein produced by different alleles of genes may influence the observed performance or **phenotype** of the animal carrying those alleles. The genetic component of phenotypic variation is the result of DNA sequence differences between chromosomes of individuals. When an animal has an EPD above the base year average for a certain trait, it means the animal has inherited a higher than average proportion of alleles for genes that favorably affect the trait. In other words, selection based on EPDs results in an increase in the average number of favorable alleles an animal can pass on to its offspring, without knowing which specific genes are involved. It should be noted that traditional EPD-based selection methods inherently tend to increase the frequency of DNA markers associated with the alleles of genes that have beneficial effects on selected traits.

With the advent of modern molecular genetics and the ability to sequence whole genomes, selection based on genetic information has become increasingly sophisticated. Meuwissen et al. (2001) suggested the use of genetic markers spread throughout the genome that could be used to accurately predict an individual's genetic merit, an approach known as **genomic selection** (GS). In combination with statistical methods, GS can combine phenotypic and genotypic information from ancestral populations to more accurately estimate the genetic potential of an individual animal.

By 2020, over 3.75 million dairy cattle and more than one million beef cattle had been genotyped at thousands of different loci with SNP chips (e.g. 50K or GGP-HD) in the United States. These genotypes are used in conjunction with the extensive phenotype databases that have been amassed to infer accurate genetic merit estimates of young animals based on their genotype, pedigree, and performance information (Wiggans et al., 2017). In beef cattle evaluations these are referred to as genomic or genomic-enhanced EPDs. Genotypic information increases the accuracy of genetic merit estimates, especially of young animals.

Cloning

Cloning is defined as making a genetic copy of an individual. Cloning has been going on for a long time. Plant breeders have been using this technique to "clonally propagate" desirable plant lines for centuries. Identical twins are clones, but more commonly the term is now used to refer to an individual that results from the transplantation of the DNA contained in a single cell into an enucleated oocyte (an egg which has had its own DNA removed). The term "cloning" became infamous following the appearance of Dolly the sheep, the first mammal cloned from DNA derived from differentiated adult somatic tissue (Campbell et al., 1996). This process is called **somatic cell nuclear transfer** (SCNT) cloning and has been successfully performed on many species including cattle.

It is important to note that prior to SCNT, two other well-established procedures were available and used to make cattle clones. Splitting or bisecting embryos, a process in which the cells of a developing embryo are split in half and placed into empty zona (the protective egg coat around early embryos) prior to transfer into different recipient mothers, was commonly used in the 1980s. Likewise, cloning by nuclear transplantation from embryonic cells was developed in the 1970s and introduced into cattle breeding programs in the 1980s, well before the appearance of Dolly. From an animal breeding perspective, the importance of the SCNT procedure that created Dolly is that it allows for the replication of adult animals with known attributes and highly accurate EPDs based on pedigree, progeny, and their own performance records.

Although clones carry exactly the same genetic information in their DNA, they may still differ from each other, in much the same way as identical twins do not look or behave in exactly the same way. In fact, it has been found that SCNT clones differ more from each other than do contemporary half-siblings. Clones do not share the same cytoplasmic inheritance of mitochondria from the donor egg, nor the same maternal environment as they are often calved and raised by different animals. It is also important to remember that most traits of economic importance

are greatly influenced by environmental factors, and so even identical twins may perform differently under varying environmental conditions.

In the case of SCNT there is an additional complicating factor, and that is the requirement for "reprogramming" of the transferred nuclear DNA as it goes from directing the cellular activities of a somatic cell, to directing the development of an entire new embryo. Currently this process is not well understood, and there appears to be an increased rate of perinatal and postnatal loss and other abnormalities in SCNT clones relative to offspring conceived in the traditional way. It may be that SCNT clones differ from the original DNA-donor in the way that their nuclear genes are expressed. These problems are not seen universally in SCNT cloned cattle, and there are reports of apparently healthy cattle that have gone on to conceive and have healthy calves. Studies comparing the performance of SCNT and other types of dairy cattle clones to their full siblings found that there were no obvious differences in performance or milk composition.

Although the performance records of SCNT clones may be different from their DNA donor, as far as we currently know they would be expected to have the same ability as their progenitor to transmit favorable alleles to their offspring. More research is required to determine if the offspring of SCNT clones perform as well as would be expected based on the predicted genetic potential of the original DNA-donor animal. Clones are in some ways a genetic stalemate because in a well-designed breeding program every successive generation would be expected to be genetically superior to the previous one.

Cloned animals may provide a "genetic insurance" policy in the case of extremely valuable animals or can be used to produce several identical bulls in production environments where AI is not a feasible option. Clones could conceptually be used to reproduce a genotype that is particularly well-suited to a given environment. The advantage of this approach is that a genotype that is proven to do especially well in a particular location could be maintained indefinitely without the genetic shuffle that normally occurs every generation with conventional reproduction. However, the disadvantage of this approach is that it freezes genetic progress at one point in time. As there is no genetic variability in a population of clones, within-herd selection no longer offers an opportunity for genetic improvement. Additionally, the lack of genetic variability could render the herd vulnerable to a catastrophic disease outbreak or singularly ill-suited to changes that may occur in the environment. There are now companies that offer bovine (and other species) cloning as a service.

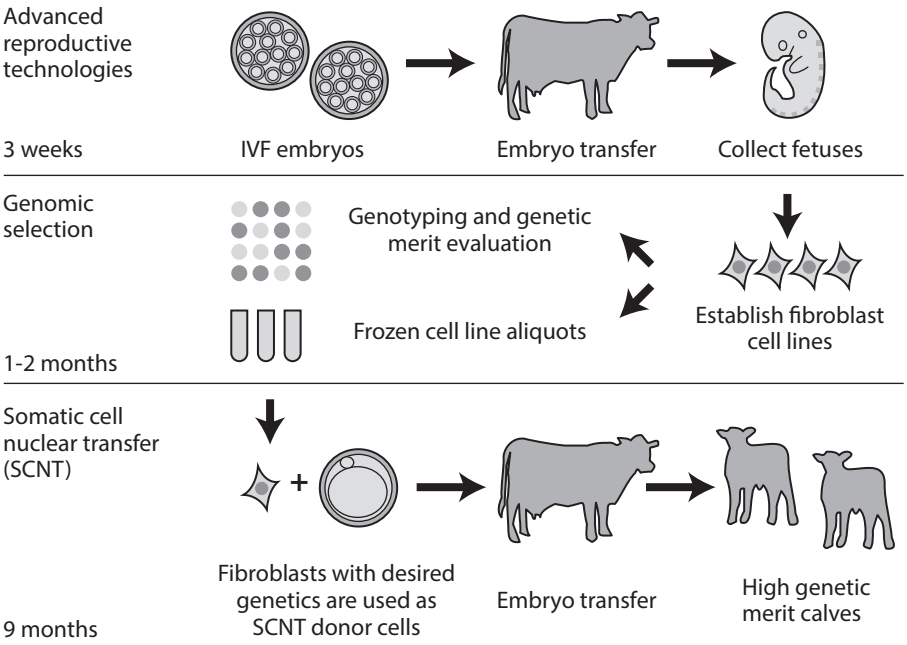

Figure 3. Production of high genetic merit calves using a range of advanced reproductive and DNA-based biotechnologies. In vitro fertilization (IVF) of multiple potentially elite embryos, followed by the brief gestation and establishment of cell lines can be used to increase the intensity of selection, genomic selection can then be used to screen cell lines for those with very best genomic breeding value, and then somatic cell nuclear transfer (SCNT) cloning can be used to realize calves from high genetic merit cell lines. Image from Kasinathan et al. (2015). Used with permission.

On January 15, 2008 the FDA published its final 968-page risk assessment on animal cloning which examined all existing data relevant to 1) the health of clones and their progeny, or 2) food consumption risks resulting from their edible products, and found that no unique food safety risks were identified in cloned animals. This report, which summarized all available data on clones and their progeny, concludes that meat and milk products from cloned cattle, swine and goats, and the offspring of any species traditionally consumed as food, are as safe to eat as food from conventionally bred animals (FDA, 2008).

A number of advanced reproductive technologies and breeding methods are being routinely combined to accelerate the rate of genetic improvement in the cattle breeding sector. Figure 3 shows how in vitro fertilization (IVF), genomic selection, and somatic cell nuclear transfer can work together to increase the intensity of selection, the reliability of the genetic merit estimate, and potentially decrease the generation interval (Kasinathan et al., 2015).

Genetic Engineering of Cattle

Genetic engineering is the process of moving a **recombinant DNA** (rDNA) sequence (i.e. a DNA sequence produced in a laboratory by joining pieces of DNA from different sources) into the genome of a living organism. What this means is that new genes, possibly derived from a different species or even kingdom, can be directed to make novel proteins in genetically engineered organisms. Genetically engineered organisms are commonly referred to as "transgenic," "genetically modified," "GMO," or simply "GE." Genetic engineering has been successfully used to make transgenic cattle, although none have been approved for commercialization or entry into the U.S. marketplace. The Food and Drug Administration (FDA) is the agency responsible for regulating genetically engineered animals (FDA, 2009).

Genetic engineering might find a place in agricultural production as a way to change the nutritional attributes or improve the safety of animal products in ways that are not possible through traditional

selection techniques. Such applications might include containing viral antigens to vaccinate calves against disease, or beef optimized for human nutrition. Genetic engineering could conceptually be used to improve production traits in cattle. It is unlikely that this will be implemented in the near future due in part to the difficulty in identifying single genes that might be good candidates to positively influence these complex traits. Additionally, genetic improvement for most production traits can be effectively achieved using traditional selection techniques on existing genetic variation, without the expense and time involved with the production and regulatory approval of genetically engineered organisms.

The previous generation of genetic engineering tools, resulting in the first transgenic livestock 35 years ago in 1985, was limited to the insertion of foreign DNA into the genome. This DNA was generally in the form of an rDNA construct comprised of a promoter and a protein coding region (protein upregulation) or an inhibitory RNA encoding region (protein downregulation). As the insertion site of the rDNA was random, there was no way of predicting all of 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.

The application of genetic engineering in cattle that is most likely to be cost-effective, at least in the near future, is the production of useful protein products such as human hormones or blood proteins in the milk or blood of genetically engineered cows. Such animals would not be destined, or permitted, to enter the food supply. Several human therapeutic proteins have been produced in cattle (Monzani et al., 2016), although none are yet commercialized.

There have been three approvals for therapeutic proteins produced by transgenic animals. These include goats producing ATryn1® (human antithrombin-III) approved to treat hereditary antithrombin deficiency by the European Commission in 2006 and by the FDA in 2009, rabbits producing RuconestTM (Rhucin® outside the EU) approved to treat hereditary angioedema in 2014, and chickens producing KanumaTM (sebelipase alfa) in their eggs for the treatment of patients with a diagnosis of lysosomal acid lipase deficiency in 2015. These applications have the potential to produce large amounts of human therapeutics at low cost relative to the current mammalian cell culture techniques.

Although cloning is not genetic engineering per se, there is a logical partnership between the two technologies. Cloning offers the opportunity to make genetically engineered or transgenic animals more efficiently from cultured somatic cells that have undergone precise, characterized modifications of the genome. The first genetically engineered mammalian clones were sheep born in 1997 carrying the coding sequences for human clotting factor IX, which is an important therapeutic for hemophiliacs (Schnieke et al., 1997). Cloning has also been used to generate genetically engineered cows that produce human polyclonal antibodies (Kuroiwa et al., 2002). It is envisioned that these unique cows will make it possible to create an efficient, safe, and steady supply of human polyclonal antibodies for the treatment of a variety of infectious human diseases and other ailments including organ transplant rejection, cancer and various autoimmune diseases, such as rheumatoid arthritis.

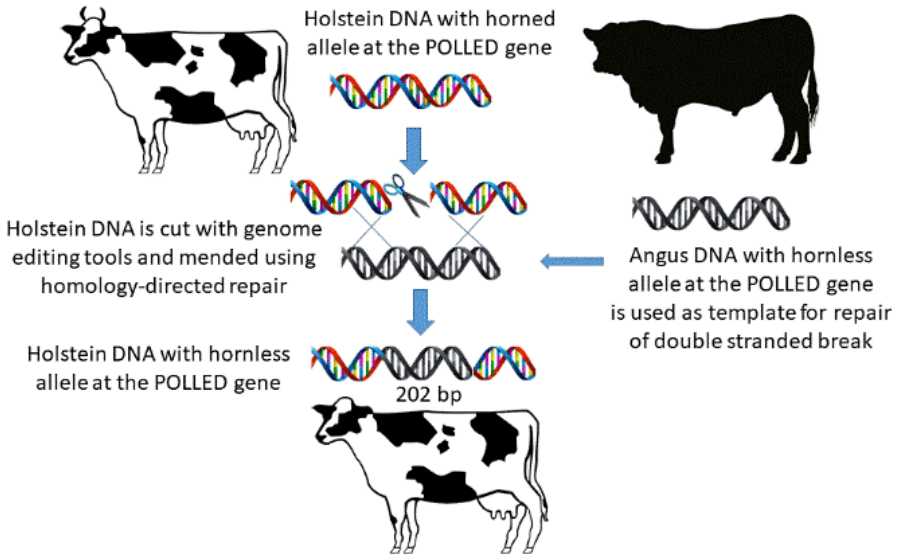

Figure 4. Genome editing induced, double-strand breaks can be repaired using a DNA template to direct the repair to mimic known, desirable genetic variants. In this example the allele that results in hornlessness was used as the homology-directed repair template to introduce a 202 bp sequence at the POLLED gene into Holstein genetics to produce dairy cattle that are naturally hornless as was described in Carlson et al. (2016).

Genome Editing of Cattle

Genome editing involves using a nuclease (e.g. Zinc finger nuclease, TALENS, CRISPR/Cas9) which cuts DNA at a targeted, specific sequence in the genome and introduces a double-stranded break (DSB) in the DNA double helix at that target site. One method that cells use to repair DSBs 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 (**indels**) at the target cleavage site due to 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 method can be used to introduce a range of genome edits, from point mutations to whole-gene insertions. Genome editing was used to move the polled allele, common in beef breeds like Angus, into dairy cattle genetics (Carlson et al., 2016) without the need for crossbreeding (Figure 4).

Genome editing presents an approach to introduce targeted modifications into existing genes and regulatory elements within a breed or species, without neces-

sarily 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 (genes introduced along with the beneficial gene during backcrossing.)

Table 1. Examples of traits that could be introduced into cattle using genome editing.

Target	Targeted Trait/Goal
Intraspecies *POLLED* allele substitution	No horns
Intraspecies *SLICK* allele substitution	Heat tolerance
Myostatin (MSTN) gene knockout	Increased lean muscle yield
Beta-lactoglobulin gene knockout	Elimination of milk allergen
Prion protein (PRNP) knockout	Elimination of prion protein
Intraspecies Calpain/Calpastatin allele substitution	Improved meat tenderness
Insertion of lysostaphin/lysozyme transgene	Resistance to mastitis
CD18 gene edit	Resistance to BRD (bovine respiratory disease)
Insertion of SP110, NRAMP1	Resistance to tuberculosis
Intraspecies SRY translocation onto X chromosome	All male offspring
NANOS gene knockout	Infertility (for germ cell transfer)

Genome editing research in cattle to date has focused primarily on monogenic (single gene) traits such as disease resistance (e.g. tuberculosis), production (e.g. myostatin knockout), generation of single sex offspring, elimination of allergens (e.g. beta-lactoglobulin knockout), and welfare traits (e.g. polled or hornlessness) (Table 1). Genome editing could be used to precisely introduce useful alleles (e.g. heat tolerance, disease resistance) and haplotypes into cattle breeds, thereby helping to improve their resilience while maintaining breed identity).

Data coming out of some of the large-scale genomic and sequencing projects are revealing situations where the sequence of one naturally occurring allele results in superior performance to that observed when animals inherit the 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 simultaneously, 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 productivity while retaining 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 (Rexroad et al., 2017).

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 toward given breeding objectives. 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.

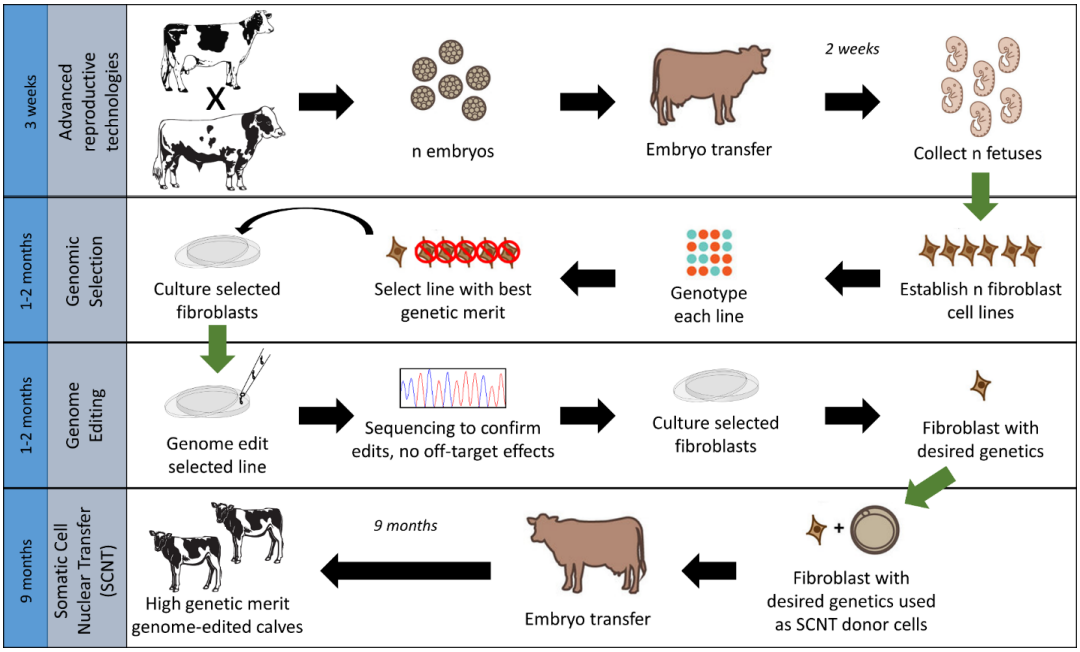

Figure 5. Production of high genetic merit calves using a range of biotechnologies and showing where genome editing might fit into the process. Gene editing was modeled as an added 1-2 month step to the elite calf production system outlined in Figure 3, which combines the use of advanced reproductive technologies and somatic cell nuclear transfer (SCNT) cloning with embryo transfer. Image from Van Eenennaam (2017). Used with permission.

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. 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, 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 unlikely that all of 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.

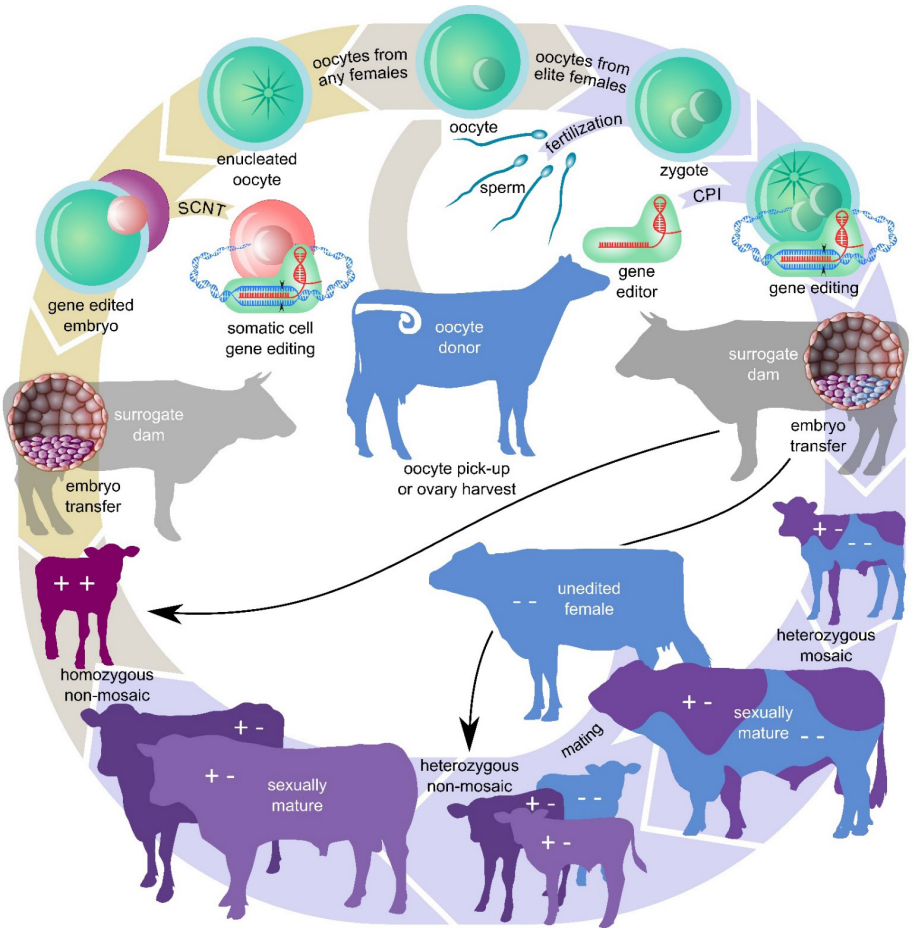

Figure 6. Steps for producing genome edited livestock through somatic cell nuclear transfer (SCNT) or zygote microinjection. Schematic showing the typical steps involved to produce homozygous, non-mosaic livestock by either somatic cell nuclear transfer (SCNT) cloning of genome-edited and screened somatic cells (yellow arrows), or cytoplasmic injection (CPI) of zygotes (purple arrows) with genome editing components. Image from Bishop and Van Eenennaam (2020). Used with permission.

Intersection with Conventional Breeding

To become an important driver of genetic change, genome editing methods must seamlessly integrate with conventional animal breeding programs (Figure 5). 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 SCNT cloning, or **cytoplasm injection** (CPI) of the gene editing reagents into early stage zygotes of the next generation of selection candidates (Figure 6).

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 prior to 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.

Direct editing of zygotes offers an alternative to cloning, but the disadvantage is that not all embryos will have the desired edit, and often embryos are mosaic—meaning the presence of two or more populations of cells with different genotypes in the one individual. However, on average fewer embryos are required to gene edit a pig, for example, using zygotic CPI as compared to SCNT due to the inefficiencies associated with cloning. Knockouts using NHEJ have been achieved through CPI of zygotes from a number of livestock species and can be obtained with relatively high frequency, with some reports of 100% efficiency. Targeted gene insertions have proven more challenging. Entire interspecies allele substitutions have been successfully knocked-in using CPI of zygotes in pigs. The birth of the first calf with a targeted gene insertion resulting from CPI of an early-stage bovine zygote occurred in 2020 (Owen et al., 2020).

Microinjection of embryos that result in mosaic offspring requires subsequent breeding to produce heterozygous or homozygous edited offspring, and this is time consuming and expensive in large food animals such as cattle (Bishop and Van Eenennaam, 2020). Many genome 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 genome editing of livestock far from routine at the current time.

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.

However, 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" (FDA, 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). Fortunately, in 2019 the FDA determined that surrogate cows, also referred to as embryo recipients, are not considered "treated" because they are extremely unlikely to contain the "intentional genomic alteration," through placental transfer or otherwise. Therefore, these cows may go into the food supply.

One procedural problem with the proposed guidance is differentiating between "intentional genomic alterations," off-target genome editing alterations, and *de novo* mutations. The 1,000 Bull Genome sequencing project found that genomic sequence data among bulls of different breeds varied by more than 84 million single-nucleotide polymorphisms (SNPs), and 2.5 million small insertion/deletions (Hayes and Daetwyler, 2019). These naturally occurring genomic alterations are the basis for all selection programs, and evolution, and are not regulated anywhere in the world.

Country	Additional Regulations?	Basis of trigger/regulation?
Argentina	No	Novel DNA sequence/transgene
Australia	Yes	Use of repair template
Brazil	No	Novel 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	No exogenous genes
New Zealand	Yes	Using of in vitro technique that modifies the genes/genetic material
United States	Yes	New Animal Drug

Figure 7. Regulatory approach to genome edited food animals in different countries. Chart indicating whether genome edited livestock carrying a naturally occurring allele introduced using genome editing and a homology-directed repair (HDR) donor template would be subjected to additional regulatory requirements relative to conventional breeding. Current as of 2020.

Further, 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 such as cattle take years and are beyond the resources of most academic laboratories, especially if the investigational animals have to 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 fit for purpose (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 "Does the final product contain a transgene?" 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 foreign rDNA constructs containing new combinations of DNA that could potentially present a hazard in the form of a new food allergen or toxin. Figure 7 reveals the 2020 disharmonious state of proposed regulations regarding genome editing in animals globally.

Conclusions

Significant improvements in the efficiency of milk and beef production have historically been accomplished through conventional breeding of superior individuals with an eye toward specific breeding objectives. A number of biotechnologies have been used to accelerate the rate of genetic gain. These include artificial insemination, embryo transfer, and genomic selection. More recent "modern" biotechnologies that could be used in breeding programs include cloning and genetic engineering. To date no genetically engineered cattle have been approved for food purposes anywhere in the world.

Genome editing is a modern biotechnology 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 they are to use this technology in their breeding programs. 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 means even SNPs and deletions introduced using editing trigger a new animal drug regulatory evaluation. This new animal drug regulatory paradigm will put the United States at a competitive disadvantage when it comes to incorporating genome editing into animal breeding programs, relative to other countries (e.g. Argentina, Canada) where novel product risk-based regulatory approaches have been implemented.

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