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Advances in breeding of dairy cattle

Edited by Professor Julius van der Werf
University of New England, Australia

Professor Jennie Pryce
Agriculture Victoria and La Trobe University, Australia

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The use of gene editing techniques in dairy cattle breeding

Alison L. Van Eenennaam and Amy E. Young, University of California-Davis, USA

1 Introduction

Livestock production is among the fastest growing agricultural subsectors, with demand projected to increase markedly in developing countries. Milk and dairy foods supply energy, protein and micronutrients, are known to be important dietary components for infants and young children and have key roles in remedying undernutrition. Per capita consumption of milk and dairy products has almost doubled since the early 1970s (Alexandratos and Bruinsma, 2012) and demand for dairy products in low- and middle-income countries is projected to increase further (FAO, 2017). As incomes and urbanization increase, expenditures on dairy products are predicted to grow more rapidly than other food items (FAO, 2013).

The current global human population is growing by 1.10% each year, meaning the annual addition of 83 million people. By 2030 there will be an estimated 8.6 billion people on the planet, 9.8 billion in 2050 and 11.2 billion by 2100. Population growth is predicted to be highest in least developed countries, presenting significant challenges in terms of hunger, malnutrition, food security and many other issues (United Nations, 2017). In order to meet increased demand, agricultural output will need to more than double in sub-Saharan Africa and South Asia by 2050 and increase by one-third in the rest of the world (FAO, 2017).

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In 2016, 6.5 billion tons of cow's milk was produced globally, twice the amount reported in 1960, with only one and a half times the number of animals, representing an overall 1.3x increase in yield (Fig. 1). Globally, there were 270 million head of dairy cows in 2016, with India being the largest holder at approximately 49 million head (FAOSTAT, 2018).

In developed countries, such as the United States, dairy cattle production has become extremely efficient, resulting in a significantly reduced dairy cattle population, reduced requirements for feed and water, land use and waste output per kg of milk in the past several decades. The US dairy cattle population peaked in 1944 at 25.6 million animals, with an annual production of 53 billion kg of milk (2074 kg of milk/cow) (USDA NASS, 2018). By 2017, the population had been reduced by more than half to 9.4 million animals but production almost doubled to a remarkable 97 billion kg of milk (10 406 kg of milk/cow) (USDA NASS, 2018). These data illustrate the incredible evolution of the US dairy industry from the low-input, low-producing, pasture-based systems of the 1940s to today's modern high-input, high-yielding systems. Advances in management practices and adoption of modern technologies, along with genetic improvements, have allowed for increased milk production while at the same time reducing the use of resources and alleviating environmental impacts (Capper et al., 2009).

The ability to generate highly productive cows relies heavily on advances in genetics and reproduction, and the ability to share elite genetics globally. Breeders select specific animals to be the parents of the next generation based on desired characteristics that contribute favourably to their breeding

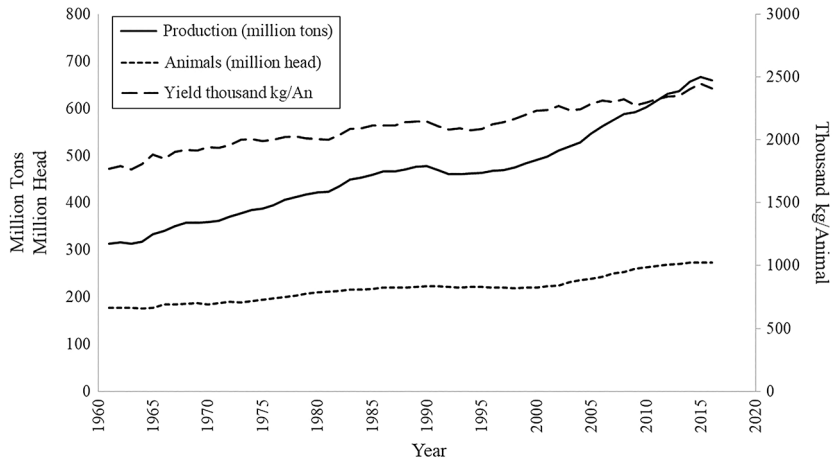

Figure 1 Dairy cattle production, head of dairy cows and milk yield from 1961 to 2016. Source: adapted from FAOSTAT (2018). Production in million tons and animals in million head are represented on the primary Y axis. Annual yield is represented by the dashed line on the secondary Y axis in 1000 kg/animal.

objectives. Adoption of new breeding methods such as artificial selection (AS), now routinely used in the dairy industry, have resulted in inflection points in the rate of genetic change. On the dam side, embryo transfer increases the number of embryos produced from elite females by moving the embryos to surrogate females with lesser genetic merit. Advances in modern molecular genetics and the ability to sequence whole genomes has led to the use of genetic markers that are located throughout the genome and can be used to predict genetic merit, an approach termed genomic selection (GS). The use of GS has been particularly effective in the dairy industry for the accurate selection of young sire candidates, and has provided the most recent inflection point in the rate of genetic gain by effectively doubling genetic progress due primarily to decreasing the age at which elite sires are first used.

A powerful new breeding method, genome or gene editing, has the potential to be the next technology that results in an inflection point in the rate of genetic improvement. This technique allows for precise, targeted alterations to the genomes of organisms. Site-directed nucleases (SDNs), including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced repeats (CRISPR) along with CRISPR-associated proteins (Cas9), induce double-stranded breaks (DSBs) at specific sites in the genome. Natural cellular machinery subsequently repairs the breaks through either the homology-directed repair (HDR) or non-homologous end-joining (NHEJ) pathways (Fig. 2). Coupled with donor templates, SDNs precisely introduce traits that may not be present in a population, or are present at very low levels, without requiring the introgression of these traits from outlying breeds or populations. Importantly, if these changes are made in the genome of embryos or germline cells, they are permanent and heritable.

1.1 Types of editors

Gene editing is possible due to the availability of programmable editing tools. The first of these tools was ZFNs, which are a fusion of specific DNA-binding zinc finger proteins and the cleavage domain of the *Fok* I endonuclease (Kim et al., 1996). Specific sites in the DNA can be targeted by modifying the DNA-binding domain. ZFNs identify target DNA in a modular fashion; each ZFN consists of at least three zinc finger domains and individual zinc fingers bind to three DNA bases. The *Fok* I nuclease domains dimerize and cleave the target DNA (Kim, 2016). Target sites for ZFNs are limited and there is no standard method for designing zinc finger proteins to recognize specific DNA sequences, so design and validation are notoriously labour intensive (Wang, 2015). Although ZFNs have been used successfully in a variety of cells and organisms, significant limitations include severe off-target effects and potential toxicity in cells (Table 1).

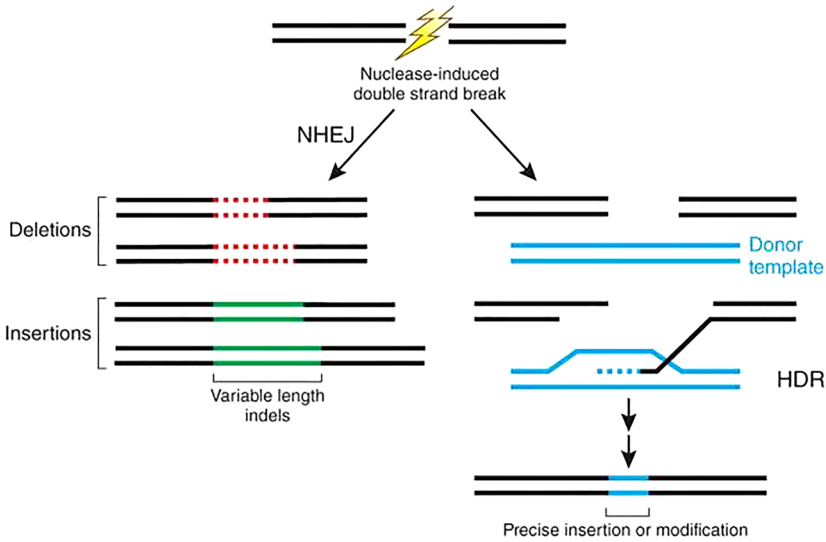

Figure 2 Nuclease-induced double-strand breaks can be repaired by non-homologous end joining (NHEJ), which can produce variable-length insertions or deletions (indels) at the break site, or homology-directed repair (HDR), which can produce precise point mutations or insertions from a single- or double-stranded nucleic acid donor template. Source: reproduced with permission from Sander and Joung (2014).

Similarly to ZFNs, TALENs also consist of the *Fok* I endonuclease, but instead fused with the TAL effector of a DNA-binding domain from *Xanthomonas*, a pathogenic bacteria (Boch et al., 2009). Whereas ZFNs recognize three bases, the individual TALE repeat domains recognize a single base, making TALENs a more flexible tool than ZFNs. They can be designed to recognize any DNA sequence with a 5′ thymine (Wright et al., 2014). TALENs are also more efficient than ZFNs, result in fewer off-target effects and show minimal cytotoxicity in cells (Mussolino et al., 2011) (Table 1).

Both of these editors sometimes require elaborate protein engineering and assembly. In contrast, CRISPR-Cas9 protein-RNA complexes instead target specific sites in the DNA through base pairing with a guide RNA, which is easily customizable. This system is based on components that are derived from prokaryotic DNA and used by bacteria to resist invasion of plasmid DNA and phages. The clustered repeats, CRISPRs, bind to viral RNA and use the Cas9 protein to disrupt it. In order to make DSBs in DNA, the CRISPR/Cas9 complex base pairs with a guide RNA (gRNA) of approximately 20 nucleotides that is followed by a 50-NGG protospacer adjacent motif (PAM) to introduce a DSB at the target locus (Ran et al., 2013). It is a relatively fast, robust, easily customizable and cost-effective system for targeted modification of genomes, hence its current popularity (Table 1).

Table 1 Comparison of ZFNs, TALENs and CRISPR-Cas9

Content	ZFN	TALEN	CRISPR-Cas9
DNA recognition	Protein-DNA	Protein-DNA	RNA-DNA
Components	ZFP-FokI	TALE-FokI	Cas9, gRNA
Efficiency	Low and variable	High and variable	High and variable
Off-target effects	Severe	Moderate	Variable
Multiple genetic loci edited	Difficult	Difficult	Yes
Target site	Guanine-rich	No limitation	PAM (NGG)
Working in dimers/pairs	Yes	Yes	No
Origin	Eukaryotes	Plant pathogen	Prokaryotes
Cost	High	Middle	Low
Vector construction	Difficult	Medium	Easy

Source: reproduced from Lee et al. (2017) under a Creative Commons licence.

1.2 Knock-ins, knockouts and base editing

A few applications of gene editing, primarily using ZFNs and TALENs since the CRISPR/Cas9 system is newer, have been reduced to practice in cattle (Table 2). Many of these have involved insertions and deletions (indels) or inversions of large DNA sequences, including whole genes.

Many of the known disease-causing mutations in cattle are single base pair changes (Ciepłoch et al., 2017), commonly referred to as single-nucleotide polymorphisms (SNPs). Most reports of single base replacement by SDNs to

Table 2 Gene editing applications in cattle

Target	Targeted trait/goal	References
Intraspecies POLLED allele substitution	No horns/welfare trait	Carlson et al. (2016)
Intraspecies SLICK allele substitution	Heat tolerance	Sonstegard et al. (2017)
Myostatin (MSTN) gene knockout	Increased lean muscle yield	Proudfoot et al. (2015)
Beta-lactoglobulin gene knockout	Elimination of milk allergen	Yu et al. (2011)
Prion protein (PRNP) knockout	Elimination of prion protein	Bevacqua et al. (2016)
Insertion of lysostaphin/lysozyme transgene	Resistance to mastitis	Liu et al. (2013, 2014)
CD18 gene edit	Resistance to bovine respiratory disease	Shanthalingam et al. (2016)
Insertion of SP110, NRAMP1	Resistance to tuberculosis	Wu et al. (2015), Gao et al. (2017)
Intraspecies SRY translocation onto Y chromosome	All male offspring	Owen et al. (2018)
NANOS gene knockout	Infertile males (for gonial cell transfer)	Ideta et al. (2016)

date in livestock have relied on systems that require DSBs in the DNA and subsequent repair by HDR, an inefficient process (Li et al., 2018). As such, base editing, making single base changes or substitutions, is challenging (Eid et al., 2018).

To overcome these issues, base editors have been designed to modify a specific DNA base while also manipulating the cell's machinery for DNA repair so the modified base is not repaired back to its original base once altered. A base editor is generally comprised of a DNA-targeting molecule, often a catalytically dead Cas9 that cannot make DSBs, and a catalytic domain that can deaminate a cytidine or adenine base. Since these systems do not require DSBs to edit DNA bases, there are limited opportunities to generate insertions and deletions (indels), meaning reduced incidences of off-target changes.

A recent report details the first use of base editors in livestock. A high-efficiency editor was microinjected into single cell embryos to induce nonsense mutations in the caprine FGF5 gene, which is associated with hair length in goats. The results showed the expected phenotypes, an efficiency of up to 39%, low indel rates and very few off-target mutations. However, the authors did observe high mosaicism upon mutation induction (Li et al., 2018).

This technology is in its infancy. Base editors can produce C to T and A to G mutations, but other point mutations are not currently feasible. In addition, the windows of activity may be narrow, precision is lacking and other Cas9 variants that are not dependent upon PAM sequences may need to be utilized (Eid et al., 2018). As base editing technology evolves, it could provide a precise method for altering known disease-causing SNPs or efficiently introducing desirable haplotypes with few unexpected off-target effects.

1.3 Off-target effects

The current genome editing tools all have the potential to induce off-target effects in the genome. Off-target effects occur when the engineered nuclease binds to genomic sites that share some homology with the target site, leading to alterations at genomic locations other than the intended target. These off-target changes can potentially lead to gene inactivation, chromosomal inversions or other mutations that could affect the health of an edited animal, but may also occur in regions that would result in no phenotypic effects.

A number of online software tools are available to perform *in silico* predictions of potential off-target sites based on sequence homology with the target site. However, these tools often do not take into account the epigenetic status of a locus *in vivo*, which can affect the accessibility of off-target sites. In recent years, successful efforts have improved the targeting specificity of TALEN and Cas9 proteins to reduce or eliminate off-target effects (Yee, 2016).

When considering off-target effects in the context of gene editing applications, it is important to recognize that spontaneously and naturally occurring DSBs occur regularly and are repaired by cellular machinery, often resulting in de novo mutations. Artificial and natural selection are in fact dependent upon the genetic variation produced by these changes. In one analysis of whole-genome sequence data from 2703 individual cattle in the 1000 Bulls Genome Project, more that 86.5 million variants were observed, comprising indels and single-nucleotide variants (Hayes et al., 2018). Another recent study found that on average every new animal will have around 65 *de novo* mutations, of which approximately five will be small indels and the remaining 60 will be single-nucleotide substitutions (Harland et al., 2017).

Most deleterious off-target mutations are selected against in plant and animal breeding programmes in a way that is not possible in human medicine applications, and neutral mutations are likely to disappear by drift (Ruan et al., 2017). A recent study that used trio sequencing in gene edited mice concluded that any off-target effects that might be caused by CRISPR mutagenesis were not statistically distinguishable from the natural background rate of de novo mutations that occur by other processes (Iyer et al., 2018).

1.4 How to get editors into mammalian cells

In order to produce gene edited organisms, SDNs need to have a way to get into cells to take advantage of the cellular machinery. There are a few options for achieving this, each with its own advantages, drawbacks and limitations.

The most commonly employed method to date has been to use somatic cell nuclear transfer (SCNT). SCNT was made famous by the birth of Dolly the sheep, who was the first animal cloned from an adult cell as opposed to an embryonic cell, showing that differentiated adult cells could be reprogrammed and induced to create a new animal that was a genetic copy of the donor. With respect to gene editing, the edit is done in primary cell lines, often fibroblasts grown in culture, and the gene edited nucleus is then removed and placed into an enucleated oocyte as the DNA donor for SCNT (Schnieke et al., 1997). The resulting offspring are all clones of the original donor cell line. Modification and selection of fibroblasts coupled with SCNT has resulted in the generation of HDR and NHEJ edited livestock. Although it is commonly used, SCNT is inefficient, with only a small per cent (3–10%) of transferred blastocysts resulting in the birth of viable offspring (Kato et al., 2000; Gurdon and Melton, 2008; Yang et al., 2007). Neonatal deaths, large offspring syndrome and cellular reprogramming issues have all been reported with bovine clones (Lamas-Toranzo et al., 2017).

An alternative to SCNT is to create fertilized embryos (zygotes) by in vitro fertilization (IVF) and directly micro-inject (MI) the gene editing

components into the ovum or one-celled embryo. The resulting edited zygotes are then transferred to surrogate dams for gestation. This approach has been successful in livestock with high rates of NHEJ being reported, although mosaicism can be a problem and targeted gene knock-ins using this approach have proven more difficult (Carlson et al., 2012; Wei et al., 2015). Both MI and SCNT can result in edited offspring, but zygote injection offers an approach to edit the next generation of sires as compared to cloning which requires the development of fibroblast cell lines from the targeted animal (Fig. 3). Figure 4 shows how gene editing and SCNT might be combined with advanced reproductive technologies and GS programmes with only a slight decrease in the generation interval as compared to the approach outlined by Kasinathan et al. (2015).

The use of embryonic stem cells (ESCs), which are self-renewing and pluripotent (the ability of a cell to generate all of the different types of cells found in an adult), has a history of successful use in mice. However, for many years the tested culture conditions did not support renewal and proliferation of livestock cells. Instead, reports detailed the derivation of induced pluripotent stem cells (iPSCs), which demonstrated long-term proliferation and pluripotency. Unfortunately, livestock iPSCs depend on the continuous expression of various reprogramming factors so it is uncertain how faithfully they represent fully reprogrammed pluripotent stem cells (PSCs). The use of PSCs is also challenging in livestock as many generations are required to produce

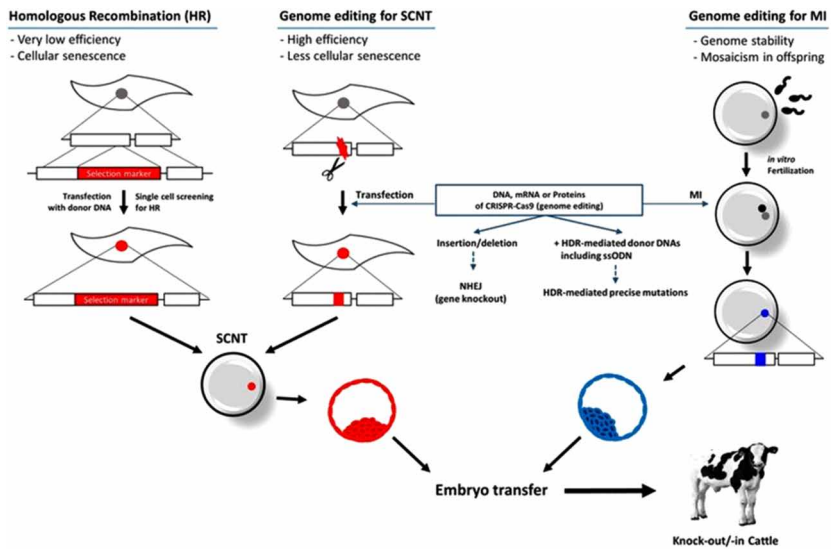

Figure 3 Illustration of knockout/knock-in cattle by SCNT and MI. Source: reproduced under a Creative Commons licence from Yum et al. (2018).

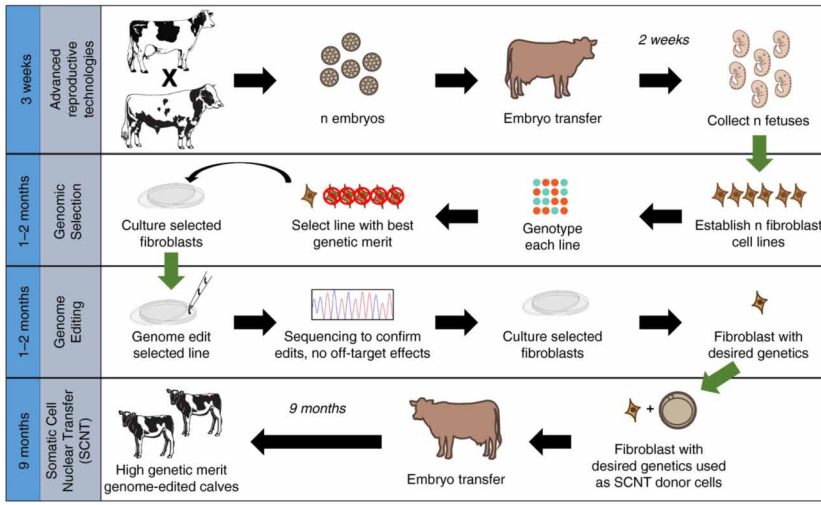

Figure 4 Production of high genetic merit calves using a range of biotechnologies and showing where gene editing might fit into the process. Source: reproduced with permission from Van Eenennaam (2017).

genetically modified founder animals (Soto and Ross, 2016) and livestock have much longer generation intervals and fewer offspring than mice. Some interest in deriving livestock PSCs stemmed from the idea of using them as donor nuclei for SCNT, which could result in higher reprogramming efficiency (Kou et al., 2010). Most reports of the use of iPSCs in livestock to date have achieved only low rates of germline transmission (West et al., 2010; Sartori et al., 2012). Fortunately, after years of research, the efficient derivation of bovine ESCs (bESCs) that can withstand extended passaging and maintain pluripotency was recently announced. The availability of these bESCs has significant implications for both agricultural and biotechnological applications (Bogliotti et al., 2018).

2 Applications of gene editing in dairy cattle

Based on the principles and tools outlined, there are a number of potential applications for gene editing to the dairy cattle industry. It can correct diseases and disorders that have a genetic basis and known causal mutations, change a less desirable allele to a more desirable allele without the need for crossbreeding or repeated backcrossing, turn genes on and off and introduce specific allelic variants or even whole genes or transgenes. In order to become successfully integrated into production systems, gene editing will need to be used alongside existing conventional selection as a synergistic accompaniment, as opposed to replacing it (Van Eenennaam, 2017).

2.1 Disease resistance

A variety of factors, including the current high level of global interconnectivity and the resulting exchange of goods and animals, modern livestock rearing systems, environmental and climate changes and the geographic spread of disease vectors into new habitats, contribute to the threat of disease in the dairy industry. Infectious diseases such as bovine viral diarrhoea, salmonellosis, tuberculosis, leptospirosis and Johne's disease are increasingly the targets of biosecurity efforts in a number of countries. These diseases can have devastating effects on production and longevity of animals in the herd (Barkema et al., 2015).

In some cases, preventative disease control measures such as vaccination and treatment options such as antibiotics are available. In others, there are no approved treatments available or pathogens have developed resistance. Additionally, increasing pressures to develop non-chemical disease management strategies and reduce the use of antibiotics will require novel disease prevention approaches.

Genetic targets have been identified for a few diseases in dairy cattle and these can be modified using gene editing to improve disease resistance. One of these diseases, which has profound economic impacts on the dairy industry, costing billions of dollars annually, is mastitis. Early genetic engineers also targeted this trait, and a number of papers detail the production of mastitis-resistant transgenic animals from as far back as 2005 (Wall et al., 2005; Maga et al., 2006).

An early report using gene editing detailed the success of inserting the *lysostaphin* gene (*LSS*) into the β-casein locus by HDR using ZFNs. This resulted in the secretion of *lysostaphin* in the milk, which is able to kill *Staphylococcus aureus*, a bacterium known to cause mastitis (Liu et al., 2013). Shortly thereafter, the same group reported a similar knock-in of the human *lysozyme* gene (*hLYZ*) using the same techniques. *Lysozyme* is an antimicrobial protein and the milk secreted by these cows also had the ability to kill *Staphylococcus aureus* (Liu et al., 2014).

Gene editing could similarly be applied to prion diseases, which are neurodegenerative diseases that affect animals and humans such as bovine spongiform encephalopathy ('mad cow disease') and Creutzfeldt–Jakob disease. The production of cattle resistant to BSE was actually achieved more than a decade ago using conventional genetic engineering (Richt et al., 2007). Deletion of the prion protein (*PRNP*) gene using TALENs was first reported in bovine fibroblasts and showed promise as an opportunity to better understand prion diseases (Choi et al., 2015). Knockout and knock-in of *PRNP* alleles was then reported in bovine foetal fibroblasts and early embryos using the CRISPR/Cas9 system, providing evidence that this system is efficient to induce indels at the *PRNP* locus (Bevacqua et al., 2016).

One of the first successful gene knock-ins in cattle using TALENs involved the insertion of the mouse SP110 nuclear body protein gene (*SP110*) into the genome of Holstein–Friesian cattle and resulted in cattle that were resistant to tuberculosis. As a zoonotic disease, tuberculosis is caused by the transmission of *Mycobacterium bovis* and infects a broad range of mammalian hosts. Widely distributed, this disease is a serious threat to global public and animal health, especially in less-developed countries. Mouse SP110 is known to control the growth of *M. bovis* in macrophages and induce apoptosis in infected cells. As such, the murine *SP110* gene emerged as a promising candidate for the control of *M. bovis* infections (Wu et al., 2015). A recent study reported the knock-in of the natural resistance-associated macrophage protein-1 (NRAMP1) gene using the CRISPR-Cas9 system that also resulted in cattle that were more resistant to tuberculosis (Gao et al., 2017).

One of the most costly diseases to both the dairy and beef industries is bovine respiratory disease (BRD). Several viruses and bacteria have been implicated in this disease. *Mannheimia haemolytica* is the bacteria most frequently associated with severe pneumonia and death. This bacteria produces a leukotoxin that binds to the CD18 signal peptide in ruminants and causes lysis of leukocytes, which in turn causes pneumonia. A reported gene edit in the integrin subunit beta 2 (ITGB2) gene to change one amino acid (Q(-5)G) in the CD18 signal peptide using ZFNs in foetal fibroblasts resulted in a foetus that produced leukocytes resistant to the *Mannheimia haemolytica* leukotoxin. The ability to produce cattle resistant to even a subset of BRD would have significant beneficial economic outcomes and would reduce the need for therapeutic and prophylactic treatments such as antibiotics (Shanthalingam et al., 2016).

It is reasonable to assume that as new diseases emerge and genome-wide studies identify causal resistance and susceptibility loci, the number of diseases that could potentially be mitigated using gene editing technology will increase. However, many of these editing applications for disease resistance involve the insertion of a transgene (genetic material that has been transferred from a different organism), and this may well become linked with the same type of regulatory cost and uncertainty, public concerns and activist opposition that were associated with genetic engineering.

2.2 Welfare

Improvements in disease resistance are directly related to the overall health, longevity and welfare of animals in the population. A variety of animal welfare issues could be alleviated, or even eliminated, through the employment of gene editing technology. The primary application to date in dairy cattle is genetic dehorning to eliminate the need for the unpleasant processes of physical dehorning and disbudding, which are currently routine management practices. This application is outlined in detail in the case study below.

2.3 Improved genetic traits

Gene editing could also be used to improve complex polygenic traits currently utilized in GS programmes, or for which promising candidate genes have been identified. Large whole-genome-sequencing projects are likely to identify additional genes and pathways involved in many important production traits such as fertility and feed efficiency.

Despite improvements in reproductive management, including advances in ovulation synchronization protocols, nutrition and the addition of fertility and longevity traits to GS programmes, dairy cattle reproductive efficiency is suboptimal, which results in economic losses (Rezende et al., 2018). In Holstein cows, GS for milk production can result in declines in fertility (Nayeri et al., 2017). Causative and candidate genes have been identified for female fertility traits such as age at puberty (Hawken et al., 2012), recessive embryonic lethals (VanRaden et al., 2011; Hoff et al., 2017; Taylor et al., 2018) and calving ease (Wickramasinghe et al., 2011), among others. These are potential targets for gene editing for improved female fertility.

On the sire side, a 10% difference in conception rate has been reported between high- and low-fertility dairy bulls (Peñagaricano et al., 2012). Genes and pathways that influence Holstein service sire fertility (Nicolini et al., 2018) and putative genes associated with Jersey sire fertility (Rezende et al., 2018) have been identified. Genes related to testis development, spermatogenesis, sperm motility, fertilization, sperm cell energy metabolism and acrosome reaction (Rezende et al., 2018) could all be potential gene editing targets to improve sire fertility.

In addition to fertility, feed efficiency is a trait that has also been a topic of interest since the introduction of GS. Feed is one of the primary costs for dairy producers. Having animals that efficiently convert feed into product has significant economic benefits. Although feed efficiency is a notoriously costly and difficult trait to measure, large genome-wide association studies (GWAS) have identified candidate genes that have a role in feed efficiency in dairy cattle (Hardie et al., 2017) and could potentially be considered as targets for gene editing. Polygenic traits, controlled by many interacting gene loci, are going to be less amenable to editing than single-gene Mendelian traits.

2.4 Environmental adaptations

In order to meet the dietary demands of the growing global human population, more food will have to be produced with fewer resources. All of this will additionally have to be achieved in the face of a changing climate. Increasing environmental temperatures are a particular threat to dairy production due to the high metabolic demands of lactation. Heat stress is already a challenge for dairy cattle in tropical and subtropical climates and will become increasingly

problematic for those in more temperate climates during summer heat waves (Davis et al., 2017).

Most of the selection for milk traits in cattle have occurred in heat-intolerant *Bos taurus* breeds. In the tropics, productivity of Holsteins is reportedly reduced by 40–60% (Usman et al., 2013). Consequently, breeders in tropical climates regularly cross high milk-producing *Bos taurus* breeds with *Bos indicus* breeds, which are lesser milk producers but have improved heat tolerance (Davis et al., 2017).

Genetic mutations have been identified that could be used to improve cattle survival and productivity in hot, tropical environments. Mutations for 'slick', characterized by short, slick-looking coats (Fig. 5), enhanced sweating ability and improved heat tolerance, have been identified in the prolactin signalling pathways (Littlejohn et al., 2014; Porto-Neto et al., 2018). Cattle with one copy of the slick gene have body temperatures that are approximately 0.5–1°C (0.9–1.8°F) lower than non-carriers.

Introgression of the slick trait into *Bos taurus* dairy breeds while maintaining acceptable levels of productive milk genetics would be a slow process. However, gene editing could be used to more rapidly add these dominant mutations to high-producing dairy breeds, enabling them to have increased heat tolerance, improved thermoregulation and less drastic drops in milk yield in the summer (Littlejohn et al., 2014). Variants for 'slick' could also be combined with known coat colour mutations for additional improvements in heat tolerance (Davis et al., 2017). For example, researchers in New Zealand have introduced a

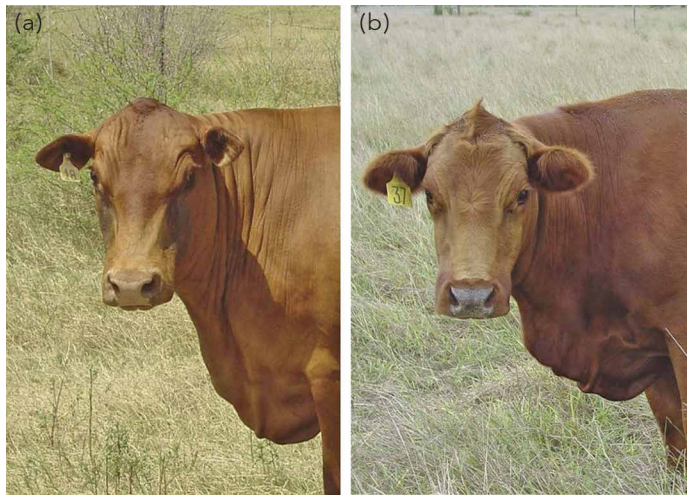

Figure 5 'Slick' coat type in crossbred cattle. (a) An animal with the 'slick' phenotype; (b) a wild-type animal. Source: reproduced under a Creative Commons licence from Littlejohn et al. (2014).

three-base pair deletion in the PMEL gene that has been associated with coat colour dilution in Highland and Galloway cattle (Schmutz and Dreger, 2013) into Friesian cattle. They produced two edited calves that displayed marked coat colour dilution compared to controls (Brophy et al., 2018).

2.5 Consumer traits

Milk is a major source of protein for human consumption. As a commercial product, it is produced worldwide. There are a number of ways that gene editing could conceivably be used to enhance milk including removing allergens, improving nutrition and increasing shelf life.

Although milk is widely consumed and offers a beneficial source of protein, many people have allergies to certain milk proteins. The allergenic potential of cow's milk can be reduced by removing β-lactoglobulin (BLG). Cows with disrupted BLG production could provide an attractive alternative to expensive processes like enzymatic hydrolysis to create hypoallergenic, BLG-free milk. Gene editing using ZFNs has been used to disrupt the *BLG* gene (Yu et al., 2011; Wei et al., 2015). Recently, the *BLG* gene was successfully knocked out using TALENs; no BLG production was detected, although a smaller form of BLG due to an in-frame deletion was observed by Western blot (Fig. 6). Whole-genome

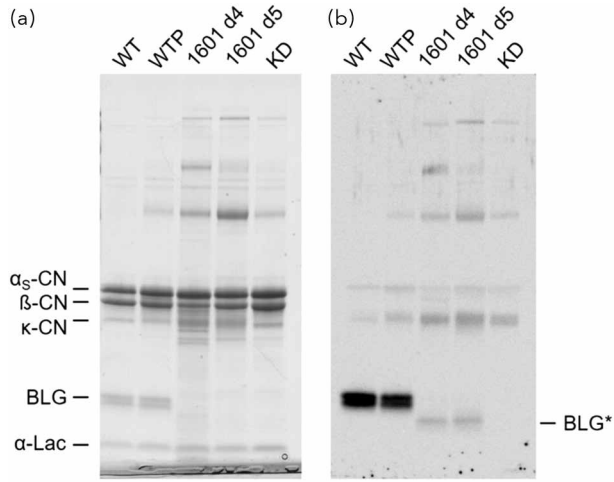

Figure 6 Analysis of BLG in milk of a female BLG knockout calf. (a) Coomassie Blue staining; (b) Western blot analysis following SDS-PAGE. WT = natural milk from a wild-type cow; WTP = pooled natural milk samples from multiple wild-type cows; 1601 d4 and d5 = milk sample from the gene edited calf on days 4 and 5, respectively, of an induced lactation; KD = milk sample from a transgenic BLG knockdown cow; αS-CN = alpha-casein; β-CN = beta-casein; κ-CN = kappa-casein; α-Lac = alpha-lactalbumin. Source: reproduced under a Creative Commons licence from Wei et al. (2018).

sequencing revealed no off-target mutations or vector integration events (Wei et al., 2018).

In addition to milk allergies, some people are lactose intolerant and consequently have to restrict their intake of dairy products and/or take lactase enzyme supplements. As an alternative, a gene for the lactase β-glycosidase from the thermophilic bacteria *Sulfolobus solfataricus* (LacS) was integrated into the genome at the bovine β-casein locus to produce a low-lactose cow using TALENs. Although this study suffered from low knock-in efficiency, it provides the first steps towards a gene editing strategy to produce milk for lactose-intolerant consumers (Su et al., 2018).

Prior to the advent of SDNs, genetic engineering was successfully used to target protein expression in milk. The dominant milk protein family is caseins, with three primary casein proteins, α-, β- and κ-. β- and κ-casein are known to improve heat stability and processing properties of milk. Genetically engineered cattle were produced that have extra copies of the β- and κ-casein genes, which resulted in nearly double the casein content compared to non-transgenic cattle (Brophy et al., 2003). Similarly, transgenic approaches were successfully employed to alter fat composition in goat milk by targeting the stearoyl-coA desaturase gene (Reh et al., 2004). These previous studies suggest that these genes may be good targets for modification by gene editing, especially considering the improved specificity of SDNs in terms of the location of transgenic integration as compared to older technologies.

2.6 Biopharming

Milk is not only a beneficial source of protein, it is also a potential bioreactor for the production of pharmaceuticals. Flexible production, simple purification and large-scale volume make milk attractive for the commercial production of bio-pharmacological proteins. Production in the mammalian mammary gland also has the advantage of post-translational modifications that produce proteins in native conformations, as opposed to alternative systems such as cell culture. Limiting factors include the long interval from birth to first lactation, investments in time and material to produce edited animals, and regulatory considerations (Monzani et al., 2016).

One possible application of gene editing to biopharming in milk is the production of human serum albumin (HSA), an abundant plasma protein widely used in human medicine. Currently, stocks of therapeutic HSA are derived from human plasma, an inconsistent source that carries the risk of spreading infection from donors to recipients. Expression of HSA in bovine milk could potentially produce larger volumes of therapeutic protein with lower risk to patients. Building upon previous work (Moghaddassi et al., 2014) that targeted

integration of the *HSA* gene into bovine fibroblasts to replace bovine serum albumin expression using TALENs, Luo et al. (2016) successfully produced calves that expressed HSA in their milk. This was achieved by using TALENs to target the *HSA* gene into the bovine *BLG* locus. Calves were produced by SCNT and the resulting HSA protein was reportedly properly folded and showed similar characteristics to the native protein (Fig. 7). This application has important implications for the reliable, low-cost production of HSA for medical use.

Based on such successes, it's easy to envision additional proteins, such as human erythropoietin (Lee et al., 2013), which stimulates red blood cell production and is used therapeutically to treat anaemia, that could be produced in the milk of gene edited cattle. Similar techniques could also be used to engineer vaccines into milk, alter milk composition to facilitate processing into various dairy products (Whitelaw et al., 2016) or produce antibodies (Bertolini et al., 2016). Given that Atryn®, a recombinant human antithrombin produced in genetically engineered goats' milk (Kling, 2009) and Ruconest, a recombinant protein produced in the mammary gland of transgenic rabbits to treat hereditary angioedema (Longhurst, 2008) have both received regulatory approval in Europe and the United States, there may be a regulatory path to market for milk-expressed exogenous therapeutic proteins introduced into the genome using gene editing.

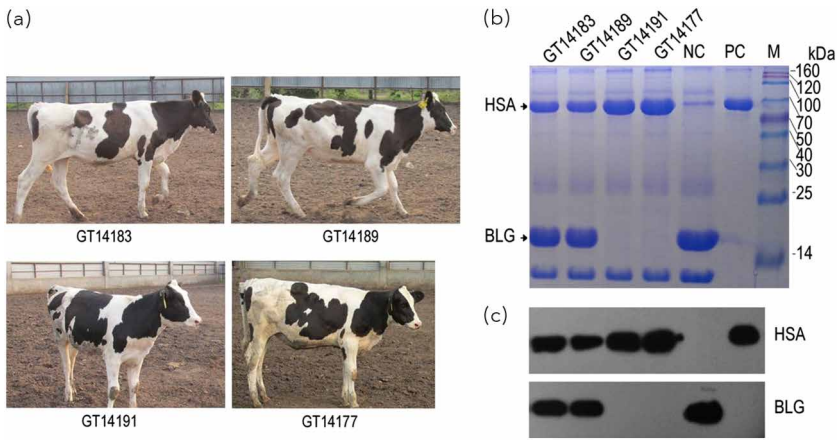

Figure 7 Recombinant HSA analysis and BLG expression in the milk of targeted cows. (a) Two heterozygous (GT14183 and GT14189) and two homozygous (GT13191 and GT14177) cows that were produced. Photographs taken by Yan Luo. (b) Expression of HSA in milk from the cows analysed by SDS-PAGE; (C) HSA and BLG expression levels analysed by Western blot. NC = negative control (i.e. non-targeted cow), PC = positive control, commercially available HSA. Source: reproduced under a Creative Commons licence from Luo et al. (2016).

2.7 Surrogate sires

The recent availability of bESCs (Bogliotti et al., 2018), combined with surrogate sire/dam technology could provide some useful applications to the commercial sector. For example, gene editing could be used to selectively inactivate an essential gene in germ cell differentiation (e.g. NANOS). This could enable the creation of commercial or even stud stock with transplanted germ or stem cells carrying elite genetics from superior donor seedstock animals (Park et al., 2017; Ideta et al., 2016).

3 Integration of editing into dairy cattle breeding programmes

Many of the gene editing applications described involve known variants with large single-gene effects, which are much easier to identify than those with small effects. However, variants of small effect influence many quantitative traits. In the coming years large datasets comprised of large numbers of individuals with sequence-level information will be available and could facilitate the identification of large numbers of these variants that influence quantitative traits. A recent study showed that genetic selection coupled with gene editing could enable a greater response to selection (1.08–4.12-fold after 20 generations) than GS approaches alone (Jenko et al., 2015). In this study, greater increases in inbreeding were noted when more edits were performed on a small number of sires as compared to fewer edits on each of a larger number of sires.

Another study similarly investigated the potential of GS in combination with gene editing to accelerate the introduction of a monogenic trait in a population compared to GS alone. The results showed a strong positive effect of gene editing on time to fixation of the allele, loss in polygenic response, reduction in long-term inbreeding when all the selection emphasis was placed on the monogenic trait and the number of animals created with the undesired phenotype before fixation of the desired allele. This approach could apply to monogenic traits that are present in the population at low frequencies, such as polled, in which increasing the frequency of such alleles by classical breeding strategies could result in increased inbreeding and decreased genetic gain towards the breeding objective. Incorporating gene editing approaches to a single target could be beneficial for disease resistance conferred by single genes or monogenic traits that reduce costs and improve animal welfare. There may be less value in editing a target that is only responsible for a small percentage of the genetic variance of a trait (Bastiaansen et al., 2018).

Genetic gain, influenced by increasing the frequency of favourable alleles, could be increased in dairy cattle breeding programmes by the use of gene drives. Gene drives are naturally occurring processes that cause a mutation on one chromosome to create a copy of itself on the homologous chromosome.

Gene editing could increase the frequency of favourable alleles in the population and gene drives could then accelerate that increase. This would result in rapid short-, medium- and long-term increases in genetic gain with no impact on inbreeding, less chance of loss of favourable alleles with lesser effect by genetic drift and eventual fixation of the favourable allele in the population (Gonen et al., 2017).

4 Regulations governing gene editing

Although the research described has many potentially useful applications, from disease resistance to improved consumer traits, there are no products from gene edited food animals on the market today. Similarly, even though the first genetically engineered food animal applications were reported in the 1980s, only the AquaBounty fast-growing AquAdvantage salmon has been able to navigate the regulatory system, albeit at great cost and after years of delays. The salmon is available commercially in Canada, and was recently allowed to enter the United States following years of legal challenges and an import ban over labelling requirements. The question remains as to whether products from gene edited animals will face the same slow, costly regulatory quagmire and how that will impact their paths to market.

In 2009, the US Food and Drug Administration's Center for Veterinary Medicine released Guidance 187, 'Regulation of Genetically Engineered Animals Containing Heritable rDNA Constructs'. The guidance outlines the evaluation of genetically engineered animals that contain heritable recombinant DNA (rDNA) constructs under the Federal Food Drug and Cosmetic Act, specifically the new animal drug provisions, with the rDNA construct being the regulated drug. This captures entire lineages of animals that descend from animals modified by rDNA techniques, regardless of their intended use. The overall evaluation is product-based, but the process that produces the genetic change (such as the use of rDNA techniques) can trigger the regulatory oversight. If new animal drugs are shown to be safe and effective with respect to their intended use, they can be approved.

With questions arising about how gene edited animals will be regulated, the FDA released a new Draft Guidance 187 in 2017 entitled, 'Regulation of Intentionally Altered Genomic DNA in Animals'. This draft guidance requires premarket new animal drug evaluation for food animals whose genomes have been 'intentionally altered' by SDNs. In this case, the 'intentional genomic alteration' is the new animal drug to be regulated, meaning even the absence of base pairs resulting from a deletion will be regulated as a drug. This is irrespective of whether novel DNA sequences were introduced and will apply to all intentional nucleotide insertions, substitutions or deletions. Each specific alteration will be treated as a 'drug' and subject to new animal drug approval

requirements, regardless of the novelty of the alteration or the presence/absence of hazards in the associated product.

Nucleotide deletions, insertions and substitutions occur every generation; they are part of normal genomic variation. Under this draft guidance, a deletion that occurs by conventional breeding would be unregulated, whereas the exact same change would be regulated if it was done using gene editing, despite no material way to distinguish between the two types of animals or their products. Although the underlying technology is the same, US regulatory agencies have announced that there are no plans to impose additional regulatory oversight on gene edited plants that do not contain novel DNA sequences (rDNA constructs) and which could have arisen through conventional breeding (backcrossing or radiation mutagenesis).

Lengthy, process-based regulations that are triggered by 'intention', rather than potential risks posed by novel products, may put a damper on the use of gene edited animal applications in the United States, as has been the case with genetically engineered animals. Globally, there is an emerging patchwork of regulations for gene edited animals, with some countries proposing no additional regulation in the absence of novel DNA sequences or product risks, and others proposing the same approach that was used for regulating genetically engineered animals. As demonstrated by the dearth of approved genetically engineered food animals, with a single exception (AquAdvantage salmon), this latter precautionary approach would effectively preclude breeder access to useful genetic variation that could provide solutions for disease resistance, animal welfare and other applications. As protocols and technology based on SDNs continue to progress rapidly, it is important that associated regulations are proportionate and based on product risk (Van Eenennaam, 2018).

5 Case study: POLLED

During the development of dairy cattle breeds, some traits unrelated to milk production became fixed. The presence or absence of horns in cattle is one example. Horns in cattle are permanent, in contrast to antlers in species such as deer and elk that shed and regrow many times over the course of an animal's life. They are made up of a bony core that is fused to the frontal bone of the skull and covered by a keratin sheath. The wild ancestors of today's domesticated cattle depended on horns for defence. During selection for improved milk traits, horns inadvertently came along as genetic hitchhikers. As a result, most of today's dairy cattle, primarily of the Holstein and Jersey breeds, are born with horns. In contrast, many beef breeds, such as Angus, which were selected for improved meat traits, do not have horns, a condition known as polled.

Modern dairy cattle live in very different environments than their horned ancestors and no longer require horns for survival. In many current production systems, horns are undesirable as they pose a danger to human handlers and other animals in the herd, and can cause economic losses due to injuries and carcass bruising. Horned animals also have additional space requirements and can be more aggressive than polled animals. As a result, horns are manually removed at a young age as part of routine management practices. Despite efforts to perform the procedure as humanely as possible, it is unpleasant for both the cattle and the producers. It has also become an animal welfare issue of increasing concern to the public. Breeding polled cattle is a possible solution to these issues.

The POLLED locus was mapped to bovine chromosome 1, where two different mutations are thought to have arisen. One is an 80 128 base pair duplication of Friesian origin (P_F); the second, simpler allele, which is of Celtic origin (P_C) is a duplication of 212 base pairs in place of a 10 base pair deletion (Medugorac et al., 2012) (Fig. 8). The polled P_C is dominant to the horned allele.

Polled genetics could be incorporated into dairy cattle breeding in three ways (Fig. 9). The first alternative, introducing polled from existing dairy genetics, is limited by the relatively small number of polled bulls in the current dairy cattle population. Additionally, polled bulls generally do not have high genetic merit for milk production traits, meaning producers could face economic losses if they use these sires. The few polled dairy sires currently available have on average approximately US$150 lower genetic merit, as estimated by the most widely used US dairy selection index, Lifetime Net Merit (NM$), than horned dairy sires (Fig. 10). In March 2018, only 3 of 3500 Holstein and 0 of 525 Jersey active (bulls with daughters and a USDA genetic evaluation) homozygous polled sires were registered with the National Association of Animal Breeders (NAAB, 2018).

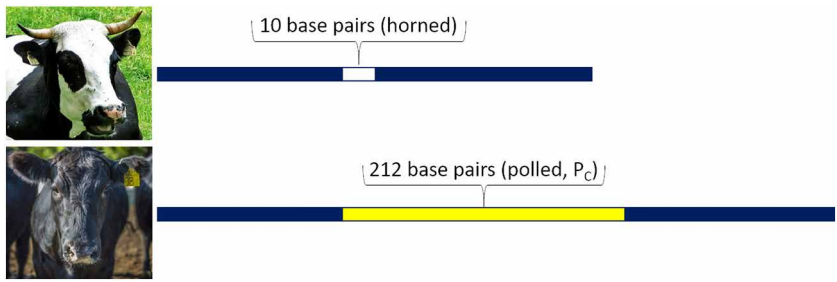

Figure 8 Graphic illustrating the nucleotide differences between the horned and polled (Pc) alleles at the POLLED locus. Source: cattle images from Needpix.com and reproduced under a Creative Commons licence.

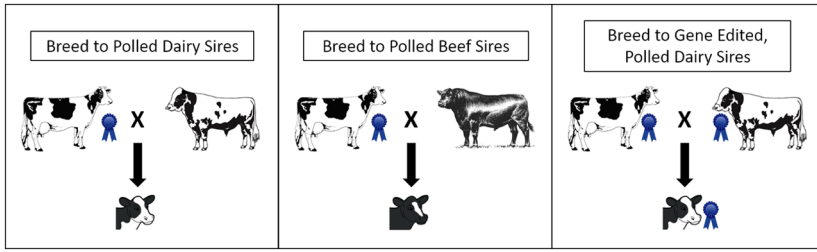

Figure 9 Three approaches to incorporate POLLED into dairy cattle populations. A cow with elite milk genetics (designated by the blue ribbon) can be used in all three scenarios. Since currently available polled sires and beef sires do not have elite genetics for milk traits, only the scenario that uses a gene edited, polled sire will result in offspring that are polled and still carry elite milk trait genetics.

Several studies have investigated the economics and different strategies of incorporating polled genetics into dairy herds using conventional breeding (Spurlock et al., 2014; Windig et al., 2015; Scheper et al., 2016). Although progress can be made in reducing the number of horned animals, it comes at a considerable loss in lifetime earnings as compared to a herd using horned sires. Gaspa et al. (2015) applied GS to polled but found that while these strategies can help to speed up the introduction process, the rate of genetic gain still decreased, and the rate of inbreeding must be monitored closely. Cole (2015) demonstrated that adding the economic value of polled to selection indices at a realistic value (US$40) or even an unreasonably high value (US$400) was

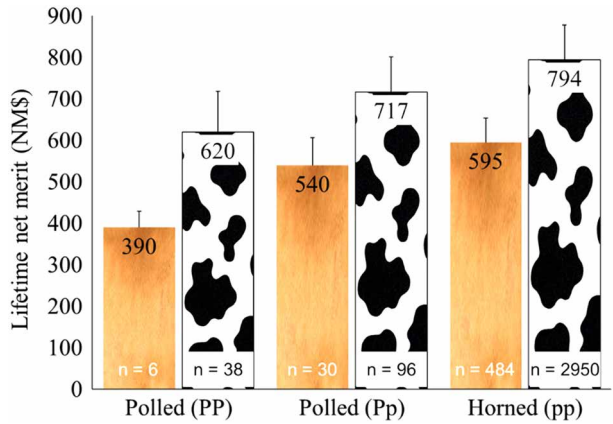

Figure 10 Average lifetime net merit (NM$) of the top 50% of polled and horned Holstein (shown in black and white) and Jersey (shown in brown) bulls, and the total number of each genotype and breed registered with the NAAB under active, foreign, genomic or limited status in March 2018. Source: adapted from NAAB (2018).

not an effective method for increasing the frequency of polled. Cole (2015) suggests that this method was ineffective because the frequency of the polled allele is so low that carriers were unlikely to be in the top-ranked bulls based on genetic merit.

Alternatively, horned dairy animals could be crossbred with polled beef animals, again since polled is dominant to horned, to create polled offspring. This would result in offspring that have some potentially good beef traits and some potentially good dairy traits, but overall are not actually well suited to either production system. Many generations of backcrossing would be required to regain the elite milk genetics that exist in the dairy population today and make it so highly productive and sustainable. This approach would likely be too costly and take too long for producers to apply.

Another alternative is to genetically dehorn dairy cattle using gene editing. In 2016, a Minnesota-based company, Recombinetics, reported using TALENs to gene edit the P_C allele into the genome of bovine embryo fibroblasts. Two cloned dairy bull calves with the polled phenotype were successfully born. Whole-genome sequencing to an average coverage of 20x did not identify any off-target insertions of the P_C allele nor any insertion-deletions attributable to unexpected cleavage by TALENs and repair by NHEJ (Carlson et al., 2016). The bulls developed normally and never grew horns. In 2017, semen from one of the bulls was used to artificially inseminate Horned Hereford cows and six heterozygous polled calves (one female, five males) were born. At the time of writing they are 1-year-old and do not have horns (Fig. 11).

Using gene editing to increase, and even fix, the polled allele in the dairy cattle population could be successful in a relatively short time (compared to conventional selection alone) due to the structure of the dairy breeding industry. A few hundred elite sires are typically bred to large numbers of cows and heifers, meaning that each sire can potentially have a very large number of offspring. The introduction of polled into a portion of these elite sires by gene

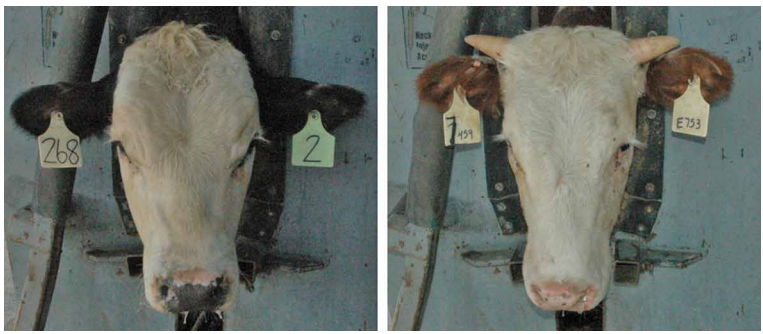

Figure 11 One of the polled offspring of the gene edited, polled bull (L) and a contemporary Horned Hereford control (R) at 1 year of age.

editing would allow the dispersal of the polled allele into a large number of offspring in one generation, while still also passing on elite milk genetics that have been under selection for generations.

A recent study that investigated the use of gene editing along with GS for polled found positive impacts on the time to fixation for polled, loss in polygenic response and fewer horned animals prior to the fixation of polled. Gene editing also reduced long-term inbreeding as compared to selection alone when all the selection emphasis was placed on the monogenic trait. However, the authors also made note that a variety of considerations, including gene editing efficiency, number of animals and ethical and welfare issues, need to be taken into account (Bastiaansen et al., 2018). Although this study used polled as an example of a monogenic trait of interest, it did not model it in the context of the current dairy population (i.e. small proportion of polled sires available, and the substantial difference in genetic merit between polled and horned sires).

Mueller et al. (2018) simulated the introduction of polled by conventional breeding using existing homozygous polled sires as compared to gene editing the top 1% of AI sires to be homozygous polled over a 20-year time frame. The change in horned allele frequency, inbreeding and genetic gain are shown in Fig. 12. Both approaches decreased the frequency of horns as compared to the baseline of no selection for polled; however, using existing polled genetics both slowed the rate of genetic gain and dramatically increased the rate of inbreeding. Editing 1% of elite AI sires to be homozygous polled maintained the rate of genetic gain and did not have such a deleterious impact on inbreeding.

Ideally, a large number of edited foundation sires would be available to propagate the beneficial edited alleles to the population and maintain background genetic variation while avoiding excessive inbreeding (Ruan

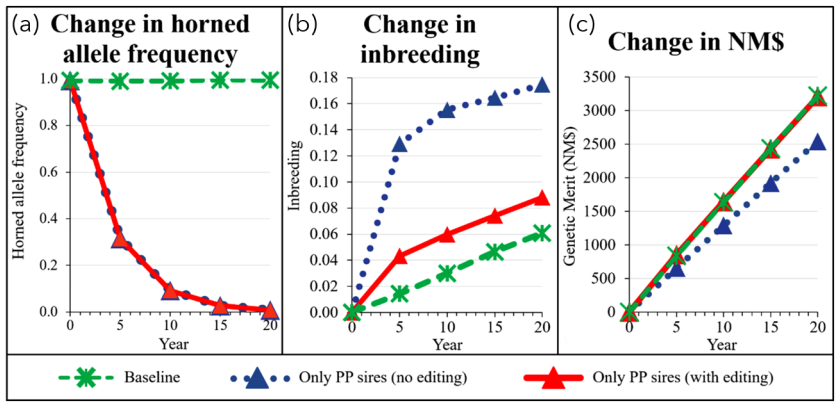

Figure 12 Comparison of breeding for polled for 20 years without gene editing (blue dotted line) using existing homozygous polled sires vs. with gene editing the top 1% of sires (red solid line). The baseline (no selection for polled) is a dashed green line. Source: reproduced with permission from Mueller et al. (2018).

Table 3 Determination of whether gene edited, polled cattle would trigger additional regulatory oversight as compared to naturally occurring polled cattle in different countries (as of September 2018)

Country	Additional regulations?	Basis of trigger/regulation?
Argentina	No	New combination of genetic material
Australia	Yes	Use of 'long' template
Canada	No	Product trait novelty
European Union	Yes	Is a GMO if used mutagenesis technique not in existence before 2001
Japan	No	Presence of exogenous genes
New Zealand	Yes	Use of *in vitro* technique that modifies the genes/genetic material
United States	Yes	Intentional modifications/new animal drug

et al., 2017). Whether this is possible very much depends upon the regulatory governance of intraspecies allele substitutions that do not introduce a new combination of genetic material into that species which currently differs markedly between countries (Table 3).

6 Conclusion

Gene editing technologies have the potential to contribute to a variety of useful applications for the dairy industry, from disease resistance to consumer traits. These technologies are more precise and easier to employ than older tools. Breeding programmes can integrate gene editing in a variety of ways to increase genetic gain in dairy cattle populations. Whether these tools will be available to breeders will be dependent upon how gene edited animals are regulated and the associated costs and timelines of moving applications through the regulatory process.

7 Future trends

Gene editing could conceivably be used to introduce useful genetic variation for a variety of applications similar to the ones that have been described such as reducing greenhouse gas emissions and producing milk with higher levels of protein to enhance neonate survival and welfare (Whitelaw et al., 2016). It could also be used to introduce a combination of traits simultaneously such as thermotolerance, lighter coat colour and tuberculosis resistance to create high milk-producing animals that are hardier for tropical environments.

Early applications are likely to be primarily associated with monogenic traits with known causal alleles of large effect. However, the frequency of favourable alleles could also be increased with SDNs for polygenic traits, which has been referred to as promotion of alleles by genome editing (PAGE). The successful

editing of large numbers of alleles at different loci has not yet been reported, but continued advances in gene editing technology, along with the generation of large datasets that can be used to identify large numbers of influential loci, could make this approach a reality in the next 5–10 years (Jenko et al., 2015).

Proposed novel breeding schemes involve successive *in vitro* cycles of GS, gene editing, gamete production and fertilization. These *in vitro* breeding schemes could reduce the generation interval and genetic lag between nucleus and commercial populations by orders of magnitude. As technology continues to improve, it is likely that improved on-farm sensors, data loggers, precision measurement techniques and other technological aids will provide precise phenotypic data that will identify additional genetic targets for gene editing that could further improve dairy cattle genetics.

8 Where to look for further information

8.1 Further reading

- A current 2018 reference that provides a comprehensive review of new breeding technologies in livestock is *Animal Biotechnology 2*, Niemann, H. and Wrenzycki, C. (Eds). https://www.springer.com/us/book/9783319923475.
- A review of reproductive and genomic technologies can be found in Fleming et al. (2018).
- See Kim (2016) for an in-depth review of gene editors and Yee (2016) for an in-depth discussion of off-target effects and efforts to minimize them.
- More information about the development of the gene edited polled bulls is available in Carlson et al. (2016) and Tan et al. (2013).
- Tan et al. (2016) provides a good history of gene targeting in livestock.
- For a discussion of regulations, see Van Eenennaam (2018).

8.2 Key journals/conferences

- PAG (Plant and Animal Genome) is an international conference attended by livestock geneticists held in January every year in San Diego, California.
- WCGALP (World Congress on Genetics Applied to Livestock Production) is a roving conference held every 4 years and brings together international experts in animal genetics and breeding.
- The Transgenic Animal Research Conference is held every other year in Northern California since 1997, on all things relating to transgenic – and now gene edited – large animals.
- The *CRISPR Journal* recently published its first issue and aims to 'deliver cutting-edge multidisciplinary peer-reviewed research, advances, and commentary on CRISPR'.

8.3 Major international research projects

- The 1000 bull genomes project (http://www.1000bullgenomes.com) aims to provide a large database for imputation of genetic variants for genomic prediction and GWAS in all cattle breeds for the bovine research community.
- ANGENMAP (https://www.animalgenome.org/community/angenmap) is an internet discussion sharing group in the broad fields of animal genome research with over 3000 subscribers from 50 countries.
- Visit www.recombinetics.com for updates on many of the editing applications discussed in this chapter, including polled, thermotolerance (slick), tuberculosis resistance and increased milk production, as well as applications in swine.

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