

## REVIEW

# Genome editing approaches to augment livestock breeding programs

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## ABSTRACT

The prospect of genome editing offers a number of promising opportunities for livestock breeders. Firstly, these tools can be used in functional genomics to elucidate gene function, and identify causal variants underlying monogenic traits. Secondly, they can be used to precisely introduce useful genetic variation into structured livestock breeding programs. Such variation may include repair of genetic defects, the inactivation of undesired genes, and the moving of useful alleles and haplotypes between breeds in the absence of linkage drag. Editing could also be used to accelerate the rate of genetic progress by enabling the replacement of the germ cell lineage of commercial breeding animals with cells derived from genetically elite lines. In the future, editing may also provide a useful complement to evolving approaches to decrease the length of the generation interval through *in vitro* generation of gametes. For editing to be adopted, it will need to seamlessly integrate with livestock breeding schemes. This will likely involve introducing edits into multiple elite animals to avoid genetic bottlenecks. It will also require editing of different breeds and lines to maintain genetic diversity, and enable structured cross-breeding. This requirement is at odds with the process-based trigger and event-based regulatory approach that has been proposed for the products of genome editing by several countries. In the absence of regulatory harmony, researchers in some countries will have the ability to use genome editing in food animals, while others will not, resulting in disparate access to these tools, and ultimately the potential for global trade disruptions.

**KEY WORDS:** Genetic improvement, Genome editing, Livestock, Food animal, Animal breeding

## Introduction

Livestock genetic improvement programs have been a powerful driving force for improving efficiencies, and thereby decreasing the environmental footprint and emission intensity of milk, meat and egg production. By combining objective phenotyping, genomic information, statistical methodologies and advanced reproductive techniques, breeders have been able to more precisely select and intensely utilize genetically superior parents for the next generation to accelerate the rate of genetic improvement programs.

The rate of genetic gain ( $\Delta G$ ) towards the breeding objective of a given production system depends upon the four components of the breeders' equation (Lush, 1937) as modified by Eberhart (1970):

$$\Delta G = \frac{i \times r \times \sigma_A}{L}, \quad (1)$$

where  $i$  is intensity (how extensively the most elite animals can be used as parents of the next generation);  $r$  is accuracy (how certain we are about the true genetic merit of selection candidates);  $\sigma_A$  is genetic diversity (as measured by the additive genetic standard deviation of the population); and  $L$  is the generation length (interval calculated as the average age of parents when progeny are born). Any technique that can cost-effectively increase  $i$ ,  $r$  or  $\sigma_A$ , or decrease  $L$ , will be adopted into breeding programs.

Each US dairy cow today produces more than four times the amount of milk of her 1944 forbearers, thereby doubling the nation's milk output even as the dairy cattle population has shrunk from 26 million to 9 million cows. These remarkable changes have been achieved using conventional selection based on naturally occurring genetic variation. As a result of this selection, the carbon footprint associated with producing a glass of milk has fallen by two-thirds over the last half century (Capper and Bauman, 2013). Likewise, from 1957 to 2005, modern broiler chicken growth increased more than fourfold (Fig. 1), with a concurrent 50% reduction in the feed consumed/unit weight gain conversion ratio (Zuidhof et al., 2014).

Breeders try to identify which animals are carrying genetic variants that are associated with traits of economic importance. This is done by extensive phenotyping of selection candidates, typically the nucleus animals at the top of the breeding pyramid (Fig. 2). For example, one poultry breeding company collects data on over 50 traits per nucleus selection candidate at various ages, and over 50% of these observations are involved with an evaluation of each bird's health, welfare and overall fitness (Katanbaf and Hardiman, 2010). Because these animals are destined to have millions of descendants, considerable investment is made to obtain an accurate estimate of their true genetic merit.

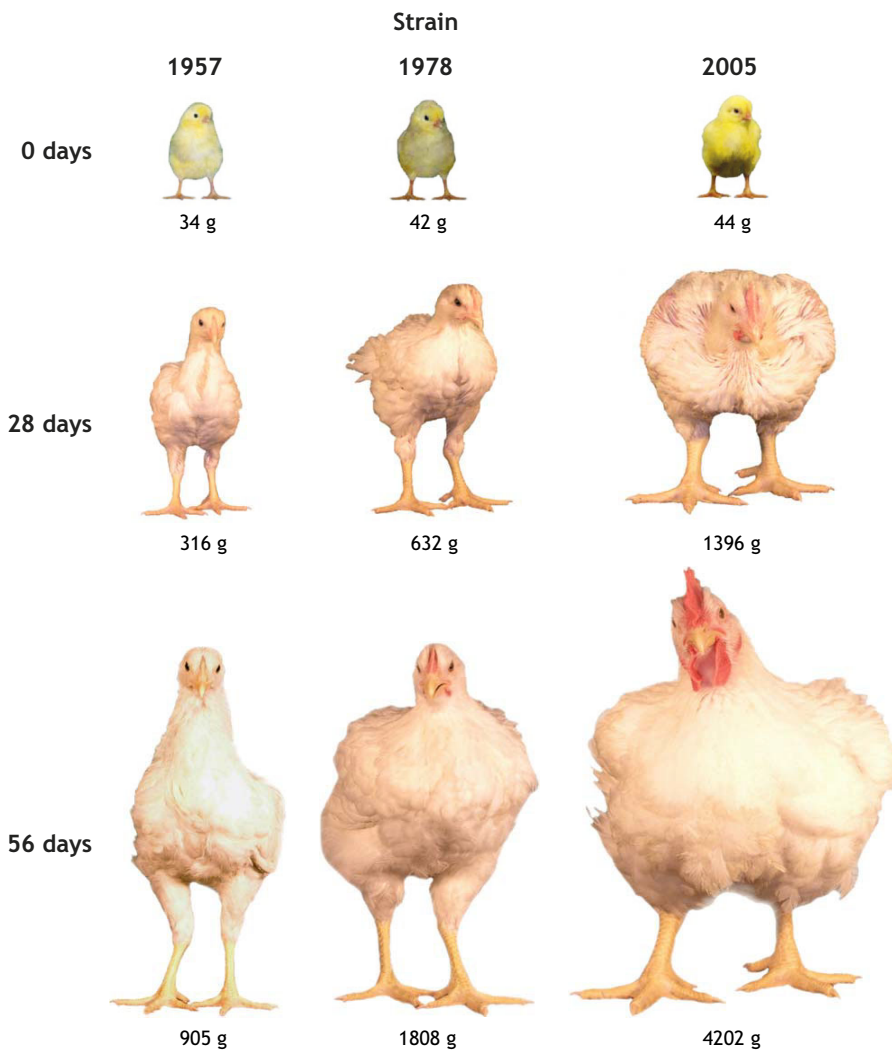
In the past decade, genomic analyses enabled by whole-genome sequence data and inexpensive genotyping platforms have provided an additional source of information for livestock improvement (Georges et al., 2019). Genomic wide association studies (GWAS) have been used to uncover associations between quantitative trait loci (QTL) for one or more phenotypes of interest, and markers. However, QTL are often associated in linkage disequilibrium with the true causative alleles. For this reason, the association between markers and QTL can differ between populations, making it difficult to generalize the results of GWAS across different populations. It is therefore of interest to identify the causative variants associated with QTL to be able to better track the alleles that are directly impacting phenotype.

The sequencing and 'resequencing' of livestock genomes has revealed a breathtaking abundance of genetic diversity among different breeds and lines. For example, when cattle geneticists sequenced over 2700 bulls of disparate breeds from around the world, they found more than 86.5 million variants between different breeds of cattle. These variants included 2.5 million insertions and deletions (indels) of one or more base pairs of DNA, and 84 million

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**Fig. 1. Age-related changes in size (mixed-sex body mass and front view photos) of University of Alberta Meat Control strains unselected since 1957 and 1978, and Ross 308 broilers (2005).** Within each strain, images are of the same bird at 0, 28 and 56 days of age. Reproduced under Creative Commons CC BY license from Zuidhof et al. (2014).

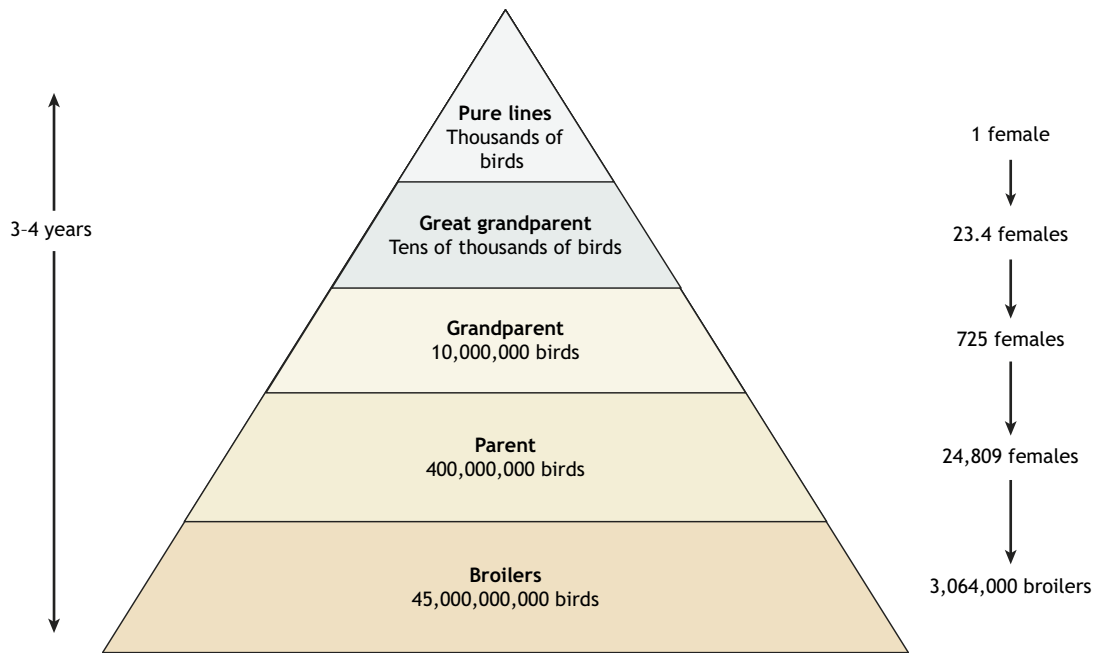
single nucleotide variants (Hayes and Daetwyler, 2019). This variation is the key to genetic improvement, and breeders routinely try to balance the extensive use of the best animals to accelerate the rate of genetic gain, with the avoidance of inbreeding to maintain genetic diversity in the population for future genetic improvement.

Genomic selection (GS) is a collection of methods to estimate the breeding values of individual animals on the basis of genome-wide single-nucleotide polymorphism genotype information. It uses genotype information to infer the genetic inheritance of each individual resulting from Mendelian sampling, and on the basis of large phenotyped and genotyped populations, enables the accurate prediction of the genetic merit of young selection candidates. In the dairy industry, this allows young bulls to be selected based on genomic-enhanced genetic merit estimates, enabling their use as sires at a younger age. An evaluation of the impact of 7 years of GS in US dairy cattle (García-Ruiz et al., 2016) shows that the rates of genetic gain per year increased by 50–100% for high-heritability traits, such as milk yield, and by 300–400% for low-heritability traits, such as somatic cell counts (a measure of udder health) and daughter pregnancy rate (a measure of female fertility). This innovation, GS, is now used in many livestock and crop genetic improvement programs (Meuwissen et al., 2013).

Typically, genetic improvement comes from selection of natural variants already existing within a breed. Other breeds may have

other useful variants, and being able to introduce them from one breed to another could further accelerate the rate of genetic improvement. Historically, this has been achieved through crossbreeding followed by repeated backcrossing to eliminate undesired traits that introgressed as selection drag from the other breed.

Genome editing offers a new opportunity to further accelerate the rate of genetic gain in livestock. Editing presents an approach to precisely introduce 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. The previous generation of genetic engineering tools, resulting in the first transgenic livestock over 30 years ago (Hammer et al., 1985), was limited to the insertion of foreign DNA into the genome. This DNA was generally in the form of a recombinant DNA (rDNA) construct comprising a promoter and a protein coding region (protein upregulation), or an inhibitory RNA encoding region (protein downregulation). As integration was random, there was no way of predicting all 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 modified founder animal had the gene inserted into a different location in the genome.



**Fig. 2. Broiler industry structure and global estimate of bird numbers alongside estimates of timeline and genetic expressions derived from a single pedigree female broiler chicken.** Reproduced with permission from Van Eenennaam et al. (2014).

Genome editing offers an opportunity for the targeted modification of existing genes and regulatory elements, without necessarily introducing foreign DNA.

**Genome editing systems**

Genome editing involves using a nuclease targeted to a specific DNA sequence to introduce a double-stranded break (DSB). 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 are reformed. This method is error-prone and often results in small indels at the 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 can be used to introduce a range of genome edits, from point mutations to whole-gene insertions. The path to repair largely depends on the stage in the cell cycle and the proteins that associate with the broken DNA ends.

There are three major genome editing systems that have been used in livestock (Table 1), each with their pros and cons. Zinc finger nucleases (ZFNs) were the first tool to allow efficient targeting of DSB to specific sites in the genome (Bibikova et al., 2002). The DNA-binding domain of ZFNs consists of an assembly of zinc finger domains, each of which recognizes three DNA base pairs. At

**Table 1. Comparison of features of three genome editing systems (adapted from Ho et al., 2018)**

Site-directed nuclease	ZFN	TALEN	CRISPR/Cas9
Type of recognition	Protein–DNA	Protein–DNA	RNA–DNA
Nuclease	FokI	FokI	Cas9
Recognition site	Typically, 18–36 bp (usually a multiple of 3) per ZFN pair	Typically, 28–40 bp per TALEN pair	22 bp followed immediately by 5′-NGG-3′ protospacer adjacent motif (PAM)
Targeting specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated, but it is easier to predict possible sites using Watson–Crick base-pairing rules
Targeting constraints	Difficult to target non-guanine rich regions	Requires offset binding proteins with defined spacing	Targeted sequence must immediately precede a PAM site
Ease of design/engineering	Difficult: may require substantial protein engineering	Moderate: requires complex molecular cloning, but TALEN design has been streamlined by the availability of modules of repeat combinations that reduce the amount of cloning	Easy: simple to design and guide sequences are easily engineered using standard cloning procedures or oligo synthesis
Multiplexing ability	No	No	Yes
Advantages	Small protein size (<1 kb)	High specificity	Enables multiplexing (targeting multiple genes)
Limitations	Length of target sequence confined to multiples of 3; difficult cloning methods	Large protein size	Limited to PAM sequences; Cas9 nuclease is large (~4.2 kb)

the C-terminus end of the DNA binding domain is a FokI cleavage domain, which dimerizes to form a non-specific nuclease. When two ZFNs bind to adjacent target sites on opposite DNA strands, the nuclease subunits combine to form a functional nuclease and create a targeted DSB (Fig. 3A). The specificities of individual zinc fingers can depend on the context of surrounding zinc fingers and target DNA, and not all possible DNA triplets have corresponding zinc finger domains available. Recent improvements in ZFN targeting include the ability to skip a nucleotide between the zinc finger units so that if a unit is not available for the adjacent 3 bp, then 1 bp can be skipped (Paschon et al., 2019). This greatly increases the odds that an available zinc finger will match the target sequence. Another modification involves the placement of the FokI cleavage domain. Previously, ZFNs had the limitation that one needed to bind to the DNA strand in each direction owing to the confines of the placement of the nuclease subunit at the C' end of the ZFN. The capability to add the nuclease to the N' end allows both ZFNs to bind the DNA in the same direction, increasing the possibility of finding a suitable ZFN-binding site on the target sequence (Fig. 3B).

Transcription activator-like effectors (TALEs) are naturally occurring proteins from *Xanthomonas*, a genus of Proteobacteria and a plant pathogen. TALEs contain a DNA-binding region consisting of a series of 33- to 35-aa-long units that each recognize a specific base pair as determined by two variable residues near the middle of each unit (Kim and Kini, 2017). The FokI DNA-cleavage domain was fused to TALEs to produce TALE nucleases (TALENs) which, like with ZFNs, are used in pairs to recognize and cleave target DNA (Fig. 3C).

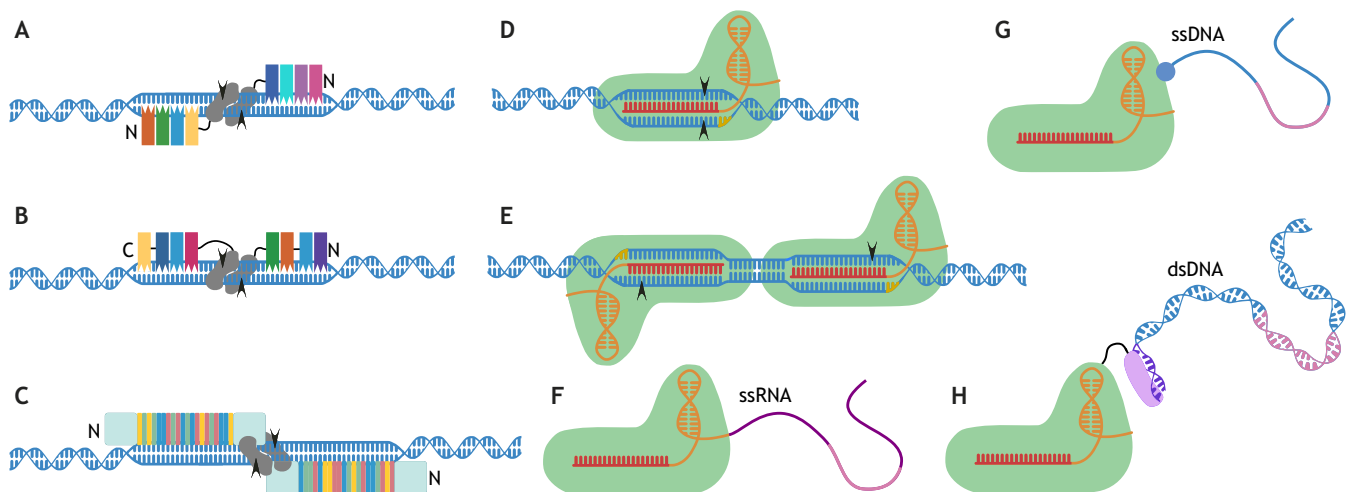
Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas is a system of adaptive immunity found in many bacterial and archaeal species, where it functions to protect against invading viruses (Jinek et al., 2012). This system uses small non-coding RNAs to target a Cas nuclease to specific DNA sequences. The most widely used system at present is CRISPR/Cas9, which is based on the system of *Streptococcus* and uses a short guide RNA (sgRNA) complexed with Cas9 nuclease. Cleavage of the target DNA by Cas9 is directed by the first 20 nucleotides at the 5' end of the sgRNA that anneal with the corresponding genomic DNA

target, which must also contain a protospacer adjacent motif (e.g. PAM: 5'-NGG-3') immediately following the sgRNA homology region (Fig. 3D). Cas9 cleaves the target DNA three nucleotides upstream of PAM, resulting in a DSB (Ran et al., 2013).

Both ZFNs and TALENs require two unique proteins to be designed and expressed for each DNA target, which can be cost-prohibitive for applications involving multiple targets. In the CRISPR/Cas9 system, one protein can be co-expressed with multiple sgRNAs, each guiding the Cas9 protein to a different target site. This feature of CRISPR-Cas makes it well suited for targeting multiple sites. One study administered CRISPR/Cas9 through microinjection of zygotes to simultaneously knock out three distinct target genes (Zhang et al., 2018a). All three targets were successfully knocked out in 14 out of 15 mice born, and the other mouse had two of the genes knocked out and was mosaic for the other. However, co-targeting multiple sites on the same chromosome can result in DNA excisions that have been reported at greater than 1 Mb in length (Kraft et al., 2015).

CRISPR-Cas recognizes a single 20 nucleotide site plus the PAM sequence; however, it can also recognize and cleave sequences with only 17 or 18 matching nucleotides and PAM (Fu et al., 2014). In addition to the target sites, CRISPR-Cas has been observed to cleave off-target sites (Fu et al., 2013). This can be limited by careful sgRNA design (Akcakeya et al., 2018), but this is not always possible when the target region is small, especially with the CRISPR/Cas9 target restraints. Cas9 can be modified into a nickase so that it only cuts one DNA strand (Fig. 3E). The creation of staggered nicks at the target site by two independent CRISPR/Cas9 complexes results in a DSB. This greatly improves the specificity of CRISPR/Cas9 as it requires two independent complexes binding in close proximity (Ren et al., 2014). The requirement for a PAM sequence adjacent to the CRISPR target sequence is a limiting factor for using this system to target specific sites for precise nucleotide changes. Cas nucleases are being produced/identified with alternate PAM sites (Leenay and Beisel, 2017), which greatly extends the targeting potential of this system.

The template for HDR is generally provided separate from the nuclease and in the form of single-stranded (ss) or double-stranded



**Fig. 3. Examples of genome editors and their modifications.** (A–E) Schematic representations of DNA-bound (A) ZFNs with dimerized C' FokI (gray) domains; (B) ZFNs with base skipping and N' FokI capability (dimerized N' and C' FokI domains shown); (C) TALENs with dimerized FokI domains; (D) sgRNA/Cas9 (sgRNA, orange/red; sgRNA target sequence, red; Cas9, green; PAM, yellow); and (E) sgRNAs/Cas9 nickases. Black arrowheads indicate DNA cleavage sites. (F) Cas9 with chimeric sgRNA: single-stranded (ss) RNA repair template. (G) Cas9 with covalently bound ssDNA repair template. (H) Cas9 with DNA binding domain (purple) and bound double-stranded (ds) DNA repair template.



(ds) DNA. Adding an RNA HDR template at the 3' end of a sgRNA improves HDR in rice (Butt et al., 2017). It was suggested that the physical presence of the HDR template at the time and location of the nuclease event facilitates a higher frequency of template-guided repair (Fig. 3F). These experiments in rice were based on previous observations of DSB-induced small RNAs (diRNAs) being generated and localizing around DSBs in plants and animals (Yamanaka and Siomi, 2014). This suggests that RNA repair template–sgRNA chimeras may also improve HDR frequency in animals and a similar approach should be tested in animal cell lines. If the physical presence of the template in close proximity to the DSB at the time of its creation improves HDR, then perhaps methods to attach a DNA repair template to Cas9 may increase HDR frequency. Indeed, it has been reported that covalently linking an ssDNA repair template to Cas9 (Fig. 3G) increased HDR frequency by up to 30 times in multiple cell lines (Aird et al., 2018; Savic et al., 2018). An alternative attachment method for dsDNA could include adding a site-specific DNA-binding domain to Cas9 and including its binding site at the end of the dsDNA repair template (Fig. 3H).

### Nuclease and repair template delivery

In animal breeding programs, germline transmission is the ultimate goal because edits must be passed on to the next generation for genetic improvement. To achieve this goal in mammalian livestock species, genome editing can be performed either in somatic cells, and the edited cell line subsequently cloned by somatic cell nuclear transfer (SCNT), or in developing zygotes. Targeted gene knock-outs, and with lesser success targeted gene insertions, in mammalian livestock have typically been achieved in cell culture, followed by SCNT (Tan et al., 2016). Making the modification in cell lines allows for more efficient identification of genetic changes, and multiple colonies of the same line can be screened so there is more chance of obtaining cells with all of the correct modifications. Zygote delivery has the advantage of producing a diversity of foundation animals as each zygote will produce a genetically distinct animal, as opposed to animals derived from a clonal cell line. The use of SCNT to derive embryos from edited cells greatly reduces the efficiency of the method owing to the low rate of healthy animals born, particularly in cattle (Akagi et al., 2014; Keefer, 2015).

Genome editing components can be delivered into target cells via physical methods or through the employment of vectors (viral or non-viral). Genome edited mammalian livestock have predominantly been produced using physical methods, which include electroporation of somatic cells (typically fetal fibroblasts) and microinjection, or more recently electroporation, of zygotes. Genome editing components can be delivered as mature proteins (or RNA/protein complexes in the case of CRISPR/Cas), or encoded in RNA or DNA. The use of proteins (and to a lesser extent RNA) allows for more precise control of the activity of the editor, which can be important for limiting possible off-target DNA cleavage events. The requirement for a repair template for HDR adds another element (DNA) to the delivery mixture, increasing its complexity. Each repair template requires homology arms that flank the desired edit, and these are generally 50–1000 bp for each of the two flanking arms. Free linear DNA can be toxic to zygotes so the delivered concentration should be limited (Brinster et al., 1985).

Electroporation uses high-voltage pulses to induce transient pore formation in the cell membrane. These pores allow the flow of genome editing components from the suspension liquid into the cell cytoplasm. Nuclear-targeting peptides can be used to aid the transport of genome editing proteins across the nuclear membrane

into the nucleus. When delivered into the cytoplasm, transcription of genome editing components from plasmid DNA occurs following nuclear membrane disassembly during mitosis/meiosis in dividing cells or following transport into the nucleus through pores (Bai et al., 2017). Nucleofection is a form of electroporation that also creates pores in the nuclear membrane, allowing more rapid delivery of genome editing components or plasmids into the nucleus (Gresch et al., 2004).

Although electroporation has traditionally been used to edit cultured cell lines, it has recently been shown to also be effective on zygotes (Chen et al., 2016; Qin et al., 2015; Tröder et al., 2018). Cytoplasmic injection (CPI) has been the go-to technique for delivering genome editing components directly into livestock zygotes. Electroporation has only recently begun to show its potential for this purpose with effective introduction of indel mutations (through NHEJ) into zygotes of pigs and cattle (Hirata et al., 2019; Miao et al., 2019; Tanihara et al., 2018, 2016). Unlike CPI, where a needle is used to deliver genome editing reagents into zygotes individually, electroporation allows the manipulation of zygotes en masse, reducing the time and expertise required. Delivery of genome editing components into the zygote avoids the shortcomings of SCNT, but has the drawback of mosaicism when the editing event occurs at a multinuclear/multicellular stage, and unknown editing success. Screening of zygotes can be done by genotyping biopsies at the blastocyst stage prior to embryo transfer into surrogate dams, but this method can decrease the viability of transplanted embryos (Cenariu et al., 2012). For mosaic animals, subsequent generations need to be produced to obtain homozygous, non-mosaic animals. Introducing genome editing reagents directly into zygotes has been a successful approach to achieve targeted knock-outs in embryos, although mosaicism can reduce germline transmission, but efficient targeted gene insertions have proven difficult. The HDR pathway is primarily restricted to actively dividing cells (S/G2-phase), and only becomes highly active towards the end of the first round of DNA replication in the one-cell zygote (Hustedt and Durocher, 2016).

Electroporation of somatic cells is typically used when HDR is required, and only a handful of examples of HDR by CPI of double-stranded DNA (dsDNA) into zygotes of livestock species have been published (Park et al., 2017b; Peng et al., 2015). However, single-strand oligo DNAs (ssODN) have been used successfully by several groups to make DNA point mutations or small fragment insertions/deletions by HDR (Lillico et al., 2016; Niu et al., 2018; Wei et al., 2018; Zhou et al., 2016b). Another approach to adding entire genes into zygotes was successfully used to create sheep expressing two proteins involved in the synthesis of the antioxidant melatonin in their milk using a  $\beta$ -casein promoter (Ma et al., 2017). Linear dsDNA without homology arms was injected directly into the cytoplasm of zygotes along with Cas9 and an sgRNA targeting the myostatin locus. The dsDNAs were integrated by homology-independent DNA reparation into the targeted site of ~35% of live lambs with ~26% of live lambs carrying both genes.

Genome editing in avian species is complicated by the inaccessibility of the avian zygote (Sang, 2004). For this reason, most genome editing studies in chickens target primordial germ cells (PGCs). Chicken PGCs circulate the bloodstream of embryos at 48–60 h of incubation as they migrate to the genital ridge of the developing gonads (Ginsburg and Eyal-Giladi, 1986; Nakamura et al., 2007). Chicken PGCs can be readily propagated *in vitro* where genome editing components can be delivered, typically in the form of DNA by lipofection. Lipofection is a non-viral vector technique that involves electrostatically complexing lipids and DNA to form

lipoplexes that are taken up by cells. Edited PGCs can be screened and injected into the bloodstream of chemically or genetically germline-ablated (Smith et al., 2015) chicken embryos through a window in the egg. Alternatively, intravenous injection can be used to directly target circulating PGCs with transfection reagents (Tyack et al., 2013). The resulting birds can be bred to produce genome edited offspring.

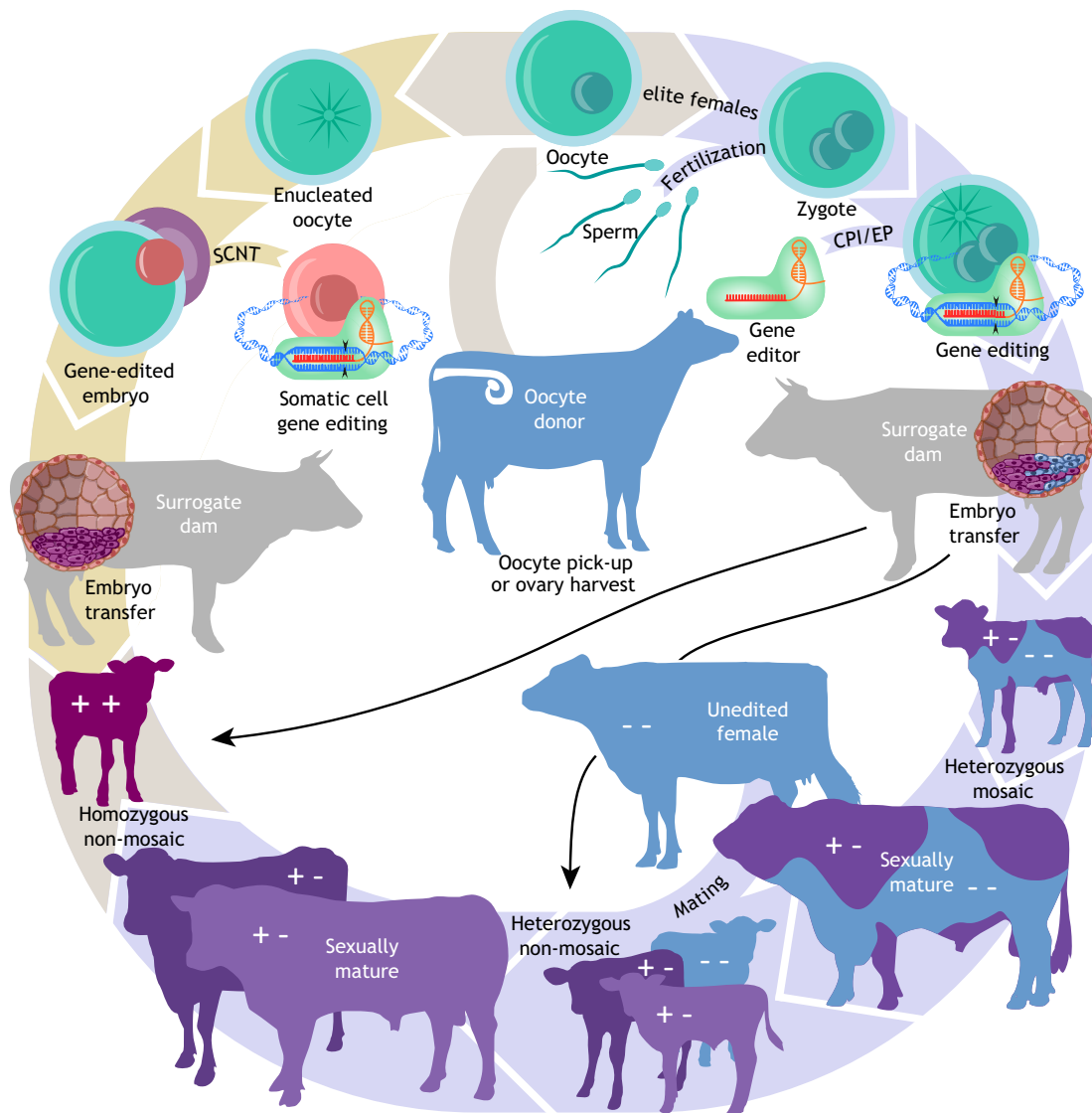
It should be emphasized that many of the processes involved in genome editing livestock are time consuming and, at present, inefficient. There are a number of steps and unpredictable biological variables including gamete collection and maturation, introduction of the editing reagents, cloning and transfer of embryos into synchronized surrogate dams, all of which have their own limitations and constraints (Fig. 4). Microinjection of embryos to produce mosaic offspring, and subsequently breeding to produce heterozygous edited offspring, is a prolonged and expensive undertaking in large food animals. 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.

**How can genome editing alter the components of the breeders' equation?**

**Intensity of selection**

Intensity of selection (*i*) is an important driver of genetic change: as shown in Fig. 2, one individual chicken in the breeding nucleus at the top of the pyramid breeding structure can give rise to millions of descendants in a well-designed breeding program. The widespread adoption of artificial insemination (AI) in dairy cattle breeding resulted in a steep change in the rate of genetic gain in the dairy industry, and as a result increasing the selection intensity of genetically elite sires (VandeHaar and St-Pierre, 2006). One bull, known as Pawnee Farm Arlinda Chief, produced 16,000 daughters, 500,000 granddaughters and more than 2 million great-



**Fig. 4. Steps for producing genome-edited livestock through somatic cell nuclear transfer (SCNT) or zygote editing.** Schematic showing the typical steps involved to produce homozygous, non-mosaic livestock by either SCNT cloning of genome-edited and screened somatic cells (yellow arrows) or cytoplasmic injection (CPI)/electroporation (EP) of zygotes (purple arrows) with genome editing components.

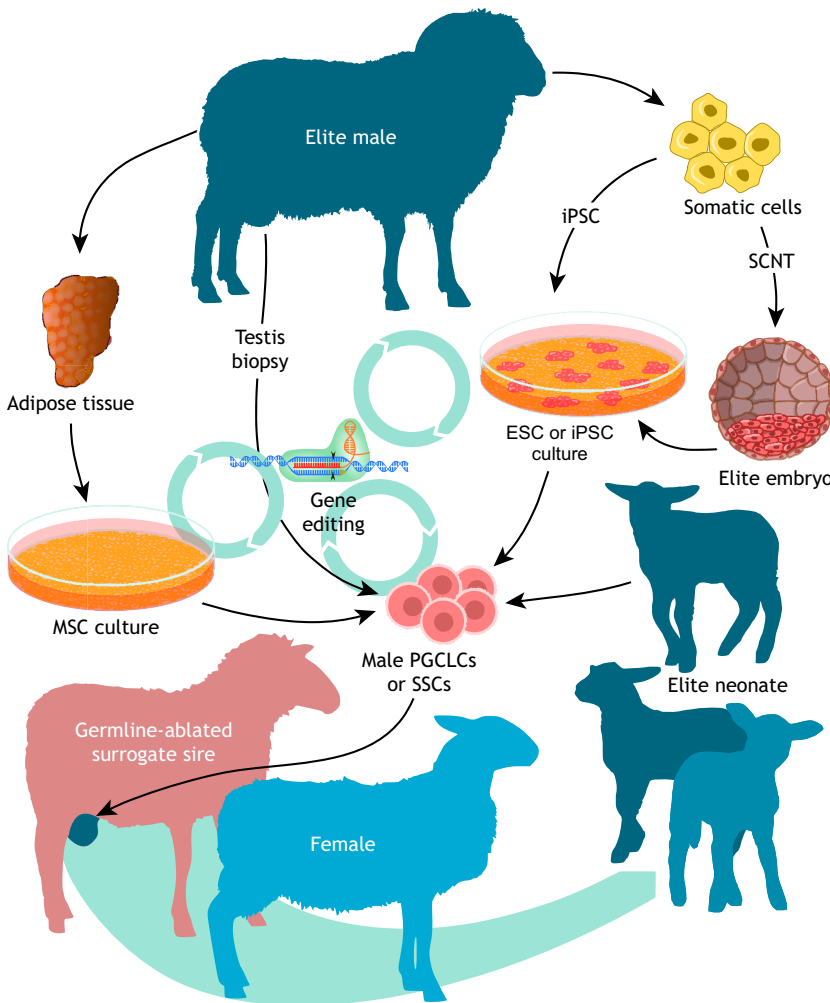
granddaughters. His sons were also popular sires. As a result, Chief's chromosomes account for almost 14% of the genome in the current Holstein population in the United States (Adams et al., 2016). The pig industry is another vertically integrated industry with a pyramid breeding structure that is well suited to the introduction of genome editing into selection programs.

Other livestock sectors are not as well suited to increasing the intensity of selection through AI. In extensive pastoral industries, such as beef cattle and sheep production, it is difficult to track and synchronize the estrous cycles of cycling females. For these reasons, implementation of AI as a breeding tool in beef cattle has been limited. Such livestock systems typically utilize a tiered breeding hierarchy to disseminate improved genetics. The top tier of this hierarchy is composed of an elite nucleus population of high-accuracy males screened by genomic selection and/or progeny testing. Their sons form the second tier (multipliers) and are used to spread the genetics of the elite nucleus either by directly siring production animals or by siring sires of production animals that form the third tier (commercial producers). Therefore, there is a lag in the genetic improvement between the elite nucleus and the commercial animals. One way to decrease this lag would be to reduce the number of tiers by making germline copies of elite animals.

This aim could be achieved through the use of surrogate sires (Gottardo et al., 2019), which involves replacing the germline of inferior males with the germline of genetically elite males by

injecting germ cells derived from the donor into the testes (Fig. 5). Spermatogonial stem cells (SSCs), which can be isolated from mature testes, represent one source of germline-competent cells with the potential to both self-renew and differentiate into daughter cells and, ultimately, spermatozoa. It was first reported in a pair of 1994 studies that SSCs transplanted from the testes of fertile donor mice into the testes of W mutant or busulfan-treated mice, which do not show spermatogenesis, are capable of producing functional spermatozoa that fertilize eggs and result in offspring (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Another study that implanted SSCs into the testes of W mutant mice also produced pups from donor cell-derived sperm (Ogawa et al., 2000). Allogeneic transplantations of SSCs into the testes of chemically germline-depleted or germline-replete recipients of several livestock species did not illicit immunologic reactions, and donor cells were shown to contribute to spermatogenesis (Herrid et al., 2006, 2019; Honaramooz et al., 2002, 2003). However, these studies all showed scattered distributions of implanted cells, likely owing to the tortuous paths and multiplicity of testicular tubules. Improved delivery methods are needed for homogeneous distribution of implanted cells.

As an alternative to chemical treatment, editing can be used to produce infertile mutants through inactivation of an essential fertility gene (e.g. NANOS2; Park et al., 2017a) that is required for germline development, creating a genetically germline-ablated surrogate sire. NANOS2 knockouts in both pigs and mice have no germline, but



**Fig. 5. Germline cloning by surrogate sire.** Schematic showing possible male germ cell sources for transfer into germline-ablated (e.g. NANOS2 knockout) surrogate sires. Tissue/cell donors are shown as elite male embryos (elite embryo), juveniles (elite neonate) and adults (elite male). Blue coloring represents different individuals of the same breed. The pink coloring represents individuals of a different breed. Green circles show potential for repeated editing and culturing. ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; MSC, mesenchymal stromal cell; PGCLC, primordial germ cell-like cell; SCNT, somatic cell nuclear transfer; SSC, spermatogonial stem cell.

the seminiferous tubules remain intact (Park et al., 2017a; Suzuki et al., 2007). The vacant developmental niche in the seminiferous tubules could then be filled by male germ cells from an elite male to produce fertile sperm. Genome editing to knock out germline development genes would mean cells could be implanted into younger animals and ensure that all offspring are made with sperm derived from donor cells. Surrogate lines could be maintained by careful selection of the knock-out target. For example, males with one intact *NANOS2* allele and *NANOS2* knockout females are fertile, thereby allowing for efficient generation of knockout males by breeding (Giassetti et al., 2019).

Other cell types could also be potential germline sources in a surrogate sire breeding system. Embryonic stem cells (ESCs) can be induced to become primordial germ cell-like cells (PGCLCs) and subsequently induced to form sperm (Hayashi et al., 2011). However, when injected into the testes of germline-ablated mice, ESC-derived PGCLCs can form teratomas (Hayashi et al., 2011), which would preclude their use for livestock breeding. Hayashi et al. (2011) used transgenic markers to select for a subpopulation of ESC-derived PGCLCs that did not form teratomas when implanted into the testes of recipient mice and produced sperm that were subsequently used to fertilize oocytes by intracytoplasmic sperm injection (ICSI). The same group later refined their culturing conditions to enhance proliferation of the subset of ESC-derived PGCLCs that does not form teratomas (Ohta et al., 2017). When PGCLCs grown under these conditions were implanted into recipient mice, they did not produce teratomas and went on to form fully functional sperm that produced offspring by both IVF and natural mating. However, they did still sort PGCLCs by expression of transgenic reporters. An alternative to selection by transgenic reporters, such as cell-surface markers, would need to be established for ESC-derived PGCLCs to have utility for livestock breeding.

Mesenchymal stromal cells (MSCs) can be isolated from a variety of adult and neonatal tissues and can transdifferentiate into multiple cell lineages including PGCLCs. MSCs have been shown to express multiple germ cell markers following treatment with retinoic acid and/or various growth factors (Dissanayake et al., 2018; Ghasemzadeh-Hasankolaei et al., 2014; Hua et al., 2009; Luo et al., 2019; Shirzeyli et al., 2017; Wei et al., 2016; Yan et al., 2015). Haploid spermatid-like cells have been observed from MSC-derived PGCLC cultures and transplants (Ghasemzadeh-Hasankolaei et al., 2016a,b; Shlush et al., 2017; Zhang et al., 2014). MSCs do not form teratomas when injected into donor testes and have even been shown to help reestablish the testicular niche following chemical damage (Cakici et al., 2013; Maghen et al., 2017; Vahdati et al., 2017). Male PGCLCs have also been produced from induced pluripotent stem cells (iPSC), which can also be derived from adult animals (Hayashi et al., 2011; Sasaki et al., 2015; Wang et al., 2016a). Further experiments are required to definitively demonstrate that these PGCLCs from different cell types can fulfill the functional definition of a germ cell, i.e. the capacity to undergo meiosis and produce a gamete.

The use of adult SSCs, MSC-derived PGCLCs or iPSCs could allow elite donor animals to be selected based on phenotypic observations and measurements, which may be especially important for species and breeds where there is no genomic way to estimate their genetic merit at a young age. One obstacle that will need to be overcome for the surrogate sires method to be of utility in livestock production is the low yield of sperm following implantation of donor cells.

The cell culture stage of germ cell replacement methods represents an ideal opportunity to introduce genome edits. If the current technical hurdles can be overcome, surrogate sire

technology could change the fundamental design of animal breeding programs, with separate population improvement and product development components (Gottardo et al., 2019).

#### Accuracy of selection

Genome editing could be used for hypothesis testing in functional genomics studies, and to help identify causative alleles by precisely editing them in or out, and testing their function in other breeds or species. Such studies could be used to discern whether alleles associated with a better quantitative trait outcome improve or impede that outcome when precisely edited in or out and tested in an otherwise homogeneous contemporary group. This information could then be used to determine the best loci to edit among the millions of genetic variants that exist when comparing individuals.

A simulation study modeled that if genome editing were used to edit 20 loci per sire of the 10,000 additive loci contributing genetic variation to a polygenic trait, this could potentially double the rate of genetic gain relative to GS alone (Jenko et al., 2015). Pipelines to discover causal variants underlying genetic variation will perhaps be the greatest challenge to implementing editing for polygenic traits (Hickey et al., 2016). Genome editing could also be used to fix favorable alleles at beneficial loci or eliminate detrimental dominant alleles by duplicating favorable recessive alleles in an F1 cross. Site-directed endonucleases could be used to create a DSB in the undesirable allele with the other allele providing the template to repair the break. This could allow new breeds to be created with desired homozygosity in key performance alleles, but enhanced heterozygosity at other loci.

#### Genetic diversity

Genome editing could be used to move genetic elements between breeds and species or alter existing genetic elements to create new ones, effectively expanding the available genetic diversity ( $\sigma_A$ ). Table 2 summarizes the peer-reviewed literature on the introduction of desired edits in food animal species that have resulted in live offspring, the transfection method used, the target gene and function, and the nuclease that was involved in the editing. Genome editing technologies have been used to address traits ranging from production efficiency to animal welfare, environmental sustainability and disease resistance. The power of editing is that it can introduce useful alleles in the absence of linkage drag of other unwanted traits. In the absence of editing, livestock producers may be resistant to the introduction of a useful new allele that is associated with having to use sires of inferior genetic merit, or sires from another breed. For example, the polled allele, which results in hornlessness (Medugorac et al., 2012), is associated with dairy sires of low genetic merit, and bulls from several beef breeds. Using genome editing, the allele has been introduced into dairy genetics in a proof of concept experiment (Carlson et al., 2016). If this allele could be edited into the genome of a number of elite dairy sires, then artificial insemination could be used to rapidly disseminate that dominant polled allele through the dairy cattle population (Mueller et al., 2019), and eliminate the need for physical horn removal, which is an animal welfare concern.

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. Some individual genes can have large effects on



**Table 2. Genome editing experiments for agricultural applications in food animal species that have resulted in live edited offspring**

Animal	Cell type	Transfection method	Gene and function	Mutation	Editor	Reference
Cattle	Bovine fetal fibroblasts (BFF), SCNT	Nucleofection	$\beta$ -lactoglobulin ( <i>PAEP</i> ): whey protein – allergen	NHEJ – knockout (KO)	ZFN	Yu et al., 2011
	BFF, SCNT	Electroporation	Lysostaphin gene (antimicrobial) inserted into $\beta$ -casein ( <i>CSN2</i> ) locus	HDR – knock-in	ZFN/ZFNickase	Liu et al., 2013
	BFF, SCNT	Electroporation	Human lysozyme gene (antimicrobial) into <i>CSN2</i> locus	HDR – knock-in	ZFN	Liu et al., 2014
	BFF, SCNT	Nucleofection	Myostatin ( <i>MSTN</i> ): negative regulator of muscle growth	NHEJ – KO	ZFN	Luo et al., 2014
Sheep	Zygote	Cytoplasmic injection (CPI)	<i>MSTN</i>	NHEJ – KO	TALEN	Proudfoot et al., 2015
	BFF, SCNT	Electroporation	Mouse <i>Sp110</i> gene (controls MTB infections) inserted into intergenic region between <i>SFTPA1</i> and <i>MAT1A</i>	HDR – knock-in	TALENNickase	Wu et al., 2015
	BFF, SCNT	Electroporation	Replace <i>horned</i> allele with <i>polled</i> allele	HDR – knock-in	TALEN	Tan et al., 2013; Carlson et al., 2016
	BFF, SCNT	Electroporation	<i>NRAMP1</i> gene (controls MTB infections) inserted into intergenic region between <i>FSCN1</i> and <i>ACTB</i>	HDR – knock-in	CRISPR/Cas9	Gao et al., 2017
Goats	BFF, SCNT	Electroporation	Repaired mutation in isoleucyl-tRNA synthetase ( <i>IARS</i> ) gene that causes IARS syndrome	HDR – single base substitution	CRISPR/Cas9 nickase	Ikeda et al., 2017
	Ear-derived fibroblast, SCNT	Electroporation	Repaired IARS syndrome mutation in <i>IARS</i> gene	HDR – single base substitution	CRISPR/Cas9	Ishino et al., 2018
	Bovine fetal skin fibroblasts, SCNT	Electroporation	<i>Suffolobus soffataricus</i> $\beta$ -glycosidase (digests lactose) into <i>CSN2</i> locus	Microhomologous-mediated end-joining (MMEJ) – knock-in	TALEN	Su et al., 2018b
	Zygote	CPI	<i>PAEP</i>	HDR – KO ssODN	TALEN	Wei et al., 2018
	Zygote	CPI	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Han et al., 2014
	Zygote	CPI	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Crispo et al., 2015
	Zygote	CPI	<i>MSTN</i>	NHEJ – KO	TALEN	Proudfoot et al., 2015
	Ovine fetal fibroblasts (OFF), SCNT	Electroporation	<i>MSTN</i>	NHEJ – KO	TALEN	Li et al., 2016
	Zygote	CPI	<i>MSTN</i> , agouti signaling protein ( <i>ASIP</i> ) and $\beta$ -carotene oxygenase 2 ( <i>BCO2</i> )	NHEJ – KO	CRISPR/Cas9	Wang et al., 2016b
	Zygote	Pronuclear injection (PNI)	Fibroblast growth factor 5 ( <i>FGF5</i> ): negative regulator of wool length	NHEJ – KO	CRISPR/Cas9	Hu et al., 2017
Goats	Zygote	CPI	<i>FGF5</i>	NHEJ – KO	CRISPR/Cas9	Li et al., 2017
	Zygote	CPI	<i>AAMAT</i> and <i>ASMT</i> (involved in synthesis of the antioxidant, melatonin) with $\beta$ -casein promoter into <i>MSTN</i> locus	homology-independent DNA repairation – knock-in & KO	CRISPR/Cas9 dsDNA	Ma et al., 2017
	Zygote	CPI	<i>ASIP</i> : coat color	NHEJ – KO	CRISPR/Cas9	Zhang et al., 2017
	Sheep ear fibroblasts	Electroporation	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Zhang et al., 2019
	Goat fetal fibroblasts	Nucleofection	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Ni et al., 2014
	(GFF), SCNT	Electroporation	Human lactoferrin ( <i>LTF</i> ): involved in iron absorption – into <i>PAEP</i> locus	HDR – knock-in & KO	TALEN	Cui et al., 2015
	GFF, SCNT	CPI	<i>MSTN</i> and <i>FGF5</i>	NHEJ – KO	CRISPR/Cas9	Wang et al., 2015b
	Zygote	CPI	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Guo et al., 2016
	GFF, SCNT	Electroporation	Human <i>LTF</i> into <i>PAEP</i> gene	HDR – knock-in & KO	TALEN	Song et al., 2016
	GFF, SCNT	Electroporation	<i>MSTN</i>	NHEJ – KO	TALEN	Yu et al., 2016
Goats	Goat ear fibroblasts (GFF) & GFF, SCNT	Electroporation	Human $\alpha$ -lactalbumin ( <i>LALBA</i> ): main whey protein in human milk – into <i>PAEP</i> locus	HDR – knock-in & KO	TALEN	Zhu et al., 2016
	Zygote	CPI	<i>PAEP</i>	NHEJ – KO	CRISPR/Cas9	Zhou et al., 2017
	Zygote	CPI	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	He et al., 2018
						Continued

**Table 2. Continued**

Animal	Cell type	Transfection method	Gene and function	Mutation	Editor	Reference
	Zygote	CPI	Introduced point mutation in growth differentiation factor 9 ( <i>GDF9</i> ) gene: increases litter size	HDR – single base substitution	CRISPR/Cas9	Niu et al., 2018
	GFF, SCNT	Electroporation	<i>C. elegans fat-1</i> gene (convert <i>n-6</i> PUFA into <i>n-3</i> PUFA) into <i>MSTN</i> locus	HDR – knock-in & KO	CRISPR/Cas9	Zhang et al., 2018b
Pigs	Zygote	CPI	<i>RELA</i> (relates to severity of African Swine Fever Virus infection)	NHEJ – KO	ZFN	Lillico et al., 2013
	Zygote	CPI	<i>RELA</i>	NHEJ – KO	TALEN	Lillico et al., 2013
	Porcine fetal fibroblasts (PFF), SCNT	Electroporation	Exon 7 of <i>CD163</i> (required for highly pathogenic-porcine reproductive and respiratory syndrome virus infection) replaced using a porcine DNA template modified to encode the same amino acid sequence as human <i>CD163L1</i> from exon 11	HDR – knock-in & KO	CRISPR/Cas9	Whitworth et al., 2014
	PFF, SCNT	Nucleofection	<i>MSTN</i>	NHEJ – KO	ZFN	Qian et al., 2015
	PFF, SCNT	Electroporation	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Wang et al., 2015a
	PFF, SCNT	Electroporation	<i>MSTN</i>	HDR – knock-in of EGFP as selectable marker, EGFP excised by Cre recombinase: KO	CRISPR/Cas9	Bi et al., 2016
	Zygote	CPI	African Swine Fever Virus-susceptible region of porcine <i>RELA</i> replaced with warthog ortholog (associated with resilience to African Swine Fever)	HDR – knock-in & KO ssODN	ZFN	Lillico et al., 2016
	Porcine dermal fibroblasts, SCNT	Electroporation	<i>MSTN</i>	NHEJ – KO	TALEN	Rao et al., 2016
	Zygote	Electroporation	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Tanihara et al., 2016
	Zygote	CPI	Exon 7 of <i>CD163</i>	NHEJ – KO	CRISPR/Cas9	Burkard et al., 2017
	Zygote	CPI	<i>NANOS2</i> : required for prospermatogonia survival	NHEJ – KO	CRISPR/Cas9	Park et al., 2017a
	PFF, SCNT	Electroporation	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Wang et al., 2017
	PFF, SCNT	Nucleofection	Mouse uncoupling protein 1 ( <i>Ucp1</i> ) (plays a key role in brown adipose tissue-mediated adaptive nonshivering thermogenesis) with adiponectin promoter into porcine endogenous <i>UCP1</i> (non-functional) locus	HR-independent integration	CRISPR/Cas9	Zheng et al., 2017
	PFF, SCNT	Electroporation	<i>fat-1</i> gene into porcine <i>ROSA26</i> locus	HDR – knock-in	CRISPR/Cas9	Li et al., 2018
	Zygote	CPI	<i>MSTN</i>	NHEJ – KO	CRISPR/Cas9	Su et al., 2018a
	Zygote	CPI	Insulin-like growth factor 2 ( <i>IGF2</i> ): promotes growth	NHEJ – KO repressor ZBED6 binding site on intron 3	CRISPR/Cas9 nickase	Xiang et al., 2018
	PFF, SCNT	Electroporation	Anti-classical swine fever virus small hairpin RNAs (shRNAs) into porcine <i>ROSA26</i> locus	HDR – knock-in	CRISPR/Cas9	Xie et al., 2018
	PFF, SCNT	Nucleofection	Exon 7 of <i>CD163</i>	NHEJ – KO	CRISPR/Cas9	Yang et al., 2018
	PFF, SCNT	Nucleofection	<i>FBXO40</i> (null mutation results in muscle hypertrophy)	NHEJ – KO	CRISPR/Cas9	Zou et al., 2018
	PFF, SCNT	Nucleofection	Exon 7 of <i>CD163</i> replaced with the corresponding exon of human <i>CD163L1</i>	HDR – knock-in and KO	CRISPR/Cas9	Chen et al., 2019
	PFF, SCNT	Electroporation	<i>IGF2</i>	NHEJ – KO repressor ZBED6 binding site on intron 3	CRISPR/Cas9	Liu et al., 2019
	Zygote	CPI	Amino peptidase N ( <i>ANPEP</i> ): receptor for transmissible gastroenteritis virus and porcine epidemic diarrhoea virus	NHEJ – KO	CRISPR/Cas9	Whitworth et al., 2019
Chickens	Primordial germ cells (PGC), transplantation	Lipofection	Ovalbumin ( <i>OVAL</i> ) and ovomucoid ( <i>SPINK7</i> ): egg white proteins – allergens	NHEJ – KO	CRISPR/Cas9	Oishi et al., 2016
	PGC, transplantation	Electroporation	<i>DDX4</i> : maternal determinant for formation of the germ cell lineage	HDR – KO	TALEN	Taylor et al., 2017

Continued

Table 2. Continued

Animal	Cell type	Transfection method	Gene and function	Mutation	Editor	Reference
	PGC, transplantation	Lipofection	Green fluorescent protein (GFP) with cytomegalovirus promoter into intergenic region of the Z chromosome (for screening male eggs)	NHEJ – knock-in	CRISPR/Cas9	Lee et al., 2019
Carp	PGC, transplantation	Lipofection	G0/G1 switch 2 gene ( <i>G0S2</i> ): positive regulator of fat deposition	NHEJ – KO	CRISPR/Cas9	Park et al., 2019
Sturgeon	Zygote	Yolk injection (YI)	Myostatin 1a ( <i>mstn</i> )	NHEJ – KO	TALEN	Zhong et al., 2016
	Zygote	YI	Dead end protein homolog 1 ( <i>dnd1</i> ): essential for migration of primordial germ cells	NHEJ – KO	CRISPR/Cas9	Baloch et al., 2019
Catfish	Zygote	YI	<i>mstna</i> : one of the duplicated myostatin genes	NHEJ – KO	ZFN	Dong et al., 2011
	Zygote	YI	<i>mstnb</i> : one of the duplicated myostatin genes	NHEJ – KO	TALEN	Dong et al., 2014
	Sperm then zygote	Double electroporation	Luteinizing hormone beta subunit ( <i>lhb</i> ) gene: central role in gametogenesis	NHEJ – KO	ZFN	Qin et al., 2016
Sole	Zygote	YI	<i>mstn</i>	NHEJ – KO	CRISPR/Cas9	Khalil et al., 2017
	Zygote	YI	<i>dmdrt1</i> : putative male determination gene (females have faster growth rates)	NHEJ – KO	TALEN	Cui et al., 2017
Salmon	Zygote	YI	<i>dnd1</i> : essential for primordial germ cell formation; and <i>slc45a2</i> : pigmentation gene	NHEJ – KO	CRISPR/Cas9	Wargelius et al., 2016

MTB, *Mycobacterium tuberculosis*.

polygenic traits; for example, mutations in the myostatin gene that result in ‘double muscle’ phenotypes greatly contribute to the polygenic trait of carcass yield. Other alleles with more modest effects on polygenic traits could still be good targets for genome editing, particularly with the inevitable improvements in genome editing that will meet the coming decades.

**Generation interval**

New advances in *in vitro* germ cell and gamete development from mouse ESCs have led to recent interest in the potential for *in vitro* breeding in livestock (Goszczynski et al., 2018; Hou et al., 2018). The advantage of this proposed method would be that it could effectively remove the wait required for animals to reach sexual maturity prior to meiosis and conception. This has the potential to dramatically decrease the generation interval component of the breeders’ equation (Eqn 1), and if both *in vitro* gametogenesis and fertilization can be successfully accomplished in a Petri dish, offers the possibility of maintaining an entire breeding population of large animals in a laboratory (Fig. 6).

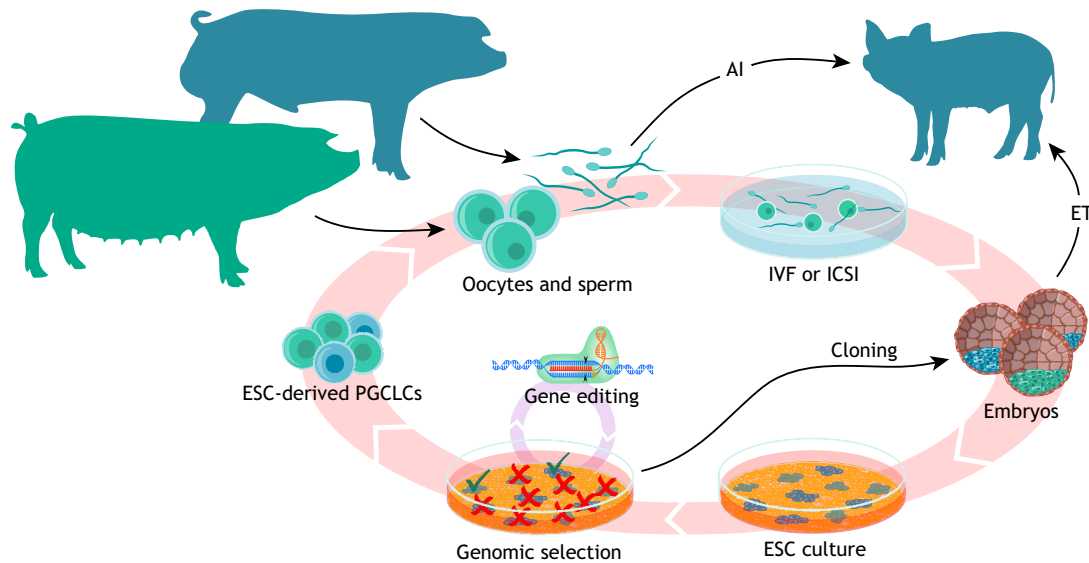
Research in mice has shown that female PGCLCs can be successfully produced *in vitro* from mouse ESCs before being further induced to become fully potent mature oocytes (Hikabe et al., 2016). Sperm have also been produced *in vitro* from mouse ESC-derived PGCLCs, but require ICSI to fertilize oocytes (Hayashi et al., 2011; Zhou et al., 2016a). The ability to produce gametes from stem cells could close the hypothetical *in vitro* reproduction loop: ESCs cultured from embryos, PGCLCs produced from ESCs, sperm and oocytes produced from germ cells and embryos produced from sperm and oocytes.

One current limitation for applying this method in livestock species is that the culture conditions to both successfully produce stable ESC cultures and derive PGCLCs and gametes have not been developed. It was recently demonstrated that stable ESC cultures can be efficiently produced from cattle blastocysts (Bogliotti et al., 2018). Advances have also been made in culturing ESCs from pigs and sheep (Brevini et al., 2010; Dattena et al., 2006; Kim et al., 2010), although to date, cattle is the only livestock species with established and stable ESC cell lines exhibiting all of the features of true ESCs.

*In vitro* breeding could potentially reduce the generation time for cattle 10-fold from approximately 2.5 to 0.25 years (Goszczynski et al., 2018). The potential increase in the rate of genetic gain is not limited to the consequences of a reduced generation time; *in vitro* breeding could also allow thousands of embryos to be produced from a single mating, thereby increasing the intensity of selection (*i*) and the chance of obtaining elite individuals from each parental pairing. This could facilitate the production of high scoring embryos from many different sire and dam combinations and help maintain genetic diversity ( $\sigma_A$ ) between generations. It could also make dams as genetically potent as sires, thereby permitting an increase in the selection intensity on the female side.

*In vitro* breeding could accelerate the rate of accumulation of alleles that contribute to polygenic traits, and it would be an ideal tool to couple with genome editing to accelerate the rate of genetic improvement programs. It would also enable a controlled environment to introduce new alleles to a breed, such as polled, without the uncertainties and inefficiencies associated with cloning and/or embryo editing.

*In vitro* breeding would rely heavily on GS, but genomic predictions have been shown to become less accurate over generations as the linkage disequilibrium between the tagging variants and causative alleles can break down (Pszczola and Calus,



**Fig. 6. *In vitro* breeding.** Schematic representation of the concept of *in vitro* breeding using gametes from an elite sow and boar. Pink circle shows potential for repeated editing and culturing. Green ticks represent ESC colonies that are selected via genomic selection, red crosses represent colonies that are not selected. AI, artificial insemination; ESC, embryonic stem cell; ET, embryo transfer; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; PGCLC, primordial germ cell-like cell.

2016). Directly genotyping the causative variants would reduce the rate of this decay over time. Without the selective pressure of having to form a fertile animal, these cell lines could lose their functionality for anything other than *in vitro* cycling. Therefore, periodic evaluations of lines derived by *in vitro* breeding by implanting embryos into surrogate females would likely be required to ensure viability, and to provide phenotypes to keep genomic prediction equations current. However, by the time the animal can be evaluated, several more generations of *in vitro* breeding could have taken place, so there would be a significant lag introduced by live animal screening.

Increasing our knowledge of causative alleles would help empower the potential of *in vitro* breeding. If all known production traits are selected for during *in vitro* breeding, several unrelated lines could be developed simultaneously to produce divergent grandparent lines that once crossed produce commercial animals with maximum heterosis. Theoretically, these crosses could be homozygous for the important production alleles, but maintain diversity in other genes, thereby accelerating the rate of gain while maintaining the genetic diversity required for long-term genetic improvement.

### Regulatory considerations

There has been an enthusiastic buzz around the prospect of genome editing in livestock breeding programs to accelerate the introgression of useful alleles. However, genome editing has not yet put a single animal product on the table for consumption by the general public. Moreover, traditional genetic engineering, which has been around for over 30 years, has only managed a single approval, the fast-growing AquAdvantage salmon. This has been due to both technical and regulatory obstacles (Van Eenennaam and Muir, 2011). As the technical obstacles of genetic modification are slowly being overcome, the regulatory aspect seems to be becoming more complicated (Van Eenennaam, 2018).

Argentina was the first country to publish its proposed regulatory approach for genome editing and other new breeding techniques (Whelan and Lema, 2015). The Argentine approach is that if there is

no 'new combination of genetic material' and the final product is free of 'transgenes', then that product will not be subject to regulation as a genetically modified organism (GMO). In this system, no distinction is drawn between genome edited plants and animals. In 2018, a genome edited line of tilapia that did not contain any foreign DNA or a new combination of genetic material received regulatory exemption by Argentina's National Advisory Commission on Agricultural Biotechnology (CONABIA) (<https://www.pnnewswire.com/news-releases/intrexon-and-aquabounty-receive-regulatory-exemption-in-development-of-gene-edited-tilapia-for-more-sustainable-production-300768053.html>). Similarly, Brazil ruled that the intraspecies allele substitution that results in hornless cattle would not be regulated as a GMO (MCTIC, 2018). And in 2018, the United States Department of Agriculture (USDA) confirmed that the 'USDA does not regulate or have any plans to regulate plants that could otherwise have been produced through traditional breeding techniques'.

However, the United States Food and Drug Administration (FDA) has taken a very different approach for edited animals, and in a 2017 draft guidance announced that 'all intentional alterations' in the genome of animals would be regulated as new animal drugs (Maxmen, 2017). The guidance elaborates that each alteration would need to go through a mandatory premarket multigenerational safety and efficacy review, irrespective of whether that alteration already exists in the target species or could have been achieved using conventional breeding. This will likely have a detrimental effect on genome editing research in food animal species. Unapproved animal drugs are not allowed to enter the food chain, rendering experimental gene-edited food animals unsaleable. The income derived from surplus animals and the milk, meat and eggs produced by university research and teaching herds and flocks is an integral offset to the sizable costs associated with large animal research. Categorizing all genome edits, irrespective of novelty, as drugs, thereby precluding saleable products from edited livestock, will increase the costs associated with this research considerably, and will dramatically increase the development costs associated with commercializing genome edited livestock.



In 2018, the Court of Justice of the European Union (CJEU) ruled that gene-edited crops should be subject to the same stringent regulations as conventional GMOs (Callaway, 2018). This will likely hinder the use of genome editing by both plant and animal researchers in the EU, and the adoption of this technology in European agriculture. A number of scientists have been vocal about the impact that these proposed regulatory approaches will have on the ability of public sector scientists and small companies, in particular, to use genome editing in food species. Petitions in the EU (Wight, 2018) and the USA (Van Eenennaam et al., 2019) calling for science-based, product risk-focused and harmonized regulations associated with genome editing in food species have been signed by hundreds of scientists.

## Conclusions

It is unclear whether genome editing will eventually be used to augment livestock breeding programs in the future. There is obviously considerable potential to use this approach to introduce useful genetic variations into livestock improvement programs, and also a multiplicity of ways in which it might accelerate the rate of genetic gain through breeding program redesign. Similar possibilities were envisioned over 30 years ago when genetic engineering was first accomplished in food animal species. Despite its extensive use in model species and experimental biology globally for three decades, genetic engineering has effectively been sidelined from animal agricultural applications due in no small part to the regulatory uncertainty and costs associated with bringing food products to market. If genome editing experiences the same fate, considerable opportunity costs in terms of forestalled improvement in the rate of genetic gain will result. Currently, some countries regulate genome editing in livestock as conventional breeding, whereas others consider it to result in a GMO or a new animal drug. Given the importance of global trade in animal products and genetics, this absence of regulatory harmony seems bound to result in global trade disruptions. The disparate regulatory approaches being proposed for genome editing in food animals fosters uncertainty as to whether this potentially valuable breeding tool will ultimately be permitted to serve as a beneficial complement to livestock genetic improvement programs.

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## Competing interests

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