© 2020. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

REVIEW

Genome editing approaches to augment livestock breeding programs

Thomas Frederick Bishop and Alison Louise Van Eenennaam*

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 (ΔG) 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); σ_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 σ_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

Department of Animal Science, University of California, Davis, Davis, CA 95616, USA.

*Author for correspondence (alvaneenennaam@ucdavis.edu)

A.L.V.E., 0000-0003-1562-162X

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

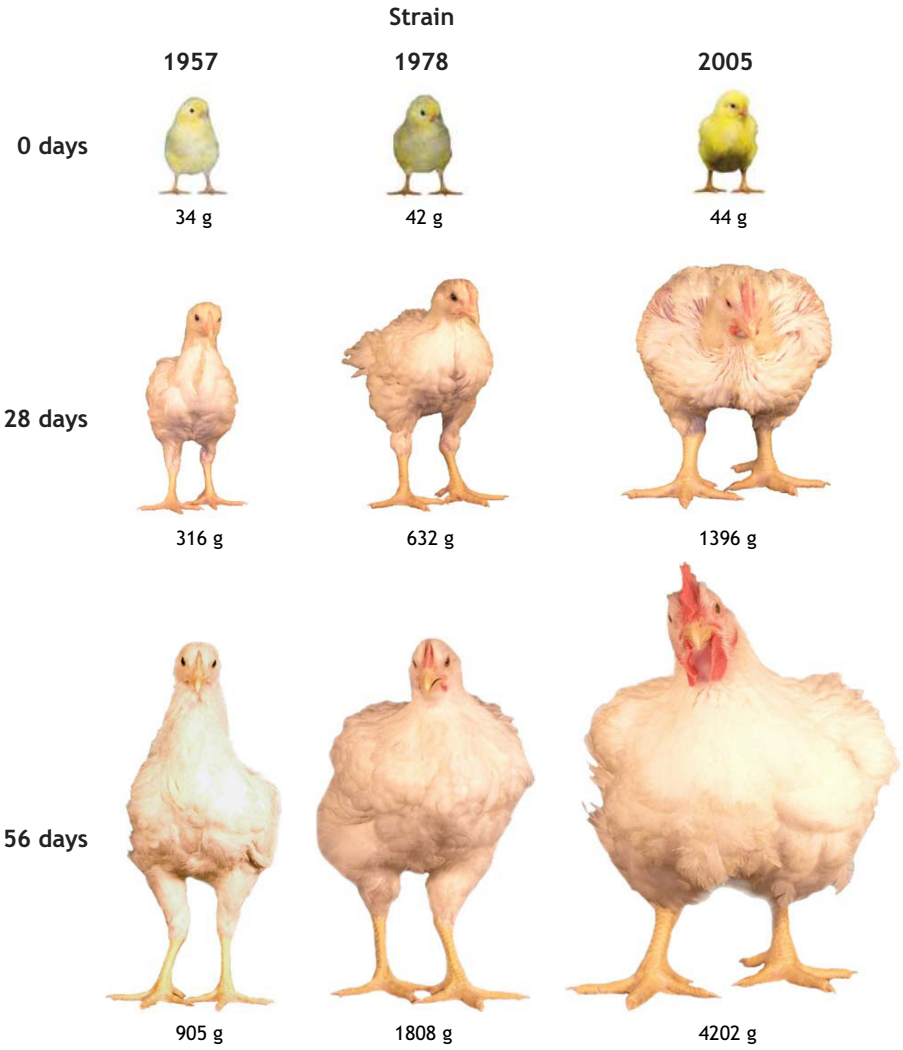

Fig. 1. Age-related changes in size (mixed-sex body mass and front view photos) of University of Alberta Meat Control strains unselected since 1957 and 1978, and Ross 308 broilers (2005). Within each strain, images are of the same bird at 0, 28 and 56 days of age. Reproduced under Creative Commons CC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

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

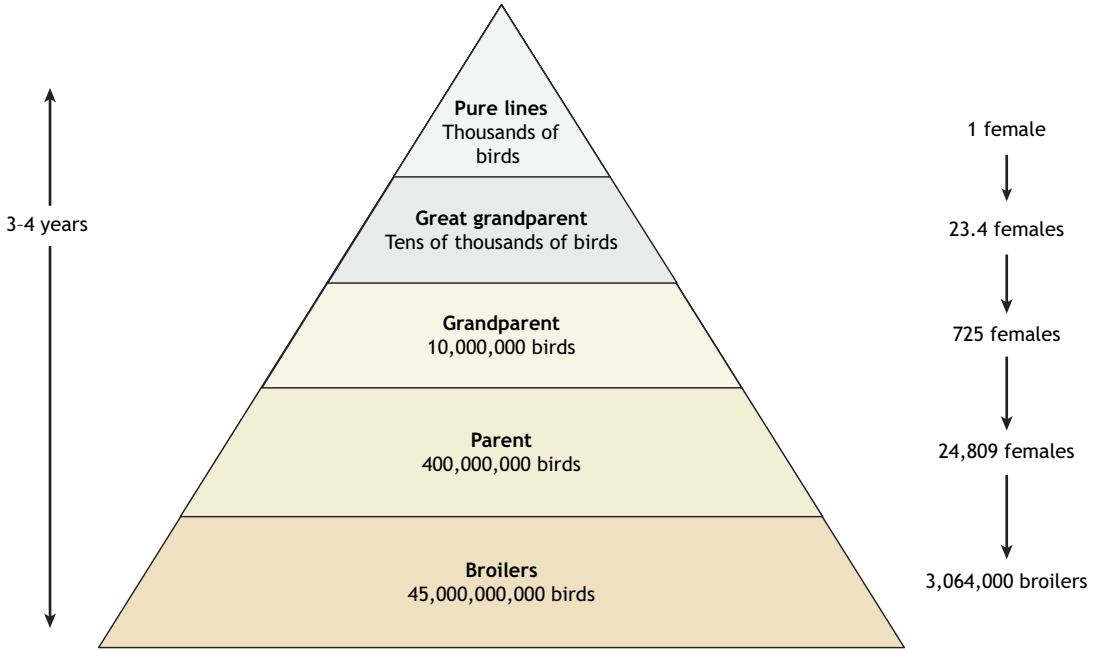

Fig. 2. Broiler industry structure and global estimate of bird numbers alongside estimates of timeline and genetic expressions derived from a single pedigree female broiler chicken. Reproduced with permission from Van Eenennaam et al. (2014).

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)

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

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 (Akcakaya 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

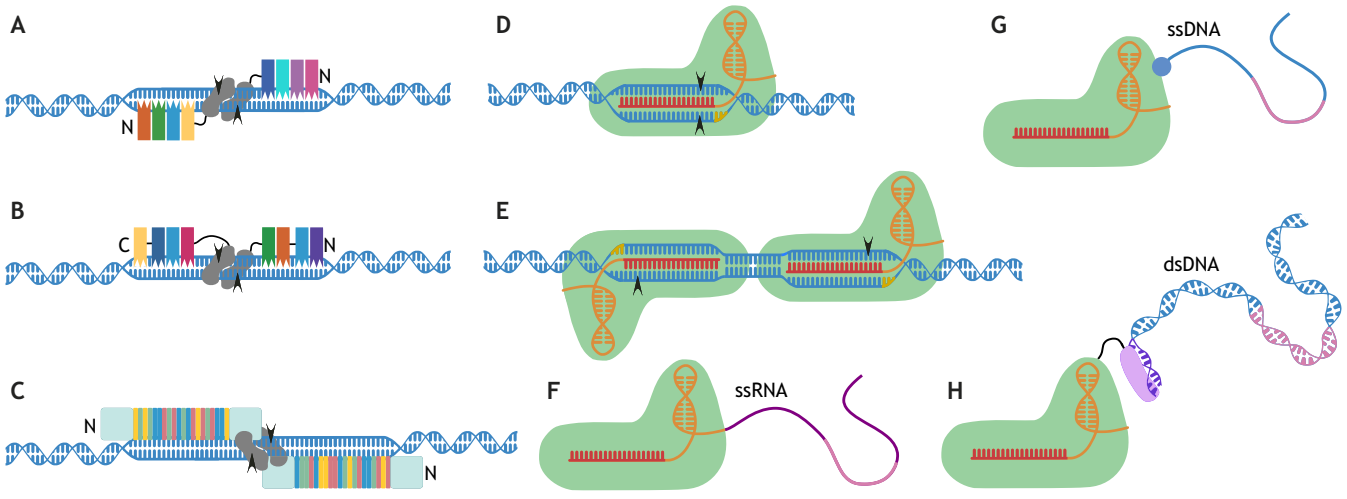

Fig. 3. Examples of genome editors and their modifications. (A–E) Schematic representations of DNA-bound (A) ZFNs with dimerized C′ FokI (gray) domains; (B) ZFNs with base skipping and N′ FokI capability (dimerized N′ and C′ FokI domains shown); (C) TALENs with dimerized FokI domains; (D) sgRNA/Cas9 (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 β-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

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

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-

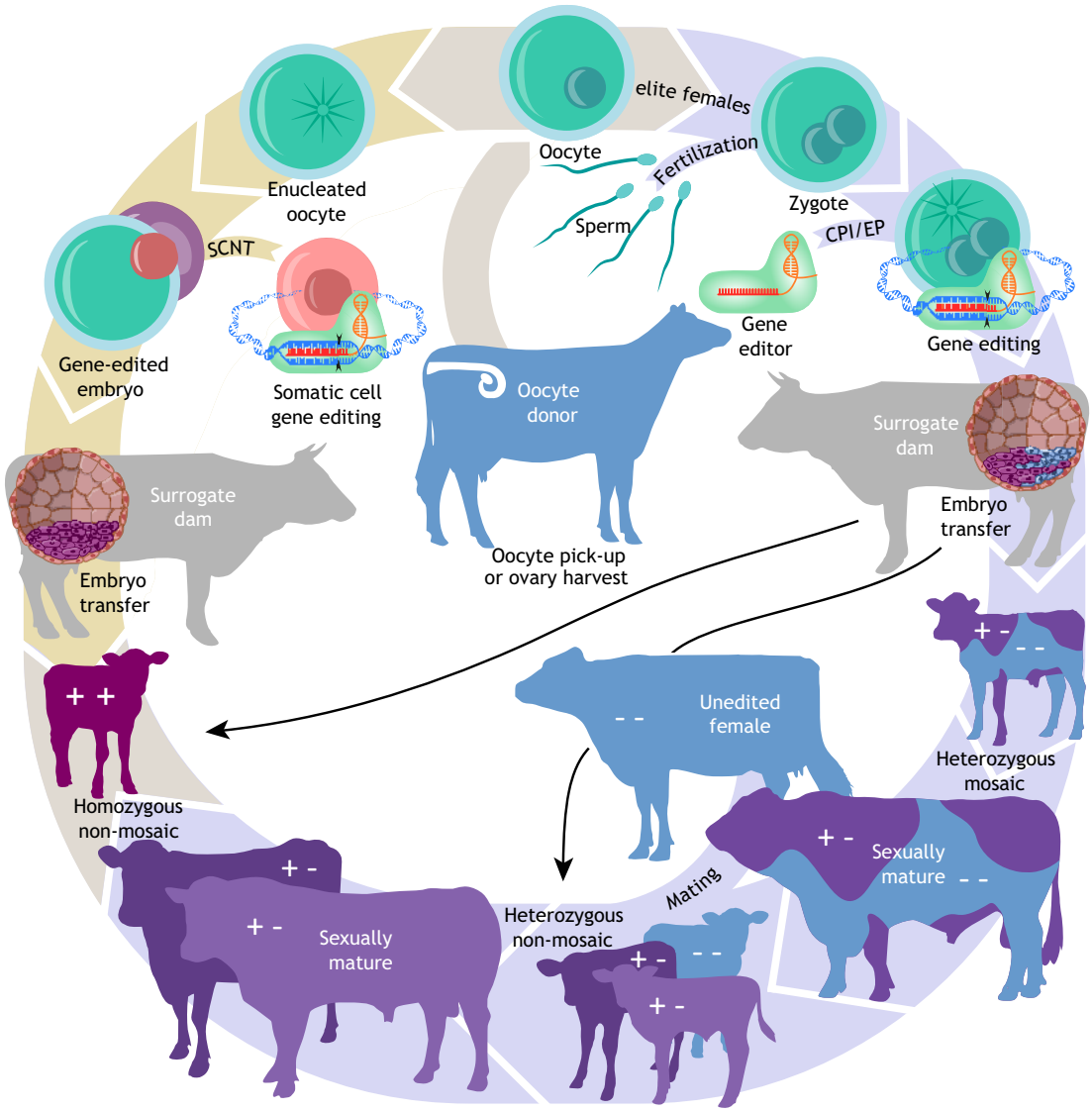

Fig. 4. Steps for producing genome-edited livestock through somatic cell nuclear transfer (SCNT) or zygote editing. Schematic showing the typical steps involved to produce homozygous, non-mosaic livestock by either SCNT cloning of genome-edited and screened somatic cells (yellow arrows) or cytoplasmic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

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

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 though 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

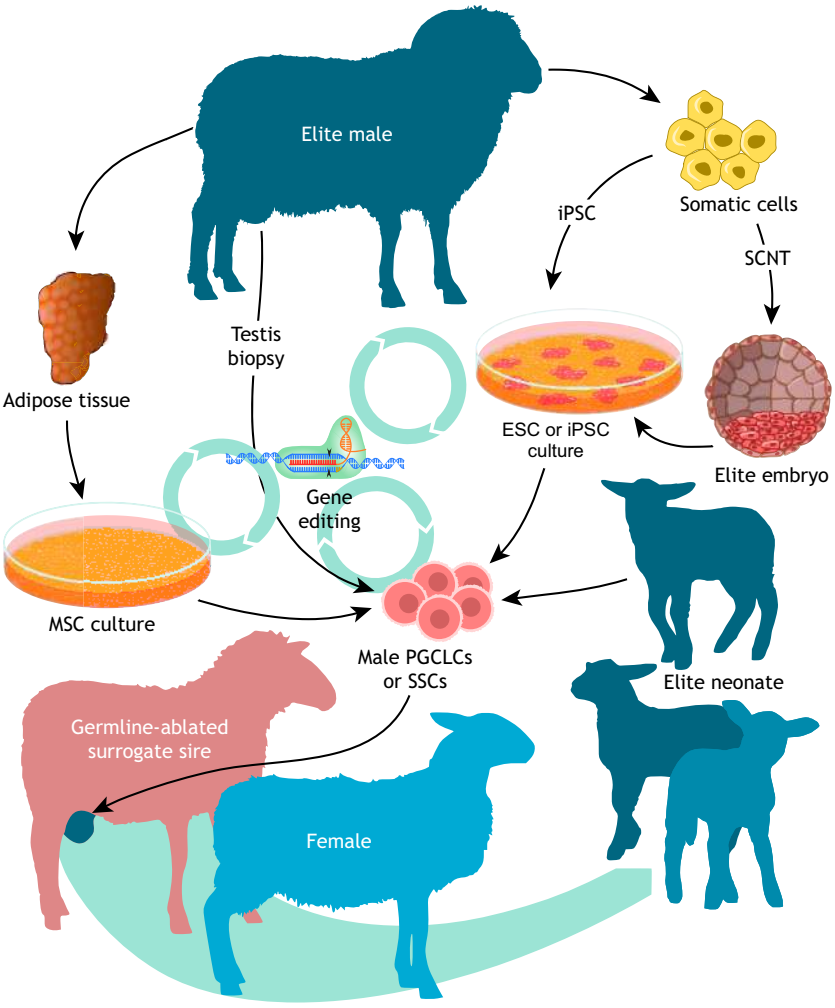

Fig. 5. Germline cloning by surrogate sire. Schematic showing possible male germ cell sources for transfer into germline-ablated (e.g. NANOS2 knockout) surrogate sires. Tissue/cell donors are shown as elite male embryos (elite embryo), juveniles (elite neonate) and adults (elite male). Blue coloring 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 (σA). 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	β-lactoglobulin (*PAEP*): whey protein – allergen	NHEJ – knockout (KO)	ZFN	Yu et al., 2011
	BFF, SCNT	Electroporation	Lysostaphin gene (antimicrobial) inserted into β-casein (*CSN2*) locus	HDR – knock-in	ZFN/ZFNickase	Liu et al., 2013
	BFF, SCNT	Electroporation	Human lysozyme gene (antimicrobial) into *CSN2* locus	HDR – knock-in	ZFN	Liu et al., 2014
	BFF, SCNT	Nucleofection	Myostatin (*MSTN*): negative regulator of muscle growth	NHEJ – KO	ZFN	Luo et al., 2014
	Zygote	Cytoplasmic injection (CPI)	*MSTN*	NHEJ – KO	TALEN	Proudfoot et al., 2015
	BFF, SCNT	Electroporation	Mouse *Sp110* gene (controls MTB infections) inserted into intergenic region between *SFTPA1* and *MAT1A*	HDR – knock-in	TALENickase	Wu et al., 2015
	BFF, SCNT	Electroporation	Replace *horned* allele with *polled* allele	HDR – knock-in	TALEN	Tan et al., 2013; Carlson et al., 2016
	BFF, SCNT	Electroporation	*NRAMP1* gene (controls MTB infections) inserted into intergenic region between *FSCN1* and *ACTB*	HDR – knock-in	CRISPR/Cas9 nickase	Gao et al., 2017
	BFF, SCNT	Electroporation	Repaired mutation in isoleucyl-tRNA synthetase (*IARS*) gene that causes IARS syndrome	HDR – single base substitution	CRISPR/Cas9	Ikeda et al., 2017
	Ear-derived fibroblast, SCNT	Electroporation	Repaired IARS syndrome mutation in *IARS* gene	HDR – single base substitution	CRISPR/Cas9	Ishino et al., 2018
	Bovine fetal skin fibroblasts, SCNT	Electroporation	*Sulfolobus solfataricus* β-glycosidase (digests lactose) into *CSN2* locus	Microhomologous-mediated end-joining (MMEJ) – knock-in	TALEN	Su et al., 2018b
	Zygote	CPI	*PAEP*	HDR – KO ssODN	TALEN	Wei et al., 2018
Sheep	Zygote	CPI	*MSTN*	NHEJ – KO	CRISPR/Cas9	Han et al., 2014
	Zygote	CPI	*MSTN*	NHEJ – KO	CRISPR/Cas9	Crispo et al., 2015
	Zygote	CPI	*MSTN*	NHEJ – KO	TALEN	Proudfoot et al., 2015
	Ovine fetal fibroblasts (OFF), SCNT	Electroporation	*MSTN*	NHEJ – KO	TALEN	Li et al., 2016
	Zygote	CPI	*MSTN*, agouti signaling protein (*ASIP*) and β-carotene oxygenase 2 (*BCO2*)	NHEJ – KO	CRISPR/Cas9	Wang et al., 2016b
	Zygote	Pronuclear injection (PNI)	Fibroblast growth factor 5 (*FGF5*): negative regulator of wool length	NHEJ – KO	CRISPR/Cas9	Hu et al., 2017
	Zygote	CPI	*FGF5*	NHEJ – KO	CRISPR/Cas9	Li et al., 2017
	Zygote	CPI	*AANAT* and *ASMT* (involved in synthesis of the antioxidant, melatonin) with β-casein promoter into *MSTN* locus	homology-independent DNA reparation – knock-in & KO	CRISPR/Cas9 dsDNA	Ma et al., 2017
	Zygote	CPI	*ASIP*: coat color	NHEJ – KO	CRISPR/Cas9	Zhang et al., 2017
	Sheep ear fibroblasts	Electroporation	*MSTN*	NHEJ – KO	CRISPR/Cas9	Zhang et al., 2019
Goats	Goat fetal fibroblasts (GFF), SCNT	Nucleofection	*MSTN*	NHEJ – KO	CRISPR/Cas9	Ni et al., 2014
	GFF, SCNT	Electroporation	Human lactoferrin (*LTF*): involved in iron absorption – into *PAEP* locus	HDR – knock-in & KO	TALEN	Cui et al., 2015
	Zygote	CPI	*MSTN* and *FGF5*	NHEJ – KO	CRISPR/Cas9	Wang et al., 2015b
	Zygote	CPI	*MSTN*	NHEJ – KO	CRISPR/Cas9	Guo et al., 2016
	GFF, SCNT	Electroporation	Human *LTF* into *PAEP* gene	HDR – knock-in & KO	TALEN	Song et al., 2016
	GFF, SCNT	Electroporation	*MSTN*	NHEJ – KO	TALEN	Yu et al., 2016
	Goat ear fibroblasts (GEF) & GFF, SCNT	Electroporation	Human α-lactalbumin (*LALBA*): main whey protein in human milk – into *PAEP* locus	HDR – knock-in & KO	TALEN	Zhu et al., 2016
	Zygote	CPI	*PAEP*	NHEJ – KO	CRISPR/Cas9	Zhou et al., 2017
	Zygote	CPI	*MSTN*	NHEJ – KO	CRISPR/Cas9	He et al., 2018

Continued

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

Table 2. Continued

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

Continued

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

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
	PGC, transplantation	Lipofection	G0/G1 switch 2 gene (*G0S2*): positive regulator of fat deposition	NHEJ – KO	CRISPR/Cas9	Park et al., 2019
Carp	Zygote	Yolk injection (YI)	Myostatin 1a (*mstn*)	NHEJ – KO	TALEN	Zhong et al., 2016
Sturgeon	Zygote	YI	Dead end protein homolog 1 (*dnd1*): essential for migration of primordial germ cells	NHEJ – KO	CRISPR/Cas9	Baloch et al., 2019
Catfish	Zygote	YI	*mstna*: one of the duplicated myostatin genes	NHEJ – KO	ZFN	Dong et al., 2011
	Zygote	YI	*mstnb*: one of the duplicated myostatin genes	NHEJ – KO	TALEN	Dong et al., 2014
	Sperm then zygote	Double electroporation	Luteinizing hormone beta subunit (*lhb*) gene: central role in gametogenesis	NHEJ – KO	ZFN	Qin et al., 2016
	Zygote	YI	*mstn*	NHEJ – KO	CRISPR/Cas9	Khalil et al., 2017
Sole	Zygote	YI	*dmrt1*: putative male determination gene (females have faster growth rates)	NHEJ – KO	TALEN	Cui et al., 2017
Salmon	Zygote	YI	*dnd1*: essential for primordial germ cell formation; and *slc45a2*: 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 (σ_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,

Journal of Experimental Biology (2020) 223, jeb207159. doi:10.1242/jeb.207159

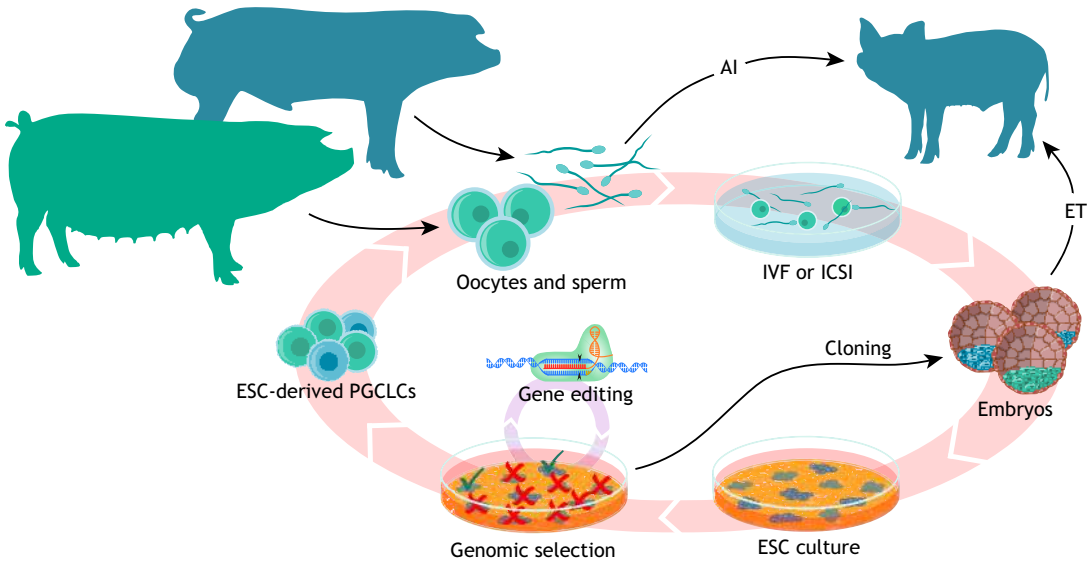

Fig. 6. *In vitro* breeding. Schematic representation of the concept of *in vitro* breeding using gametes from an elite sow and boar. Pink circle shows potential for repeated editing and culturing. Green ticks represent ESC colonies that are selected via genomic selection, red crosses represent colonies 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.prnewswire.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 polled 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.

Acknowledgements

A.L.V.E. thanks Michael Dickinson and Michaela Handel for the invitation to participate in the 2019 JEB Symposium on Genome Editing for Comparative Physiology.

Competing interests

The authors declare no competing or financial interests.

Funding

A.L.V.E. thanks The Company of Biologists for support to attend the Genome Editing for Comparative Physiology symposium. Her work on genome editing was funded by the National Institute of Food and Agriculture and the Biotechnology Risk Assessment Grant (BRAG) program, U.S. Department of Agriculture (USDA), under award numbers 2015-67015-23316, 2015-33522-24106 and 2017-33522-27097. T.F.B. was funded as a postdoctoral fellow by USDA Agriculture and Food Research Initiative (AFRI) competitive award number 2018-67030-28360.

References

Adams, H. A., Sonstegard, T. S., VanRaden, P. M., Null, D. J., Van Tassell, C. P., Larkin, D. M. and Lewin, H. A. (2016). Identification of a nonsense mutation in *APAF1* that is likely causal for a decrease in reproductive efficiency in Holstein dairy cattle. *J. Dairy Sci.* **99**, 6693-6701. doi:10.3168/jds.2015-10517

Aird, E. J., Lovendahl, K. N., St Martin, A., Harris, R. S. and Gordon, W. R. (2018). Increasing Cas9-mediated homology-directed repair efficiency through covalent tethering of DNA repair template. *Commun. Biol.* **1**, 54. doi:10.1038/s42003-018-0054-2

Akagi, S., Matsukawa, K. and Takahashi, S. (2014). Factors affecting the development of somatic cell nuclear transfer embryos in cattle. *J. Reprod. Dev.* **60**, 329-335. doi:10.1262/jrd.2014-057

Akcakaya, P., Bobbin, M. L., Guo, J. A., Malagon-Lopez, J., Clement, K., Garcia, S. P., Fellows, M. D., Porritt, M. J., Firth, M. A., Carreras, A. et al. (2018). *In vivo* CRISPR editing with no detectable genome-wide off-target mutations. *Nature* **561**, 416-419. doi:10.1038/s41586-018-0500-9

Bai, H., Lester, G. M. S., Petishnok, L. C. and Dean, D. A. (2017). Cytoplasmic transport and nuclear import of plasmid DNA. *Biosci. Rep.* **37**, BSR20160616. doi:10.1042/BSR20160616

Baloch, A. R., Franěk, R., Tichopád, T., Fučíková, M., Rodina, M. and Pšenička, M. (2019). Dnd1 knockout in sturgeons by CRISPR/Cas9 generates germ cell free host for surrogate production. *Animals* **9**, 174. doi:10.3390/ani9040174

Bi, Y., Hua, Z., Liu, X., Hua, W., Ren, H., Xiao, H., Zhang, L., Li, L., Wang, Z., Laible, G. et al. (2016). Isozygous and selectable marker-free MSTN knockout cloned pigs generated by the combined use of CRISPR/Cas9 and Cre/LoxP. *Sci. Rep.* **6**, 31729. doi:10.1038/srep31729

Bibikova, M., Golic, M., Golic, K. G. and Carroll, D. (2002). Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics* **161**, 1169-1175.

Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., Sakurai, M., Sampaio, R. V., Suzuki, K., Izpisua Belmonte, J. C. et al. (2018). Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proc. Natl. Acad. Sci. USA* **115**, 2090. doi:10.1073/pnas.1716161115

Brevini, T. A. L., Pennarossa, G., Attanasio, L., Vanelli, A., Gasparrini, B. and Gandolfi, F. (2010). Culture conditions and signalling networks promoting the establishment of cell lines from parthenogenetic and biparental pig embryos. *Stem Cell Rev.* **6**, 484-495. doi:10.1007/s12015-010-9153-2

Brinster, R. L. and Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11303-11307. doi:10.1073/pnas.91.24.11303

Brinster, R. L. and Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11298-11302. doi:10.1073/pnas.91.24.11298

Brinster, R. L., Chen, H. Y., Trumbauer, M. E., Yagle, M. K. and Palmiter, R. D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci. USA* **82**, 4438-4442. doi:10.1073/pnas.82.13.4438

Burkard, C., Lillico, S. G., Reid, E., Jackson, B., Mileham, A. J., Ait-Ali, T., Whitelaw, C. B. A. and Archibald, A. L. (2017). Precision engineering for PRRSV resistance in pigs: macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. *PLoS Pathog.* **13**, e1006206. doi:10.1371/journal.ppat.1006206

Butt, H., Eid, A., Ali, Z., Atia, M. A. M., Mokhtar, M. M., Hassan, N., Lee, C. M., Bao, G. and Mahfouz, M. M. (2017). Efficient CRISPR/Cas9-mediated genome editing using a chimeric single-guide RNA molecule. *Front.Plant Sci.* **8**, 1441. doi:10.3389/fpls.2017.01441

Cakici, C., Buyrukcu, B., Duruksu, G., Haliloglu, A. H., Aksoy, A., Isik, A., Uludag, O., Ustun, H., Subası, C. and Karaoz E. (2013). Recovery of fertility in azoospermia rats after injection of adipose-tissue-derived mesenchymal stem cells: the sperm generation. *Biomed Res Int.* **2013**, 529589. doi:10.1155/2013/529589

Callaway, E. (2018). CRISPR plants now subject to tough GM laws in European Union. *Nature* **560**, 16. doi:10.1038/d41586-018-05814-6

Capper, J. L. and Bauman, D. E. (2013). The role of productivity in improving the environmental sustainability of ruminant production systems. *Annu. Rev. Anim. Biosci.* **1**, 469-489. doi:10.1146/annurev-animal-031412-103727

Carlson, D. F., Lancto, C. A., Zang, B., Kim, E.-S., Walton, M., Oldeschulte, D., Seabury, C., Sonstegard, T. S. and Fahrenkrug, S. C. (2016). Production of hornless dairy cattle from genome-edited cell lines. *Nat. Biotechnol.* **34**, 479-481. doi:10.1038/nbt.3560

Cenariu, M., Pall, E., Cernea, C. and Groza, I. (2012). Evaluation of bovine embryo biopsy techniques according to their ability to preserve embryo viability. *J. Biomed.Biotechnol.* **2012**, 541384. doi:10.1155/2012/541384

Chen, S., Lee, B., Lee, A. Y.-F., Modzelewski, A. J. and He, L. (2016). Highly efficient mouse genome editing by CRISPR ribonucleoprotein electroporation of zygotes. *J. Biol. Chem.* **291**, 14457-14467. doi:10.1074/jbc.M116.733154

Chen, J., Wang, H., Bai, J., Liu, W., Liu, X., Yu, D., Feng, T., Sun, Z., Zhang, L., Ma, L. et al. (2019). Generation of pigs resistant to highly pathogenic-porcine reproductive and respiratory syndrome virus through gene editing of CD163. *Int. J. Biol. Sci.* **15**, 481-492. doi:10.7150/ijbs.25862

Crispo, M., Mulet, A. P., Tesson, L., Barrera, N., Cuadro, F., dos Santos-Neto, P. C., Nguyen, T. H., Créneguy, A., Brusselle, L., Anegón, I. et al. (2015). Efficient generation of myostatin knock-out sheep using CRISPR/Cas9 technology and microinjection into zygotes. *PLoS ONE* **10**, e0136690. doi:10.1371/journal.pone.0136690

Cui, C., Song, Y., Liu, J., Ge, H., Li, Q., Huang, H., Hu, L., Zhu, H., Jin, Y. and Zhang, Y. (2015). Gene targeting by TALEN-induced homologous recombination in goats directs production of β-lactoglobulin-free, high-human lactoferrin milk. *Sci. Rep.* **5**, 10482. doi:10.1038/srep10482

Cui, Z., Liu, Y., Wang, W., Wang, Q., Zhang, N., Lin, F., Wang, N., Shao, C., Dong, Z., Li, Y. et al. (2017). Genome editing reveals dmrt1 as an essential male sex-determining gene in Chinese tongue sole (*Cynoglossus semilaevis*). *Sci. Rep.* **7**, 42213. doi:10.1038/srep42213

Dattena, M., Chessa, B., Lacerenza, D., Accardo, C., Pilichi, S., Mara, L., Chessa, F., Vincenti, L. and Cappai, P. (2006). Isolation, culture, and characterization of embryonic cell lines from vitrified sheep blastocysts. *Mol.Reprod. Dev.* **73**, 31-39. doi:10.1002/mrd.20378

Dissanayake, D., Patel, H. and Wijesinghe, P. S. (2018). Differentiation of human male germ cells from Wharton's jelly-derived mesenchymal stem cells. *Clin. Exp. Reprod. Med.* **45**, 75-81. doi:10.5653/cerm.2018.45.2.75

Dong, Z., Ge, J., Li, K., Xu, Z., Liang, D., Li, J., Li, J., Jia, W., Li, Y., Dong, X. et al. (2011). Heritable targeted inactivation of myostatin gene in yellow catfish (*Pelteobagrus fulvidraco*) using engineered zinc finger nucleases. *PLoS ONE* **6**, e28897. doi:10.1371/journal.pone.0028897

Dong, Z., Ge, J., Xu, Z., Dong, X., Cao, S., Pan, J. and Zhao, Q. (2014). Generation of myostatin B knockout yellow catfish (*Tachysurus fulvidraco*) using transcription activator-like effector nucleases. *Zebrafish* **11**, 265-274. doi:10.1089/zeb.2014.0974

Eberhart, S. (1970). Factors effecting efficiencies of breeding methods. *African Soils* **15**, 655-680.

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K. and Sander, J. D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822-826. doi:10.1038/nbt.2623

Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M. and Joung, J. K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* **32**, 279-284. doi:10.1038/nbt.2808

Gao, Y., Wu, H., Wang, Y., Liu, X., Chen, L., Li, Q., Cui, C., Liu, X., Zhang, J. and Zhang, Y. (2017). Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. *Genome Biol.* **18**, 13. doi:10.1186/s13059-016-1144-4

García-Ruiz, A., Cole, J. B., VanRaden, P. M., Wiggans, G. R., Ruiz-López, F. J. and Van Tassell, C. P. (2016). Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. *Proc. Natl. Acad. Sci. USA* **113**, E3995-E4004. doi:10.1073/pnas.1519061113

Georges, M., Charlier, C. and Hayes, B. (2019). Harnessing genomic information for livestock improvement. *Nat. Rev. Genet.* **20**, 135-156. doi:10.1038/s41576-018-0082-2

Ghasemzadeh-Hasankolaei, M., Eslaminejad, M. B., Batavani, R. and Sedighi-Gilani, M. (2014). Comparison of the efficacy of three concentrations of retinoic acid for transdifferentiation induction in sheep marrow-derived mesenchymal stem cells into male germ cells. *Andrologia* **46**, 24-35. doi:10.1111/and.12037

Ghasemzadeh-Hasankolaei, M., Batavani, R., Eslaminejad, M. B. and Sayahpour, F. (2016a). Transplantation of autologous bone marrow mesenchymal stem cells into the testes of infertile male rats and new germ cell formation. *Int. J. Stem Cells* **9**, 250-263. doi:10.15283/ijsc16010

Ghasemzadeh-Hasankolaei, M., Eslaminejad, M. B. and Sedighi-Gilani, M. (2016b). Derivation of male germ cells from ram bone marrow mesenchymal stem cells by three different methods and evaluation of their fate after transplantation into the testis. *In Vitro Cell. Dev. Biol. Anim.* **52**, 49-61. doi:10.1007/s11626-015-9945-4

Giassetti, M. I., Ciccarelli, M. and Oatley, J. M. (2019). Spermatogonial stem cell transplantation: insights and outlook for domestic animals. *Annu. Rev. Anim. Biosci.* **7**, 385-401. doi:10.1146/annurev-animal-020518-115239

Ginsburg, M. and Eyal-Giladi, H. (1986). Temporal and spatial aspects of the gradual migration of primordial germ cells from the epiblast into the germinal crescent in the avian embryo. *J. Embryol. Exp. Morphol.* **95**, 53-71.

Goszczynski, D. E., Cheng, H., Demyda-Peyrás, S., Medrano, J. F., Wu, J. and Ross, P. J. (2018). In vitro breeding: application of embryonic stem cells to animal production. *Biol. Reprod.* **100**, 885-895. doi:10.1093/biolre/ioy256

Gottardo, P., Gorjanc, G., Battagin, M., Gaynor, R. C., Jenko, J., Ros-Freixedes, R., Bruce, A., Whitelaw, C., Mileham, A. J., Herring, W. O. et al. (2019). A strategy to exploit surrogate sire technology in livestock breeding programs. *G3* **9**, 203-215. doi:10.1534/g3.118.200890

Gresch, O., Engel, F. B., Nesic, D., Tran, T. T., England, H. M., Hickman, E. S., Körner, I., Gan, L., Chen, S., Castro-Obregon, S. et al. (2004). New non-viral method for gene transfer into primary cells. *Methods* **33**, 151-163. doi:10.1016/j.ymeth.2003.11.009

Guo, R., Wan, Y., Xu, D., Cui, L., Deng, M., Zhang, G., Jia, R., Zhou, W., Wang, Z., Deng, K. et al. (2016). Generation and evaluation of Myostatin knock-out rabbits and goats using CRISPR/Cas9 system. *Sci. Rep.* **6**, 29855. doi:10.1038/srep29855

Hammer, R. E., Pursel, V. G., Rexroad, C. E., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D. and Brinster, R. L. J. N. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315**, 680-683. doi:10.1038/315680a0

Han, H., Ma, Y., Wang, T., Lian, L., Tian, X., Hu, R., Deng, S., Li, K., Wang, F., Li, N. et al. (2014). One-step generation of myostatin gene knockout sheep via the CRISPR/Cas9 system. *Front. Agr. Sci. Eng.* **1**, 2-5. doi:10.15302/J-FASE-2014007

Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S. and Saitou, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell* **146**, 519-532. doi:10.1016/j.cell.2011.06.052

Hayes, B. J. and Daetwyler, H. D. (2019). 1000 bull genomes project to map simple and complex genetic traits in cattle: applications and outcomes. *Annu. Rev. Anim. Biosci.* **7**, 89-102. doi:10.1146/annurev-animal-020518-115024

He, Z., Zhang, T., Jiang, L., Zhou, M., Wu, D., Mei, J. and Cheng, Y. (2018). Use of CRISPR/Cas9 technology efficiently targeted goat myostatin through zygotes microinjection resulting in double-muscled phenotype in goats. *Biosci. Rep.* **38**, BSR20180742. doi:10.1042/BSR20180742

Herrid, M., Vignarajan, S., Davey, R., Dobrinski, I. and Hill, J. R. (2006). Successful transplantation of bovine testicular cells to heterologous recipients. *Reproduction* **132**, 617-624. doi:10.1530/rep.1.01125

Herrid, M., Nagy, P., Juhasz, J., Morrell, J. M., Billah, M., Khazanehdari, K. and Skidmore, J. A. (2019). Donor sperm production in heterologous recipients by testis germ cell transplantation in the dromedary camel. *Reprod. Fertil. Dev.* **31**, 538-546. doi:10.1071/RD18191

Hickey, J. M., Bruce, C., Whitelaw, A. and Gorjanc, G. (2016). Promotion of alleles by genome editing in livestock breeding programmes. *J. Anim. Breed. Genet.* **133**, 83-84. doi:10.1111/jbg.12206

Hikabe, O., Hamazaki, N., Nagamatsu, G., Obata, Y., Hirao, Y., Hamada, N., Shimamoto, S., Imamura, T., Nakashima, K., Saitou, M. et al. (2016). Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature* **539**, 299. doi:10.1038/nature20104

Hirata, M., Tanihara, F., Wittayarat, M., Hirano, T., Nguyen, N. T., Le, Q. A., Namula, Z., Nii, M. and Otoi, T. (2019). Genome mutation after introduction of the gene editing by electroporation of Cas9 protein (GEEP) system in matured oocytes and putative zygotes. *In Vitro Cell. Dev. Biol. Anim.* **55**, 237-242. doi:10.1007/s11626-019-00338-3

Ho, B. X., Loh, S. J. H., Chan, W. K. and Soh, B. S. (2018). *In vivo* genome editing as a therapeutic approach. *Int. J. Mol. Sci.* **19**, 2721. doi:10.3390/ijms19092721

Honaramooz, A., Megee, S. O. and Dobrinski, I. (2002). Germ cell transplantation in pigs. *Biol. Reprod.* **66**, 21-28. doi:10.1095/biolreprod66.1.21

Honaramooz, A., Behboodi, E., Blash, S., Megee, S. O. and Dobrinski, I. (2003). Germ cell transplantation in goats. *Mol. Reprod. Dev.* **64**, 422-428. doi:10.1002/mrd.10205

Hou, Z., An, L., Han, J., Yuan, Y., Chen, D. and Tian, J. (2018). Revolutionize livestock breeding in the future: an animal embryo-stem cell breeding system in a dish. *J. Anim. Sci. Biotechnol.* **9**, 90. doi:10.1186/s40104-018-0304-7

Hu, R., Fan, Z. Y., Wang, B. Y., Deng, S. L., Zhang, X. S., Zhang, J. L., Han, H. B. and Lian, Z. X. (2017). Generation of FGF5 knockout sheep via the CRISPR/Cas9 system12. *J. Anim. Sci.* **95**, 2019-2024. doi:10.2527/jas.2017.1503

Hua, J., Pan, S., Yang, C., Dong, W., Dou, Z. and Sidhu, K. S. (2009). Derivation of male germ cell-like lineage from human fetal bone marrow stem cells. *Reprod. Biomed. Online* **19**, 99-105. doi:10.1016/S1472-6483(10)60052-1

Hustedt, N. and Durocher, D. (2016). The control of DNA repair by the cell cycle. *Nat. Cell Biol.* **19**, 1-9. doi:10.1038/ncb3452

Ikeda, M., Matsuyama, S., Akagi, S., Ohkoshi, K., Nakamura, S., Minabe, S., Kimura, K. and Hosoe, M. (2017). Correction of a disease mutation using CRISPR/Cas9-assisted genome editing in Japanese black cattle. *Sci. Rep.* **7**, 17827. doi:10.1038/s41598-017-17968-w

Ishino, T., Hashimoto, M., Amagasa, M., Saito, N., Dochi, O., Kirisawa, R. and Kitamura, H. (2018). Establishment of protocol for preparation of gene-edited bovine ear-derived fibroblasts for somatic cell nuclear transplantation. *Biomed. Res.* **39**, 95-104. doi:10.2220/biomedres.39.95

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821. doi:10.1126/science.1225829

Jenko, J., Gorjanc, G., Cleveland, M. A., Varshney, R. K., Whitelaw, C. B. A., Woolliams, J. A. and Hickey, J. M. (2015). Potential of promotion of alleles by genome editing to improve quantitative traits in livestock breeding programs. *Genet. Sel. Evol.* **47**, 55. doi:10.1186/s12711-015-0135-3

Katanbaf, M. N. and Hardiman, J. W. (2010). Primary broiler breeding – striking a balance between economic and well-being traits. *Poult. Sci.* **89**, 822-824. doi:10.3382/ps.2009-00439

Keefer, C. L. (2015). Artificial cloning of domestic animals. *Proc. Natl. Acad. Sci. USA* **112**, 8874. doi:10.1073/pnas.1501718112

Khalil, K., Elayat, M., Khalifa, E., Daghash, S., Elaswad, A., Miller, M., Abdelrahman, H., Ye, Z., Odin, R., Drescher, D. et al. (2017). Generation of myostatin gene-edited channel catfish (*Ictalurus punctatus*) via zygote injection of CRISPR/Cas9 system. *Sci. Rep.* **7**, 7301. doi:10.1038/s41598-017-07223-7

Kim, M.-S. and Kini, A. G. (2017). Engineering and application of zinc finger proteins and TALEs for biomedical research. *Mol. Cells* **40**, 533-541. doi:10.14348/molcells.2017.0139

Kim, S., Kim, J. H., Lee, E., Jeong, Y. W., Hossein, M. S., Park, S. M., Park, S. W., Lee, J. Y., Jeong, Y. I., Kim, H. S. et al. (2010). Establishment and

characterization of embryonic stem-like cells from porcine somatic cell nuclear transfer blastocysts. *Zygote* **18**, 93-101. doi:10.1017/S0967199409990372

Kraft, K., Geuer, S., Will, A. J., Chan, W. L., Paliou, C., Borschiwer, M., Harabula, I., Wittler, L., Franke, M., Ibrahim, D. M. et al. (2015). Deletions, inversions, duplications: engineering of structural variants using CRISPR/Cas in mice. *Cell Rep.* **10**, 833-839. doi:10.1016/j.celrep.2015.01.016

Lee, H. J., Yoon, J. W., Jung, K. M., Kim, Y. M., Park, J. S., Lee, K. Y., Park, K. J., Hwang, Y. S., Park, Y. H., Rengaraj, D. et al. (2019). Targeted gene insertion into Z chromosome of chicken primordial germ cells for avian sexing model development. *FASEB J.* **33**, 8519-8529. doi:10.1096/fj.201802671R

Leenay, R. T. and Beisel, C. L. (2017). Deciphering, communicating, and engineering the CRISPR PAM. *J. Mol. Biol.* **429**, 177-191. doi:10.1016/j.jmb.2016.11.024

Li, H., Wang, G., Hao, Z., Zhang, G., Qing, Y., Liu, S., Qing, L., Pan, W., Chen, L., Liu, G. et al. (2016). Generation of biallelic knock-out sheep via gene-editing and somatic cell nuclear transfer. *Sci. Rep.* **6**, 33675. doi:10.1038/srep33675

Li, W.-R., Liu, C.-X., Zhang, X.-M., Chen, L., Peng, X.-R., He, S.-G., Lin, J.-P., Han, B., Wang, L.-Q., Huang, J.-C. et al. (2017). CRISPR/Cas9-mediated loss of FGF5 function increases wool staple length in sheep. *FEBS J.* **284**, 2764-2773. doi:10.1111/febs.14144

Li, M., Ouyang, H., Yuan, H., Li, J., Xie, Z., Wang, K., Yu, T., Liu, M., Chen, X., Tang, X. et al. (2018). Site-specific Fat-1 knock-in enables significant decrease of n-6PUFAs/n-3PUFAs ratio in pigs. *G3* **8**, 1747-1754. doi:10.1534/g3.118.200114

Lillico, S. G., Proudfoot, C., Carlson, D. F., Stverakova, D., Neil, C., Blain, C., King, T. J., Ritchie, W. A., Tan, W., Mileham, A. J. et al. (2013). Live pigs produced from genome edited zygotes. *Sci. Rep.* **3**, 2847. doi:10.1038/srep02847

Lillico, S. G., Proudfoot, C., King, T. J., Tan, W., Zhang, L., Mardjuki, R., Paschon, D. E., Rebar, E. J., Urnov, F. D., Mileham, A. J. et al. (2016). Mammalian interspecies substitution of immune modulatory alleles by genome editing. *Sci. Rep.* **6**, 21645. doi:10.1038/srep21645

Liu, X., Wang, Y., Guo, W., Chang, B., Liu, J., Guo, Z., Quan, F. and Zhang, Y. (2013). Zinc-finger nickase-mediated insertion of the lysostaphin gene into the beta-casein locus in cloned cows. *Nat. Commun.* **4**, 2565. doi:10.1038/ncomms3565

Liu, X., Wang, Y., Tian, Y., Yu, Y., Gao, M., Hu, G., Su, F., Pan, S., Luo, Y., Guo, Z. et al. (2014). Generation of mastitis resistance in cows by targeting human lysozyme gene to β-casein locus using zinc-finger nucleases. *Proc. Biol. Sci.* **281**, 20133368. doi:10.1098/rspb.2013.3368

Liu, X., Liu, H., Wang, M., Li, R., Zeng, J., Mo, D., Cong, P., Liu, X., Chen, Y. and He, Z. (2019). Disruption of the ZBED6 binding site in intron 3 of IGF2 by CRISPR/Cas9 leads to enhanced muscle development in Liang Guang small spotted pigs. *Trans. Res.* **28**, 141-150. doi:10.1007/s11248-018-0107-9

Luo, J., Song, Z., Yu, S., Cui, D., Wang, B., Ding, F., Li, S., Dai, Y. and Li, N. (2014). Efficient generation of Myostatin (MSTN) biallelic mutations in cattle using zinc finger nucleases. *PLoS ONE* **9**, e95225. doi:10.1371/journal.pone.0095225

Luo, Y., Xie, L., Mohsin, A., Ahmed, W., Xu, C., Peng, Y., Hang, H., Zhuang, Y., Chu, J. and Guo, M. (2019). Efficient generation of male germ-like cells derived during co-culturing of adipose-derived mesenchymal stem cells with Sertoli cells under retinoic acid and testosterone induction. *Stem. Cell Res. Ther.* **10**, 91. doi:10.1186/s13287-019-1181-5

Lush, J. L. (1937). *Animal Breeding Plans*. Ames, IA: Collegiate Press, Inc.

Ma, T., Tao, J., Yang, M., He, C., Tian, X., Zhang, X., Zhang, J., Deng, S., Feng, J., Zhang, Z. et al. (2017). An AANAT/ASMT transgenic animal model constructed with CRISPR/Cas9 system serving as the mammary gland bioreactor to produce melatonin-enriched milk in sheep. *J. Pineal Res.* **63**, e12406. doi:10.1111/jpi.12406

Maghen, L., Shlush, E., Gat, I., Filice, M., Barretto, T. A., Jarvi, K., Lo, K., Gauthier-Fisher, A. and Librach, C. L. (2017). Human umbilical perivascular cells (HUCPVCs): a novel source of mesenchymal stromal-like (MSC) cells to support the regeneration of the testicular niche. *Reproduction* **153**, 85-95. doi:10.1530/REP-16-0220

Maxmen, A. (2017). Gene-edited animals face US regulatory crackdown. *Nature News*. doi:10.1038/nature.2017.21331

MCTIC (Ministry of Science, Technology, Innovation and Communications) (2018). Technical Opinion 6125/2018. Consultation on the application of Normative Resolution 16 in animal products developed with innovative precision improvement techniques - TIMP. http://sei.mctic.gov.br/verifica.html. Validation code 3509624 and CRC code FFECC97F.

Medugorac, I., Seichter, D., Graf, A., Russ, I., Blum, H., Göpel, K. H., Rothammer, S., Förster, M. and Krebs, S. (2012). Bovine polledness – an autosomal dominant trait with allelic heterogeneity. *PLoS ONE* **7**, e39477. doi:10.1371/journal.pone.0039477

Meuwissen, T., Hayes, B. and Goddard, M. (2013). Accelerating improvement of livestock with genomic selection. *Annu. Rev. Anim. Biosci.* **1**, 221-237. doi:10.1146/annurev-animal-031412-103705

Miao, D., Giassetti, M. I., Ciccarelli, M., Lopez-Biladeau, B. and Oatley, J. M. (2019). Simplified pipelines for genetic engineering of mammalian embryos by CRISPR-Cas9 electroporation. *Biol. Reprod.* **101**, 177-187. doi:10.1093/biolre/ioz075

Mueller, M. L., Cole, J. B., Sonstegard, T. S. and Van Eenennaam, A. L. (2019). Comparison of gene editing versus conventional breeding to introgress the *POLLED* allele into the US dairy cattle population. *J. Dairy Sci.* **102**, 4215-4226. doi:10.3168/jds.2018-15892

Nakamura, Y., Yamamoto, Y., Usui, F., Mushika, T., Ono, T., Setioko, A. R., Takeda, K., Nirasawa, K., Kagami, H. and Tagami, T. (2007). Migration and proliferation of primordial germ cells in the early chicken embryo. *Poult. Sci.* **86**, 2182-2193. doi:10.1093/ps/86.10.2182

Ni, W., Qiao, J., Hu, S., Zhao, X., Regouski, M., Yang, M., Polejaeva, I. A. and Chen, C. (2014). Efficient gene knockout in goats using CRISPR/Cas9 system. *PLoS ONE* **9**, e106718. doi:10.1371/journal.pone.0106718

Niu, Y., Zhao, X., Zhou, J., Li, Y., Huang, Y., Cai, B., Liu, Y., Ding, Q., Zhou, S., Zhao, J. et al. (2018). Efficient generation of goats with defined point mutation (I397V) in *GDF9* through CRISPR/Cas9. *Reprod. Fertil. Dev.* **30**, 307-312. doi:10.1071/RD17068

Ogawa, T., Dobrinski, I., Avarbock, M. R. and Brinster, R. L. (2000). Transplantation of male germ line stem cells restores fertility in infertile mice. *Nat. Med.* **6**, 29-34. doi:10.1038/71496

Ohta, H., Kurimoto, K., Okamoto, I., Nakamura, T., Yabuta, Y., Miyauchi, H., Yamamoto, T., Okuno, Y., Hagiwara, M., Shirane, K. et al. (2017). In vitro expansion of mouse primordial germ cell-like cells recapitulates an epigenetic blank slate. *EMBO J.* **36**, 1888-1907. doi:10.15252/embj.201695862

Oishi, I., Yoshii, K., Miyahara, D., Kagami, H. and Tagami, T. (2016). Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Sci. Rep.* **6**, 23980. doi:10.1038/srep23980

Park, K.-E., Kaucher, A. V., Powell, A., Waqas, M. S., Sandmaier, S. E. S., Oatley, M. J., Park, C.-H., Tibary, A., Donovan, D. M., Blomberg, L. A. et al. (2017a). Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. *Sci. Rep.* **7**, 40176. doi:10.1038/srep40176

Park, K.-E., Powell, A., Sandmaier, S. E. S., Kim, C.-M., Mileham, A., Donovan, D. M. and Telugu, B. P. (2017b). Targeted gene knock-in by CRISPR/Cas ribonucleoproteins in porcine zygotes. *Sci. Rep.* **7**, 42458. doi:10.1038/srep42458

Park, T. S., Park, J., Lee, J. H., Park, J.-W. and Park, B.-C. (2019). Disruption of G0/G1 switch gene 2 (G0S2) reduced abdominal fat deposition and altered fatty acid composition in chicken. *FASEB J.* **33**, 1188-1198. doi:10.1096/fj.201800784R

Paschon, D. E., Lussier, S., Wangzor, T., Xia, D. F., Li, P. W., Hinkley, S. J., Scarlott, N. A., Lam, S. C., Waite, A. J., Truong, L. N. et al. (2019). Diversifying the structure of zinc finger nucleases for high-precision genome editing. *Nat. Commun.* **10**, 1133. doi:10.1038/s41467-019-08867-x

Peng, J., Wang, Y., Jiang, J., Zhou, X., Song, L., Wang, L., Ding, C., Qin, J., Liu, L., Wang, W. et al. (2015). Production of human albumin in pigs through CRISPR/Cas9-mediated knockin of human cDNA into swine albumin locus in the zygotes. *Sci. Rep.* **5**, 16705. doi:10.1038/srep16705

Proudfoot, C., Carlson, D. F., Huddart, R., Long, C. R., Pryor, J. H., King, T. J., Lillico, S. G., Mileham, A. J., McLaren, D. G., Whitelaw, C. B. A. et al. (2015). Genome edited sheep and cattle. *Trans. Res.* **24**, 147-153. doi:10.1007/s11248-014-9832-x

Pszczola, M. and Calus, M. P. L. (2016). Updating the reference population to achieve constant genomic prediction reliability across generations. *Animal* **10**, 1018-1024. doi:10.1017/S1751731115002785

Qian, L., Tang, M., Yang, J., Wang, Q., Cai, C., Jiang, S., Li, H., Jiang, K., Gao, P., Ma, D. et al. (2015). Targeted mutations in myostatin by zinc-finger nucleases result in double-muscled phenotype in Meishan pigs. *Sci. Rep.* **5**, 14435. doi:10.1038/srep14435

Qin, W., Dion, S. L., Kutny, P. M., Zhang, Y., Cheng, A. W., Jillette, N. L., Malhotra, A., Geurts, A. M., Chen, Y.-G. and Wang, H. (2015). Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. *Genetics* **200**, 423-430. doi:10.1534/genetics.115.176594

Qin, Z., Li, Y., Su, B., Cheng, Q., Ye, Z., Perera, D. A., Fobes, M., Shang, M. and Dunham, R. A. (2016). Editing of the luteinizing hormone gene to sterilize channel catfish, *Ictalurus punctatus*, using a modified zinc finger nuclease technology with electroporation. *Mar. Biotech.* **18**, 255-263. doi:10.1007/s10126-016-9687-7

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281-2308. doi:10.1038/nprot.2013.143

Rao, S., Fujimura, T., Matsunari, H., Sakuma, T., Nakano, K., Watanabe, M., Asano, Y., Kitagawa, E., Yamamoto, T. and Nagashima, H. (2016). Efficient modification of the myostatin gene in porcine somatic cells and generation of knockout piglets. *Mol. Reprod. Dev.* **83**, 61-70. doi:10.1002/mrd.22591

Ren, X., Yang, Z., Mao, D., Chang, Z., Qiao, H.-H., Wang, X., Sun, J., Hu, Q., Cui, Y., Liu, L.-P. et al. (2014). Performance of the Cas9 nickase system in *Drosophila melanogaster*. *G3* **4**, 1955-1962. doi:10.1534/g3.114.013821

Sang, H. (2004). Prospects for transgenesis in the chick. *Mech. Dev.* **121**, 1179-1186. doi:10.1016/j.mod.2004.05.012

Sasaki, K., Yokobayashi, S., Nakamura, T., Okamoto, I., Yabuta, Y., Kurimoto, K., Ohta, H., Moritoki, Y., Iwatani, C., Tsuchiya, H. et al. (2015). Robust *in vitro* induction of human germ cell fate from pluripotent stem cells. *Cell Stem Cell* **17**, 178-194. doi:10.1016/j.stem.2015.06.014

Savic, N., Ringnalda, F. C. A. S., Lindsay, H., Berk, C., Bargsten, K., Li, Y., Neri, D., Robinson, M. D., Ciaudo, C., Hall, J. et al. (2018). Covalent linkage of the DNA repair template to the CRISPR-Cas9 nuclease enhances homology-directed repair. *eLife* **7**, e33761. doi:10.7554/eLife.33761

Shirzeyli, M. H., Khanlarkhani, N., Amidi, F., Shirzeyli, F. H., Aval, F. S. and Sobhani, A. (2017). Bones Morphogenic Protein-4 and retinoic acid combined treatment comparative analysis for in vitro differentiation potential of murine mesenchymal stem cells derived from bone marrow and adipose tissue into germ cells. *Microsc. Res. Tech.* **80**, 1151-1160. doi:10.1002/jemt.22880

Shlush, E., Maghen, L., Swanson, S., Kenigsberg, S., Moskovtsev, S., Barretto, T., Gauthier-Fisher, A. and Librach, C. L. (2017). In vitro generation of Sertoli-like and haploid spermatid-like cells from human umbilical cord perivascular cells. *Stem. Cell Res. Ther.* **8**, 37. doi:10.1186/s13287-017-0491-8

Smith, L. B., O'Shaughnessy, P. J. and Rebourcet, D. (2015). Cell-specific ablation in the testis: what have we learned? *Andrology* **3**, 1035-1049. doi:10.1111/andr.12107

Song, S., Zhu, M., Yuan, Y., Rong, Y., Xu, S., Chen, S., Mei, J. and Cheng, Y. (2016). [BLG gene knockout and hLF gene knock-in at BLG locus in goat by TALENs]. *Sheng Wu Gong Cheng Xue Bao* **32**, 329-338.

Su, X., Cui, K., Du, S., Li, H., Lu, F., Shi, D. and Liu, Q. (2018a). Efficient genome editing in cultured cells and embryos of Debao pig and swamp buffalo using the CRISPR/Cas9 system. *In Vitro Cell. Dev. Biol. Anim.* **54**, 375-383. doi:10.1007/s11626-018-0236-8

Su, X., Wang, S., Su, G., Zheng, Z., Zhang, J., Ma, Y., Liu, Z., Zhou, H., Zhang, Y. and Zhang, L. (2018b). Production of microhomologous-mediated site-specific integrated LacS gene cow using TALENs. *Theriogenology* **119**, 282-288. doi:10.1016/j.theriogenology.2018.07.011

Suzuki, A., Tsuda, M. and Saga, Y. (2007). Functional redundancy among Nanos proteins and a distinct role of Nanos2 during male germ cell development. *Development* **134**, 77. doi:10.1242/dev.02697

Tan, W., Carlson, D. F., Lancto, C. A., Garbe, J. R., Webster, D. A., Hackett, P. B. and Fahrenkrug, S. C. (2013). Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proc. Natl. Acad. Sci. USA* **110**, 16526-16531. doi:10.1073/pnas.1310478110

Tan, W., Proudfoot, C., Lillico, S. G. and Whitelaw, C. B. (2016). Gene targeting, genome editing: from Dolly to editors. *Trans. Res.* **25**, 273-287. doi:10.1007/s11248-016-9932-x

Tanihara, F., Takemoto, T., Kitagawa, E., Rao, S., Do, L. T. K., Onishi, A., Yamashita, Y., Kosugi, C., Suzuki, H., Sembon, S. et al. (2016). Somatic cell reprogramming-free generation of genetically modified pigs. *Sci. Adv.* **2**, e1600803. doi:10.1126/sciadv.1600803

Tanihara, F., Hirata, M., Nguyen, N. T., Le, Q. A., Hirano, T., Takemoto, T., Nakai, M., Fuchimoto, D.-I. and Otoi, T. (2018). Generation of a TP53-modified porcine cancer model by CRISPR/Cas9-mediated gene modification in porcine zygotes via electroporation. *PLoS ONE* **13**, e0206360. doi:10.1371/journal.pone.0206360

Taylor, L., Carlson, D. F., Nandi, S., Sherman, A., Fahrenkrug, S. C. and McGrew, M. J. (2017). Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development* **144**, 928-934. doi:10.1242/dev.145367

Tröder, S. E., Ebert, L. K., Butt, L., Assenmacher, S., Schermer, B. and Zevnik, B. (2018). An optimized electroporation approach for efficient CRISPR/Cas9 genome editing in murine zygotes. *PLoS ONE* **13**, e0196891. doi:10.1371/journal.pone.0196891

Tyack, S. G., Jenkins, K. A., O'Neil, T. E., Wise, T. G., Morris, K. R., Bruce, M. P., McLeod, S., Wade, A. J., McKay, J., Moore, R. J. et al. (2013). A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells. *Trans. Res.* **22**, 1257-1264. doi:10.1007/s11248-013-9727-2

Vahdati, A., Fathi, A., Hajihoseini, M., Aliborzi, G. and Hosseini, E. (2017). The regenerative effect of bone marrow-derived stem cells in spermatogenesis of infertile hamster. *World J. Plast. Surg.* **6**, 18-25.

Van Eenennaam, A. L. (2018). The importance of a novel product risk-based trigger for gene-editing regulation in food animal species. *CRISPR J.* **1**, 101-106. doi:10.1089/crispr.2017.0023

Van Eenennaam, A. L. and Muir, W. M. (2011). Transgenic salmon: a final leap to the grocery shelf? *Nat. Biotechnol.* **29**, 706-710. doi:10.1038/nbt.1938

Van Eenennaam, A. L., Weigel, K. A., Young, A. E., Cleveland, M. A. and Dekkers, J. C. M. (2014). Applied animal genomics: results from the field. *Annu. Rev. Anim. Biosci.* **2**, 105-139. doi:10.1146/annurev-animal-022513-114119

Van Eenennaam, A. L., Wells, K. D. and Murray, J. D. (2019). Proposed U.S. regulation of gene-edited food animals is not fit for purpose. *NPJ Sci. Food* **3**, 3. doi:10.1038/s41538-019-0035-y

VandeHaar, M. J. and St-Pierre, N. (2006). Major advances in nutrition: relevance to the sustainability of the dairy industry. *J. Dairy Sci.* **89**, 1280-1291. doi:10.3168/jds.S0022-0302(06)72196-8

Wang, K., Ouyang, H., Xie, Z., Yao, C., Guo, N., Li, M., Jiao, H. and Pang, D. (2015a). Efficient generation of myostatin mutations in pigs using the CRISPR/Cas9 system. *Sci. Rep.* **5**, 16623. doi:10.1038/srep16623

Wang, X., Yu, H., Lei, A., Zhou, J., Zeng, W., Zhu, H., Dong, Z., Niu, Y., Shi, B., Cai, B. et al. (2015b). Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. *Sci. Rep.* **5**, 13878. doi:10.1038/srep13878

Wang, H., Xiang, J., Zhang, W., Li, J., Wei, Q., Zhong, L., Ouyang, H. and Han, J. (2016a). Induction of germ cell-like cells from porcine induced pluripotent stem cells. *Sci. Rep.* **6**, 27256. doi:10.1038/srep27256

Wang, X., Niu, Y., Zhou, J., Yu, H., Kou, Q., Lei, A., Zhao, X., Yan, H., Cai, B., Shen, Q. et al. (2016b). Multiplex gene editing via CRISPR/Cas9 exhibits desirable muscle hypertrophy without detectable off-target effects in sheep. *Sci. Rep.* **6**, 32271. doi:10.1038/srep32271

Wang, K., Tang, X., Xie, Z., Zou, X., Li, M., Yuan, H., Guo, N., Ouyang, H., Jiao, H. and Pang, D. (2017). CRISPR/Cas9-mediated knockout of myostatin in Chinese indigenous Erhualian pigs. *Trans. Res.* **26**, 799-805. doi:10.1007/s11248-017-0044-z

Wargelius, A., Leininger, S., Skaftnesmo, K. O., Kleppe, L., Andersson, E., Taranger, G. L., Schulz, R. W. and Edvardsen, R. B. (2016). Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *Sci. Rep.* **6**, 21284. doi:10.1038/srep21284

Wei, Y., Fang, J., Cai, S., Lv, C., Zhang, S. and Hua, J. (2016). Primordial germ cell-like cells derived from canine adipose mesenchymal stem cells. *Cell Prolif.* **49**, 503-511. doi:10.1111/cpr.12271

Wei, J., Wagner, S., Maclean, P., Brophy, B., Cole, S., Smolenski, G., Carlson, D. F., Fahrenkrug, S. C., Wells, D. N. and Laible, G. (2018). Cattle with a precise, zygote-mediated deletion safely eliminate the major milk allergen beta-lactoglobulin. *Sci. Rep.* **8**, 7661. doi:10.1038/s41598-018-25654-8

Whelan, A. I. and Lema, M. A. (2015). Regulatory framework for gene editing and other new breeding techniques (NBTs) in Argentina. *GM Crops Food* **6**, 253-265. doi:10.1080/21645698.2015.1114698

Whitworth, K. M., Lee, K., Benne, J. A., Beaton, B. P., Spate, L. D., Murphy, S. L., Samuel, M. S., Mao, J., O'Gorman, C., Walters, E. M. et al. (2014). Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. *Biol. Reprod.* **91**, 78. doi:10.1095/biolreprod.114.121723

Whitworth, K. M., Rowland, R. R. R., Petrovan, V., Sheahan, M., Cino-Ozuna, A. G., Fang, Y., Hesse, R., Mileham, A., Samuel, M. S., Wells, K. D. et al. (2019). Resistance to coronavirus infection in amino peptidase N-deficient pigs. *Trans. Res.* **28**, 21-32. doi:10.1007/s11248-018-0100-3

Wight, A. J. (2018). Strict EU ruling on gene-edited crops squeezes science. *Nature* **563**, 15-16. doi:10.1038/d41586-018-07166-7

Wu, H., Wang, Y., Zhang, Y., Yang, M., Lv, J., Liu, J. and Zhang, Y. (2015). TALE nickase-mediated SP110 knockin endows cattle with increased resistance to tuberculosis. *Proc. Natl. Acad. Sci. USA* **112**, E1530. doi:10.1073/pnas.1421587112

Xiang, G., Ren, J., Hai, T., Fu, R., Yu, D., Wang, J., Li, W., Wang, H. and Zhou, Q. (2018). Editing porcine IGF2 regulatory element improved meat production in Chinese Bama pigs. *Cell. Mol. Life Sci.* **75**, 4619-4628. doi:10.1007/s00018-018-2917-6

Xie, Z., Pang, D., Yuan, H., Jiao, H., Lu, C., Wang, K., Yang, Q., Li, M., Chen, X., Yu, T. et al. (2018). Genetically modified pigs are protected from classical swine fever virus. *PLoS Pathog.* **14**, e1007193. doi:10.1371/journal.ppat.1007193

Yamanaka, S. and Siomi, H. (2014). diRNA-Ago2-RAD51 complexes at double-strand break sites. *Cell Res.* **24**, 511-512. doi:10.1038/cr.2014.45

Yan, G., Fan, Y., Li, P., Zhang, Y. L. and Wang, F. (2015). Ectopic expression of DAZL gene in goat bone marrow-derived mesenchymal stem cells enhances the trans-differentiation to putative germ cells compared to the exogenous treatment of retinoic acid or bone morphogenetic protein 4 signalling molecules. *Cell Biol. Int.* **39**, 74-83. doi:10.1002/cbin.10348

Yang, H., Zhang, J., Zhang, X., Shi, J., Pan, Y., Zhou, R., Li, G., Li, Z., Cai, G. and Wu, Z. (2018). CD163 knockout pigs are fully resistant to highly pathogenic porcine reproductive and respiratory syndrome virus. *Antivir. Res.* **151**, 63-70. doi:10.1016/j.antiviral.2018.01.004

Yu, S., Luo, J., Song, Z., Ding, F., Dai, Y. and Li, N. (2011). Highly efficient modification of beta-lactoglobulin (BLG) gene via zinc-finger nucleases in cattle. *Cell Res.* **21**, 1638-1164. doi:10.1038/cr.2011.153

Yu, B., Lu, R., Yuan, Y., Zhang, T., Song, S., Qi, Z., Shao, B., Zhu, M., Mi, F. and Cheng, Y. (2016). Efficient TALEN-mediated myostatin gene editing in goats. *BMC Dev. Biol.* **16**, 26. doi:10.1186/s12861-016-0126-9

Zhang, D., Liu, X., Peng, J., He, D., Lin, T., Zhu, J., Li, X., Zhang, Y. and Wei, G. (2014). Potential spermatogenesis recovery with bone marrow mesenchymal stem cells in an azoospermic rat model. *Int. J. Mol. Sci.* **15**, 13151-13165. doi:10.3390/ijms150813151

Zhang, X., Li, W., Liu, C., Peng, X., Lin, J., He, S., Li, X., Han, B., Zhang, N., Wu, Y. et al. (2017). Alteration of sheep coat color pattern by disruption of ASIP gene via CRISPR Cas9. *Sci. Rep.* **7**, 8149. doi:10.1038/s41598-017-08636-0

Zhang, H., Pan, H., Zhou, C., Wei, Y., Ying, W., Li, S., Wang, G., Li, C., Ren, Y., Li, G. et al. (2018a). Simultaneous zygotic inactivation of multiple genes in mouse through CRISPR/Cas9-mediated base editing. *Development* **145**, dev168906. doi:10.1242/dev.168906

Zhang, J., Cui, M.-L., Nie, Y.-W., Dai, B., Li, F.-R., Liu, D.-J., Liang, H. and Cang, M. (2018b). CRISPR/Cas9-mediated specific integration of fat-1 at the goat MSTN locus. *FEBS J.* **285**, 2828-2839. doi:10.1111/febs.14520

Zhang, Y., Wang, Y., Yulin, B., Tang, B., Wang, M., Zhang, C., Zhang, W., Jin, J., Li, T., Zhao, R. et al. (2019). CRISPR/Cas9-mediated sheep MSTN gene

knockout and promote sSMSCs differentiation. *J. Cell. Biochem.* **120**, 1794-1806. doi:10.1002/jcb.27474

Zheng, Q., Lin, J., Huang, J., Zhang, H., Zhang, R., Zhang, X., Cao, C., Hambly, C., Qin, G., Yao, J. et al. (2017). Reconstitution of *UCP1* using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and improves thermogenic capacity. *Proc. Natl. Acad. Sci. USA* **114**, E9474-E9482. doi:10.1073/pnas.1707853114

Zhong, Z., Niu, P., Wang, M., Huang, G., Xu, S., Sun, Y., Xu, X., Hou, Y., Sun, X., Yan, Y. et al. (2016). Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp. *Sci. Rep.* **6**, 22953. doi:10.1038/srep22953

Zhou, Q., Wang, M., Yuan, Y., Wang, X., Fu, R., Wan, H., Xie, M., Liu, M., Guo, X., Zheng, Y. et al. (2016a). Complete meiosis from embryonic stem cell-derived germ cells *in vitro*. *Cell Stem Cell* **18**, 330-340. doi:10.1016/j.stem.2016.01.017

Zhou, X., Wang, L., Du, Y., Xie, F., Li, L., Liu, Y., Liu, C., Wang, S., Zhang, S., Huang, X. et al. (2016b). Efficient generation of gene-modified pigs harboring precise orthologous human mutation via CRISPR/Cas9-induced homology-directed repair in zygotes. *Hum. Mutat.* **37**, 110-118. doi:10.1002/humu.22913

Zhou, W., Wan, Y., Guo, R., Deng, M., Deng, K., Wang, Z., Zhang, Y. and Wang, F. (2017). Generation of beta-lactoglobulin knock-out goats using CRISPR/Cas9. *PLoS ONE* **12**, e0186056. doi:10.1371/journal.pone.0186056

Zhu, H., Liu, J., Cui, C., Song, Y., Ge, H., Hu, L., Li, Q., Jin, Y. and Zhang, Y. (2016). Targeting human α-lactalbumin gene insertion into the goat β-lactoglobulin locus by TALEN-mediated homologous recombination. *PLoS ONE* **11**, e0156636. doi:10.1371/journal.pone.0156636

Zou, Y., Li, Z., Zou, Y., Hao, H., Li, N. and Li, Q. (2018). An FBXO40 knockout generated by CRISPR/Cas9 causes muscle hypertrophy in pigs without detectable pathological effects. *Biochem. Biophys. Res. Commun.* **498**, 940-945. doi:10.1016/j.bbrc.2018.03.085

Zuidhof, M. J., Schneider, B. L., Carney, V. L., Korver, D. R. and Robinson, F. E. (2014). Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. *Poult. Sci.* **93**, 2970-2982. doi:10.3382/ps.2014-04291