Livestock breeding for the twenty-first century: the promise of the editing revolution

Front. Agr. Sci. Eng.
https://doi.org/10.15302/J-FASE-2019304 Available online at http://journal.hep.com.cn/fase

REVIEW

Livestock breeding for the 21st century: the promise of the editing revolution

Chris PROUDFOOT, Gus MCFARLANE, Bruce WHITELAW, Simon LILLICO (✉)

The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK

Abstract In recent years there has been a veritable explosion in the use of genome editors to create site-specific changes, both *in vitro* and *in vivo*, to the genomes of a multitude of species for both basic research and biotechnology. Livestock, which form a vital component of most societies, are no exception. While selective breeding has been hugely successful at enhancing some production traits, the rate of progress is often slow and is limited to variants that exist within the breeding population. Genome editing provides the potential to move traits between breeds, in a single generation, with no impact on existing productivity or to develop *de novo* phenotypes that tackle intractable issues such as disease. As such, genome editors provide huge potential for ongoing livestock development programs in light of increased demand and disease challenge. This review will highlight some of the more notable agricultural applications of this technology in livestock.

Keywords cattle, pig, sheep, chicken, aquaculture, CRISPR

1 Introduction

Genetic selection was for millennia an unintended consequence of domestication, with sustained pressure linking reproductive fitness to desirable traits inevitably resulting in domesticated animals becoming both physically and genetically distinct from their wild ancestors. It is only relatively recently that humans have come to understand the sometimes-complex relationship between genotype and phenotype and exploit this in a more direct manner. Genomic selection tools now form the basis for many livestock breeding programs, and the associated productivity gains seen across the industry have been astounding. In parallel to genetic improvement programs, molecular biology tools have been developed that allow the introduction of precise alterations to the DNA *in situ*, enabling intentional *de novo* modification of the genome as opposed to selection for observed variants.

These are tools commonly referred to as genome editors and can be divided into three groups: ZFNs (zinc finger nucleases), TALENs (transcription activator-like effector nucleases) and CRISPR-Cas (clustered regularly interspaced short palindromic repeats—CRISPR-associated). ZFNs are proteins composed of a short array of zinc finger (ZF) DNA binding motifs fused to one half of the dimeric FokI nuclease. ZFNs are usually applied in pairs, with each of the ZF arrays binding one strand of the DNA, facilitating dimerization of the nuclease and cutting of the intervening DNA. ZFNs typically have good sequence specificity but are both challenging and expensive to produce, and as such are no longer the reagent of choice for most research groups. TALENs, like ZFNs, are composed of an array of programmable DNA binding domains fused to FokI, and like ZFNs are employed as pairs in order to cleave the target DNA. Unlike ZFNs, TALENs are relatively easy to design and cost-effective to construct using basic molecular biology reagents, and as such saw significant uptake. However, they have been largely supplanted by the CRISPR-Cas system. Unlike ZFNs and TALENs that rely on designed proteins to confer specificity for the DNA target sequence, CRISPR-Cas relies on a small RNA complexed to the Cas nuclease for target site recognition. As the binding specificity relies only on Watson-Crick base pairing, guide RNA design is simple and computational tools predicting both on- and off-target specificity abound[1]. Importantly, because the Cas nuclease component of the CRISPR-Cas system remains constant it is conceptually easy to multiplex editing events, making this toolset ideal for tackling genetic alterations involving multiple loci. CRISPR-like systems are being discovered

Received October 30, 2019; accepted November 18, 2019

Correspondence: simon.lillico@roslin.ed.ac.uk

in an ever increasing number of bacteria and archea and it is likely that initial discoveries have only scratched the surface of the available toolbox[2,3].

The number of publications reporting application of editing in model species broadly correlates with discovery of the tools and their ease of use. Before 2010, editing was almost a niche technique, limited to laboratories that could afford to work with ZFNs. Following the first description of the utility of TALENs[4] there was a dramatic increase in the utilization of editing relative to the more proven transgenic technologies, but it was not until the advent of the CRISPR system[5–7] that editing became a molecular biology mainstay. In the 7 years since the initial reports there has been continuous development of CRISPR tools, improving activity, specificity and utility and resulting in unprecedented uptake of the technology in laboratories across the globe. In the first 6 months of 2019 there were over 2500 research articles that relied on CRISPR as a core technique to deliver their scientific output.

2 Application in livestock

Given the extended generation time and increased costs associated with livestock research it is unsurprising that examples of genome editing in this field have lagged slightly behind the rodent community. While not the focus of this review, it is worth noting the first demonstration of editing an endogenous gene in a livestock species was for the purpose of developing pigs for xenotransplantation. In 2011, Hauschild et al.[8] reported ZFN-mediated disruption of the *GGTA1* gene encoding the enzyme α1,3-galactosyltransferase that adds Gal epitopes to the porcine cell surface. The researchers were able to employ negative selection for the presence of Gal on the transfected primary fibroblasts, dramatically improving the probability that the cloned piglets would be biallelic for gene disruption. As an alternative to cloning, edited animals have been produced by directly microinjecting mRNAs encoding the genome editor reagents into the cytoplasm of an early zygote[9]. Litter sizes and viability are good with this approach, and while early demonstrations had relatively low editing frequency, evolution of the tools and refinement in delivery has resulted in substantive improvements[10,11]. A combination of SCNT followed by zygote microinjection with Cas9 ribonucleoprotein (Cas9 protein pre-complexed with sgRNA) has also been shown to be a very efficient strategy for generation of genome edited pigs[12].

Disrupting genes by way of targeted non-homologous end joining (NHEJ) has proven a robust strategy for achieving defined phenotypic outcomes. However, the number of agricultural traits for which this will be a viable approach is likely to be limited, with more subtle changes to gene function a desirable outcome in most cases. In an age where access to large data sets comprising sequence information and/or biometrics is becoming increasingly common, understanding of the genome-phenome relationship for many traits is expanding. This will undoubtedly yield opportunities to move relevant allelic variants directly between breeds without the genetic drag associated with conventional breeding. Editor-mediated allele modification has been used to introduce natural variants observed in other breeds[13,14] and even other species[15], making these tools viable additions to ongoing breeding projects.

3 Welfare

Livestock in modern agricultural systems often engage in behaviors natural to their wild forebears. While some of these activities are perceived as beneficial to the welfare of the individuals and therefore often actively encouraged (e.g., active foraging or nesting behavior in pigs), others can be detrimental to both the animals and the farmers. This is exemplified in cattle, where natural behavior includes head butting. In beef cattle breeds that lack horns (termed polled) this is rarely problematic. However, in dairy breeds, where most cattle are naturally horned, significant physical harm can ensue. Consequently, it is common to burn out the horn buds of young calves with either heat or caustic pastes, or to physically cut the horns from older animals. In addition to being painful for the animals and causing stress to both the cattle and their handlers, these interventions incur a monetary cost. It is estimated that 14 million cattle are disbudded or dehorned annually in the USA alone. The genetics of polled are well understood, and it is possible to propagate polled alleles through a population using standard breeding processes. The problem with this approach is that dissemination of elite genetics in the dairy industry is predominantly through AI, and the dairy bulls with the highest breeding values are horned. Introduction of polled would, in the short-term at least, likely be at the detriment of productivity. Genome editing offers a potential solution to this tradeoff. TALENs have been used to introduce the Celtic polled (Pc) allele (duplication of 212 bp that replaces 10 bp) into fibroblasts derived from a horned dairy bull[16]. Cloning from these cells resulted in calves that were genetically identical to the father with the exception of the targeted locus, and crucially were phenotypically polled[13]. Subsequent whole-genome sequence analysis of polled bull RCI002 revealed that in addition to the biallelic introgression of Pc, one allele also contained an unintentional insertion of the plasmid HDR vector proximal to the target site[17]. This issue of unexpected plasmid insertion associated with editor-mediated targeting has been reported previously[15], and can be resolved simply by selection among second generation segregants. Indeed, semen from RCI002 was used to artificially inseminate a cohort of horned Hereford cows, resulting in all six calves displaying the polled phenotype (Pc is dominant). The Pc

and Pc-plasmid alleles segregated as expected during breeding, resulting in two offspring with Pc and four with Pc-plasmid[17].

4 Production traits

Myostatin is a negative regulator of skeletal muscle growth, and when it comes to monogenic livestock traits of large effect there are few that can compete. Breeds that have been selected for increased muscle mass, such as Belgian Blue cattle, have mutations within the coding sequence of the myostatin gene[18], while others, such as Texel sheep, have reduced myostatin expression due to altered non-coding sequence[19]. Given the striking phenotype that can be achieved by perturbing myostatin expression, coupled with its perceived value for agriculture, it is not surprising that it has now been edited in most major livestock species including cattle[20], sheep[21], pig[22] and goat[11]. It is important to note that such animals require a more energy-dense diet than comparators and as such are not suitable for all agricultural systems.

Several genes have been associated with prolificacy in sheep, including *FecB*, *FecG* and *FecX*. While allelic variants of *FecG* and *FecX* have both been associated with increased lambing rates in heterozygous females, homozygote females are infertile. By contrast, *FecB* has additive effect with homozygote females having a higher lambing rate than their heterozygous counterparts[23]. A mutation in the 3′UTR of the *FecB* gene has similarly been associated with increased prolificacy in goats[24]. Improved lambing rate is desirable in some husbandry systems, and a CRISPR/Cas-induced point mutation has been used to introduce the Q249R mutation into the *FecB* of Chinese Tan sheep[14].

Milk is an excellent source of nutrition, but milk from livestock contains a number of proteins known to be associated with allergies (as opposed to lactose, which is associated with intolerance), including the most abundant proteins in milk such as the caseins, α-lactalbumin and β-lactoglobulin. Bovine milk is one of the most common food allergens, affecting up to 3% of infants globally, with milk from sheep, goats and buffalo also having the potential to elicit an adverse immune response. A cow lacking β-lactoglobulin was engineered by inducing a small, targeted deletion and a premature stop codon proximal to translation start of the gene[25]. Analysis of milk from this animal confirmed that β-lactoglobulin was no longer expressed. Similar abrogation of β-lactoglobulin expression has been demonstrated in goats[26,27]. It is likely implausible at this time to remove all potential allergens from milk, as simple deletion of the respective genes would likely cause significant detriment to milk production. However, the work above clearly demonstrates the potential for generating milk and milk products with reduced allergenic potential.

5 Disease resistance

Disease burden is one of the most significant challenges to livestock husbandry. Where available, appropriate vaccines can significantly reduce losses, but cost implications and the presence of a reliable cold-chain can prove significant barriers to application in some regions. There are additional logistical challenges to vaccination out-with commercial settings. Many viral infections are associated with secondary bacterial infections, contributing significantly to use of antimicrobials in agriculture.

Porcine reproductive and respiratory syndrome (PRRS) is a global disease of pigs resulting in significant economic losses to the swine industry. The two main genotypes of PRRS virus (PRRSV) share about 60% sequence identity, with further genetic disparity between isolates within each genotype. Control is generally with modified live vaccines, but cross-protection between viral strains is poor[28]. *In vitro* studies identified CD163, a member of the scavenger receptor cysteine-rich superfamily, as necessary for establishment of a productive viral infection[29]. Production of edited pigs with a functional knockout of porcine CD163 demonstrated for the first time *in vivo* a robust genetic approach to confer resistance to PRRSV infection[30]. The CD163 protein has a number of important biological functions including hemoglobin/haptoglobin recycling. CD163 presents nine extracellular domains on the surface of monocytes and macrophages, with the virus interacting specifically with domain number five. By deleting exon 7, which encodes the entirety of domain five, it proved possible to produce edited pigs that expressed a modified CD163 protein that retained these functions. Crucially, these pigs were also resistant to challenge with PRRSV[31].

Aminopeptidase N (AP-N, also known as CD13), a zinc-dependent type II metalloprotease situated on the plasma membrane of many cell types, cleaves N-terminal neutral amino acids. It is involved in a variety of cellular processes including peptide metabolism, angiogenesis, cell motility and adhesion, and has elevated expression in many cancers. It is enriched on the apical membrane of enterocytes where it has a role in the digestion of peptides. AP-N was identified as a putative receptor for the porcine coronavirus transmissible gastroenteritis virus (TGEV)[32], and the human coronavirus 229E[33]. Despite subsequent *in vitro* experiments supporting these associations, *in vivo* biological relevance remained unproven. Injection of porcine zygotes with CRISPR/Cas9 reagents targeting the first coding exon of *ANPEP*, the gene encoding *AP-N*, resulted in piglets lacking *AP-N* expression[34]. Challenge of these animals with TGEV confirmed that AP-N was indeed required for productive viral infection. AP-N is involved in a several normal biological processes, and while no adverse effect associated with *AP-N* knockout was observed in the null pigs it is likely that an approach akin to the CD163 domain deletion, whereby viral entry is

inhibited and the functions of the remaining protein retained, would be beneficial for production animals.

In vitro studies also identified AP-N as the putative entry receptor for porcine epidemic diarrhea virus (PEDV)[35,36], while other research contradicted these findings[37,38]. Challenge of the AP-N null piglets categorically demonstrated that AP-N was not required for PEDV infection in pigs.

6 Aquaculture

The field of aquaculture was quick to adopt genome editor technology, with many groups reporting on its application in a diverse range of species (reviewed in Gratacap et al.[39]). Li and colleagues were among the first groups to highlight this potential[40], demonstrating efficient editing of a number of genes in Nile tilapia, one of the most important aquaculture species globally. The establishment of effective culture conditions for pluripotent tilapia spermatogonial stem cells (SSCs) could prove to be a significant development[41], as it offers the opportunity to sequentially introduce more complex changes into the germline than would be practical by the direct injection of the reagents into zygotes.

As discussed above for mammals, myostatin has also proven a popular target in fish. Knockout of myostatin a (*MSTNa*) in channel catfish[42] or myostatin ba (*MSTNba*) in common carp[43] resulted in an enhanced growth rate, with corresponding increases in size and number of muscle fibers. It should be noted that while mammals have a single *MSTN* gene, tetraploid species such as the carp have a duplication(s) resulting in two or more copies of myostatin. In addition to expression in skeletal muscle, myostatin in fish has been reported in numerous other tissues, suggesting functions beyond regulation of muscle growth. Indeed, knockout of both genes in zebrafish is associated with impaired function of the immune system[44]. Encouragingly for those working in this sector, the Argentine National Advisory Commission on Agricultural Biotechnology has recently ruled that AquaBounty's FLT 01 line of gene-edited tilapia will not be regulated as a GMO, as they contain no new DNA.

Biosecurity is a longstanding concern in aquaculture, with the genetic identity and diversity of wild populations facing threat from escapees. Genome editing could further exacerbate this risk, but also offers potential solutions. By using the CRISPR/Cas9 system to knockout dead end (*dnd*), a gene required for primordial germ cell (PGC) formation, genetically infertile Atlantic salmon have been produced[45].

In the field of aquaculture, genome editing has not been limited to fish. Shrimp farming contributes billions of dollars to the global economy, and genome editing has been demonstrated in the ridgetail white prawn[46]. By microinjecting zygotes with Cas9 mRNA and a guide targeting the gene encoding molt-inhibiting hormone, researchers demonstrated a significantly reduced metamorphosis time in larvae and an increase in body length in adults. Proof-of-concept has also been demonstrated in oysters, with editing of either myostatin or the transcription factor TWIST2[47]. These diverse examples highlight the range of opportunities genome editing could offer in an agricultural sector that is rapidly expanding.

7 *In vitro* germline editing

In chickens the egg is laid about 24 h post fertilization, at which point the blastodermal disk is composed of around 60000 cells. Accessing the embryo before lay poses significant challenges, and this constraint to modification of the single-celled zygote has proven an obstacle to genetic alteration of poultry. At 48–60 h post-incubation chicken PGCs, the precursor cells of the gametes, migrate through the blood stream from the germinal crescent and begin to populate the developing gonads. These cells can be isolated, and *in vitro* culture conditions have been developed that allow their long-term propagation with retention of *in vivo* germline competence[48]. Germline transmission of transplanted PGCs ranged from $< 1\%$ to 86%, at least in part due to inherent competition with the endogenous PGCs of the recipient. Editor-mediated targeting of the *DDX4* gene in cultured PGCs enabled the production of sterile female chickens[49] and transfer of cultured PGCs to these surrogates *in ovo* resulted in all subsequent offspring being derived from the transplanted cells[50]. As with the tilapia SSCs discussed above, the ability to culture poultry PGCs for extended periods potentiates sequential editing of multiple genes.

In mammals, SSCs can be isolated from the testes of adult livestock. While there are numerous reports detailing culture and characterization of these cells, the definitive proof—functional colonization of a recipient gonad—remains lacking. As a step toward this goal, CRISPR-mediated disruption of the *Nanos2* gene was used to generate male pigs with an empty SSC niche[10]. As with the *DDX4* knockout chickens, these animals could improve frequency of germline transmission of transplanted SSCs by circumventing competition with endogenous cells.

8 Summary and future perspective

Genome editing has rapidly become a mainstream tool in biological research. The ability to generate site-specific changes to the genome allows researchers to ask fundamental questions about gene function, to move allelic variants between breeds or species, or to create novel phenotypes. Breaking a gene with these reagents to create functional knockouts is relatively simple (e.g., myostatin or ANPEP), as is inducing modest changes at the target site

Fig. 1 Genome editing in livestock. Genome editing has been reported in many major livestock species, enabling enhancement of the genetics underlying traits associated with welfare, production and disease resistance.

(e.g., CD163 exon 7 removal or Celtic polled insertion). The agricultural traits exploited with this technology thus far have involved single target loci with large effects, and while these have served as great exemplars of its potential there are a finite number of such low-hanging fruits. Many production traits are known to be influenced by a multitude of genes with much smaller effect. As understanding of the genome/phenome relationship develops, it seems likely that polygenic modification will become a desirable goal. Given the current momentum in both academic and commercial sectors to develop new and improved tools, multiplexed editing is edging closer to being a tenable solution with clear potential to positively impact genetic gain[51]. Time will tell if achieving robust multiplex editing will require cell-based protocols or if direct embryo injection will remain an optimal approach.

While genome editing clearly holds great potential, there remain relatively few examples of manipulation of endogenous genes of agricultural significance. This is, at least in part, due to a lack of verified targets. Many research groups are now employing genome-wide CRISPR-based screens to identify host genes that can be manipulated to improve disease resistance. Many different screens can be devised, from knocking out gene function through to elevating gene activity. In addition, to screen the entire gene repertoire, selective screens can be developed that test specific types of regulatory control elements or are combined with selective overexpression platforms. It is tempting to predict these approaches will quickly throw up many candidate loci, while recognizing that each candidate will probably require *in vivo* validation either through extensive genetic studies or by editing the candidate gene in the target animal. While application of such screens for disease resistance traits is conceptually simple, developing appropriate assays for other agricultural traits could prove significantly more challenging.

Genome editing technology attracts considerable public interest with voices both for and against. Opinions are likely to differ substantially depending on societal perspectives including but not limited to affluence, age, religion and geography. For many, issues related to technology do not elicit a yes or no response but a case-by-case deliberation. Reports of surveys of public opinion are starting to emerge[52–54]. These show general support for application of the technology, with a parallel cry for more information so that informed opinions can be developed[55]. Recognized as a topic of public interest, the Nuffield Council on Bioethics (UK) is currently preparing a report on 'Genome Editing and Farmed Animals' which will provide a yardstick on the ethical aspect of this technology in livestock.

Genome editing is here to stay. Initial projects have shown biological success with some progressing through the regulatory landscape. It is now time to expand the cohort of traits that can benefit from this transformative technology.

Compliance with ethics guidelines Chris Proudfoot, Gus McFarlane, Bruce Whitelaw, and Simon Lillico declare that they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

1. Torres-Perez R, Garcia-Martin J A, Montoliu L, Oliveros J C, Pazos F. WeReview: CRISPR tools—live repository of computational tools for assisting CRISPR/Cas experiments. *Bioengineering*, 2019, **6**(3): 63
2. Chaudhary K, Chattopadhyay A, Pratap D. The evolution of CRISPR/Cas9 and their cousins: hope or hype? *Biotechnology Letters*, 2018, **40**(3): 465–477
3. Pickar-Oliver A, Gersbach C A. The next generation of CRISPR-Cas technologies and applications. *Nature Reviews: Molecular Cell Biology*, 2019, **20**(8): 490–507
4. Christian M, Cermak T, Doyle E L, Schmidt C, Zhang F, Hummel A, Bogdanove A J, Voytas D F. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics*, 2010, **186**(2): 757–761
5. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna J A, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 2012, **337**(6096): 816–821
6. Cong L, Ran F A, Cox D, Lin S, Barretto R, Habib N, Hsu P D, Wu

X, Jiang W, Marraffini L A, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*, 2013, **339** (6121): 819–823

7. Mali P, Yang L, Esvelt K M, Aach J, Guell M, DiCarlo J E, Norville J E, Church G M. RNA-guided human genome engineering via Cas9. *Science*, 2013, **339**(6121): 823–826
8. Hauschild J, Petersen B, Santiago Y, Queisser A L, Carnwath J W, Lucas-Hahn A, Zhang L, Meng X, Gregory P D, Schwinzer R, Cost G J, Niemann H. Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proceedings of the National Academy of Sciences of the United States of America*, 2011, **108**(29): 12013–12017
9. 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, McLaren D G, Fahrenkrug S C, Whitelaw C B. Live pigs produced from genome edited zygotes. *Scientific Reports*, 2013, **3**(1): 2847
10. Park K E, Kaucher A V, Powell A, Waqas M S, Sandmaier S E, Oatley M J, Park C H, Tibary A, Donovan D M, Blomberg L A, Lillico S G, Whitelaw C B, Mileham A, Telugu B P, Oatley J M. Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. *Scientific Reports*, 2017, **7**(1): 40176
11. He Z, Zhang T, Jiang L, Zhou M, Wu D, Mei J, Cheng Y. Use of CRISPR/Cas9 technology efficiently targetted goat myostatin through zygotes microinjection resulting in double-muscled phenotype in goats. *Bioscience Reports*, 2018, **38**(6): BSR20180742
12. Sheets T P, Park C H, Park K E, Powell A, Donovan D M, Telugu B P. Somatic cell nuclear transfer followed by CRIPSR/Cas9 microinjection results in highly efficient genome editing in cloned pigs. *International Journal of Molecular Sciences*, 2016, **17**(12): 2031
13. Carlson D F, Lancto C A, Zang B, Kim E S, Walton M, Oldeschulte D, Seabury C, Sonstegard T S, Fahrenkrug S C. Production of hornless dairy cattle from genome-edited cell lines. *Nature Biotechnology*, 2016, **34**(5): 479–481
14. Zhou S, Yu H, Zhao X, Cai B, Ding Q, Huang Y, Li Y, Li Y, Niu Y, Lei A, Kou Q, Huang X, Petersen B, Ma B, Chen Y, Wang X. Generation of gene-edited sheep with a defined Booroola fecundity gene ($FecB^B$) mutation in bone morphogenetic protein receptor type 1B (*BMPR1B*) via clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) 9. *Reproduction, Fertility, and Development*, 2018, **30**(12): 1616–1621
15. 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, McLaren D G, Whitelaw C B. Mammalian interspecies substitution of immune modulatory alleles by genome editing. *Scientific Reports*, 2016, **6** (1): 21645
16. Tan W, Carlson D F, Lancto C A, Garbe J R, Webster D A, Hackett P B, Fahrenkrug S C. Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proceedings of the National Academy of Sciences of the United States of America*, 2013, **110**(41): 16526–16531
17. Young A E, Mansour T A, McNabb B R, Owen J R, Trott J F, Brown C T, van Eenennaam A L. Genomic and phenotypic analyses of six offspring of a genome-edited hornless bull. *Nature Biotechnology*, 2019 [Published Online] doi: 10.1038/s41587-019-0266-0
18. Grobet L, Royo Martin L J, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Ménissier F, Massabanda J, Fries R, Hanset R, Georges M. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nature Genetics*, 1997, **17**(1): 71–74
19. Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibé B, Bouix J, Caiment F, Elsen J M, Eychenne F, Larzul C, Laville E, Meish F, Milenkovic D, Tobin J, Charlier C, Georges M. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nature Genetics*, 2006, **38**(7): 813–818
20. 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, Fahrenkrug S C. Genome edited sheep and cattle. *Transgenic Research*, 2015, **24**(1): 147–153
21. Crispo M, Mulet A P, Tesson L, Barrera N, Cuadro F, dos Santos-Neto P C, Nguyen T H, Crénéguy A, Brusselle L, Anegón I, Menchaca A. Efficient generation of myostatin knock-out sheep using CRISPR/Cas9 technology and microinjection into zygotes. *PLoS One*, 2015, **10**(8): e0136690
22. Wang K, Ouyang H, Xie Z, Yao C, Guo N, Li M, Jiao H, Pang D. Efficient generation of myostatin mutations in pigs using the CRISPR/Cas9 system. *Scientific Reports*, 2015, **5**(1): 16623
23. Davis G H. Fecundity genes in sheep. *Animal Reproduction Science*, 2004, **82**–**83**: 247–253
24. Yue C, Bai W L, Zheng Y Y, Hui T Y, Sun J M, Guo D, Guo S L, Wang Z Y. Correlation analysis of candidate gene SNP for high-yield in Liaoning cashmere goats with litter size and cashmere performance. *Animal Biotechnology*, 2019 [Published Online] doi: 10.1080/10495398.2019.1652188
25. Wei J, Wagner S, Maclean P, Brophy B, Cole S, Smolenski G, Carlson D F, Fahrenkrug S C, Wells D N, Laible G. Cattle with a precise, zygote-mediated deletion safely eliminate the major milk allergen β-lactoglobulin. *Scientific Reports*, 2018, **8**(1): 7661
26. Cui C, Song Y, Liu J, Ge H, Li Q, Huang H, Hu L, Zhu H, Jin Y, Zhang Y. Gene targeting by TALEN-induced homologous recombination in goats directs production of β-lactoglobulin-free, high-human lactoferrin milk. *Scientific Reports*, 2015, **5**(1): 10482
27. Zhou W, Wan Y, Guo R, Deng M, Deng K, Wang Z, Zhang Y, Wang F. Generation of β-lactoglobulin knock-out goats using CRISPR/Cas9. *PLoS One*, 2017, **12**(10): e0186056
28. Nan Y, Wu C, Gu G, Sun W, Zhang Y J, Zhou E M. Improved vaccine against PRRSV: current progress and future perspective. *Frontiers in Microbiology*, 2017, **8**: 1635
29. Calvert J G, Slade D E, Shields S L, Jolie R, Mannan R M, Ankenbauer R G, Welch S K. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *Journal of Virology*, 2007, **81**(14): 7371–7379
30. Whitworth K M, Rowland R R, Ewen C L, Trible B R, Kerrigan M A, Cino-Ozuna A G, Samuel M S, Lightner J E, McLaren D G, Mileham A J, Wells K D, Prather R S. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. *Nature Biotechnology*, 2016, **34**(1): 20–22
31. Burkard C, Opriessnig T, Mileham A J, Stadejek T, Ait-Ali T, Lillico S G, Whitelaw C B A, Archibald A L. Pigs lacking the scavenger receptor cysteine-rich domain 5 of CD163 are resistant to porcine reproductive and respiratory syndrome virus 1 infection.

Journal of Virology, 2018, **92**(16): e0045-18

32. Delmas B, Gelfi J, L'Haridon R, Vogel L K, Sjöström H, Norén O, Laude H. Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature*, 1992, **357**(6377): 417–420
33. Yeager C L, Ashmun R A, Williams R K, Cardellichio C B, Shapiro L H, Look A T, Holmes K V. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature*, 1992, **357**(6377): 420–422
34. 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, Prather R S. Resistance to coronavirus infection in amino peptidase N-deficient pigs. *Transgenic Research*, 2019, **28**(1): 21–32
35. Oh J S, Song D S, Park B K. Identification of a putative cellular receptor 150 kDa polypeptide for porcine epidemic diarrhea virus in porcine enterocytes. *Journal of Veterinary Science*, 2003, **4**(3): 269–275
36. Kamau A N, Park J E, Park E S, Yu J E, Rho J, Shin H J. Porcine amino peptidase N domain VII has critical role in binding and entry of porcine epidemic diarrhea virus. *Virus Research*, 2017, **227**: 150–157
37. Shirato K, Maejima M, Islam M T, Miyazaki A, Kawase M, Matsuyama S, Taguchi F. Porcine aminopeptidase N is not a cellular receptor of porcine epidemic diarrhea virus, but promotes its infectivity via aminopeptidase activity. *Journal of General Virology*, 2016, **97**(10): 2528–2539
38. Li W, Luo R, He Q, van Kuppeveld F J M, Rottier P J M, Bosch B J. Aminopeptidase N is not required for porcine epidemic diarrhea virus cell entry. *Virus Research*, 2017, **235**: 6–13
39. Gratacap R L, Wargelius A, Edvardsen R B, Houston R D. Potential of genome editing to improve aquaculture breeding and production. *Trends in Genetics*, 2019, **35**(9): 672–684
40. Li M, Yang H, Zhao J, Fang L, Shi H, Li M, Sun Y, Zhang X, Jiang D, Zhou L, Wang D. Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. *Genetics*, 2014, **197**(2): 591–599
41. Santos Nassif Lacerda S M, Costa G M J, da Silva M A, Almeida Campos-Junior P H, Segatelli T M, Peixoto M T D, Resende R R, de França L R. Phenotypic characterization and *in vitro* propagation and transplantation of the Nile tilapia (*Oreochromis niloticus*) spermatogonial stem cells. *General and Comparative Endocrinology*, 2013, **192**: 95–106
42. Khalil K, Elayat M, Khalifa E, Daghash S, Elaswad A, Miller M, Abdelrahman H, Ye Z, Odin R, Drescher D, Vo K, Gosh K, Bugg W, Robinson D, Dunham R. Generation of myostatin gene-edited channel catfish (*Ictalurus punctatus*) via zygote injection of CRISPR/Cas9 system. *Scientific Reports*, 2017, **7**(1): 7301
43. Zhong Z, Niu P, Wang M, Huang G, Xu S, Sun Y, Xu X, Hou Y, Sun X, Yan Y, Wang H. Targeted disruption of *sp7* and *myostatin* with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp. *Scientific Reports*, 2016, **6**(1): 22953
44. Wang C, Chen Y L, Bian W P, Xie S L, Qi G L, Liu L, Strauss P R, Zou J X, Pei D S. Deletion of MSTNa and MSTNb impairs the immune system and affects growth performance in zebrafish. *Fish & Shellfish Immunology*, 2018, **72**: 572–580
45. Wargelius A, Leininger S, Skaftnesmo K O, Kleppe L, Andersson E, Taranger G L, Schulz R W, Edvardsen R B. *Dnd* knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *Scientific Reports*, 2016, **6**(1): 21284
46. Zhang J, Song F, Sun Y, Yu K, Xiang J. CRISPR/Cas9-mediated deletion of *EcMIH* shortens metamorphosis time from mysis larva to postlarva of *Exopalaemon carinicauda*. *Fish & Shellfish Immunology*, 2018, **77**: 244–251
47. Yu H, Li H, Li Q, Xu R, Yue C, Du S. Targeted gene disruption in pacific oyster based on CRISPR/Cas9 ribonucleoprotein complexes. *Marine Biotechnology*, 2019, **21**(3): 301–309
48. van de Lavoir M C, Diamond J H, Leighton P A, Mather-Love C, Heyer B S, Bradshaw R, Kerchner A, Hooi L T, Gessaro T M, Swanberg S E, Delany M E, Etches R J. Germline transmission of genetically modified primordial germ cells. *Nature*, 2006, **441**(7094): 766–769
49. Taylor L, Carlson D F, Nandi S, Sherman A, Fahrenkrug S C, McGrew M J. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development*, 2017, **144**(5): 928–934
50. Woodcock M E, Gheyas A A, Mason A S, Nandi S, Taylor L, Sherman A, Smith J, Burt D W, Hawken R, McGrew M J. Reviving rare chicken breeds using genetically engineered sterility in surrogate host birds. *Proceedings of the National Academy of Sciences of the United States of America*, 2019, **116**(42): 20930–20937
51. Jenko J, Gorjanc G, Cleveland M A, Varshney R K, Whitelaw C B A, Woolliams J A, Hickey J M. Potential of promotion of alleles by genome editing to improve quantitative traits in livestock breeding programs. *Genetics, Selection, Evolution*, 2015, **47**(1): 55
52. Eriksson S, Jonas E, Rydhmer L, Röcklinsberg H. Invited review: breeding and ethical perspectives on genetically modified and genome edited cattle. *Journal of Dairy Science*, 2018, **101**(1): 1–17
53. McConnachie E, Hötzel M J, Robbins J A, Shriver A, Weary D M, von Keyserlingk M A G. Public attitudes towards genetically modified polled cattle. *PLoS One*, 2019, **14**(5): e0216542
54. Yunes M C, Teixeira D L, von Keyserlingk M A G, Hötzel M J. Is gene editing an acceptable alternative to castration in pigs? *PLoS One*, 2019, **14**(6): e0218176
55. McPhetres J, Rutjens B T, Weinstein N, Brisson J A. Modifying attitudes about modified foods: increased knowledge leads to more positive attitudes. *Journal of Environmental Psychology*, 2019, **64**: 21–29