Genetic Modification of Food Animals

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Abstract 100-120 words

Animal breeders have used a variety of methods in selective breeding programs to genetically improve food animal species. Recently this has included the use of both genetic engineering and genome editing, particularly for targeting improvement in traits for which there is no within-species or within-breed genetic variation. Both intra- and interspecies allele substitutions and gene knock-ins have been accomplished with genome editing tools, targeting a number of important traits. The regulatory status of such animals is unclear as the definition of a regulated article is not consistent among different regulatory agencies and organizations. In the absence of a harmonized global regulatory approach to the genetic improvement of animals, it will be difficult for breeders to effectively achieve sustainable breeding objectives.

Introduction

Animal breeders have been directing livestock evolution since animals were first domesticated. Initially, the tools available to breeders were simply observations on what was perceived to be the desired appearance and the selective mating of chosen parents. One only needs to look at the differences between a Chihuahua and a Great Dane, relative to their wild ancestor the wolf, to appreciate that conventional selection is a powerful force for genetic change. Over time, the tools and methods used to make genetic improvement have advanced, and this has accelerated the rate of genetic change. Subjective observations were replaced by objective measurements, and sophisticated statistical methods were implemented to isolate heritable genetic effects from environmental influences [1]. The rate of genetic improvement was further accelerated by combining genetic selection methods with advanced reproductive technologies (ART) such as artificial insemination (AI) and embryo transfer (ET).

The impact of selective breeding programs on the footprint of agriculture and food production is difficult to overstate. It has been estimated that historic genetic improvement in selected traits (e.g. milk/meat output, growth efficiency) has resulted in a 1% per year reduction in greenhouse gas (GHG) emissions per unit food produced (e.g., a tonne of beef/sheep meat) [2]. Capper, et al. [3] compared the environmental footprint of US dairy production between 1944 and 2007 and reported that although the carbon footprint per individual cow had increased over time, the carbon footprint per unit of milk was 63% lower in 2007 than it was in 1944. Perhaps this point is best illustrated by calculating that, *sans* genetic improvement, we would need in excess of 30 million dairy cows in the United States to produce the amount of milk that 9 million cows were able to produce in 2014. Genetic improvement is undoubtedly one of the most powerful drivers of agricultural sustainability.

Given this history and the fact that conventional, or “artificial”, selection is largely uncontroversial, it might be expected that innovations in breeding methods would be mostly undisputed as self-evident approaches to help meet the projected increase in global animal protein demand. However, this is clearly not the case given the decades-old global debate [4] over the use of one particular breeding method, genetic engineering. Plants and animals that are destined for food and which have been genetically modified using this particular breeding method, although interestingly not those modified for medical purposes using exactly the same techniques, generate absolute moral opposition [5] and have been at the epicenter of a controversial and rancorous scientific debate. This review will discuss recent developments in the use of modern biotechnologies in food animal breeding programs.

Genetic Engineering

One definition of genetic engineering (GE) is a process in which recombinant DNA (rDNA) technology is used to introduce desirable traits into an organism. The real power of this technology is in enabling breeders to access genetic variation that is not otherwise normally present in the target species, especially for traits such as disease resistance. Genetically engineered animals were first produced in the late 1970s, and the first GE livestock were produced in 1985 [6]. Thirty years later, GE animals have been produced by researchers globally in many different food animal species and for a variety of target traits (Table 1), although to date none have successfully moved all the way from the laboratory to retail food outlets.

Species	Transgene	Origin	Trait/Goal
Cattle	Lysozyme, Lactoferrin	Human	Milk composition; animal health; mastitis resistance
	Prion Protein (PrP) shRNA	Knockout	Animal health
	α - κ -Casein	Bovine	Milk composition
	Omega-3 (Fat-1)	Nematode	Milk composition
	β -Casein miRNA	Cattle	Milk composition
	Lysostaphin	Bacterial	Mastitis resistance
	SP110	Murine	Bovine Tuberculosis resistance
	Myostatin shRNA	Knockout	Increased muscle yield
Chicken	alv6 envelope glycoprotein	Viral	Disease resistance
	short hairpin RNA	Viral	Disease resistance
	LacZ	Bacterial	Animal Health
Carp	Growth Hormone	Piscine	Growth rate
	Lactorferrin	Human	Disease resistance
Catfish	Cercopin B	Insect	Disease resistance
Goat	Lysozyme	Human-Bovine	Animal Health
	Stearoyl-CoA desaturase	Rat-Bovine	Mastitis resistance
	Lactoferrin	Human	Prophylactic treatment
	Human beta-defensin 3	Human	Milk composition
	Myostatin shRNA	Knockout	Increased muscle yield
	Prion Protein (PrP) shRNA	Knockout	Animal health
Pig	Phytase	<i>E. coli</i> -Mouse	Feed uptake; decreased phosphorus in manure
	Growth hormone, growth hormone releasing factor, insulin-like growth factor-1	Human-Porcine	Growth rate
	cSKI	Chicken	Muscle development
	Lysozyme	Human	Piglet survival
	Unsat. fat. acid (FAD2)	Spinach	Meat composition
	Omega-3 (Fat-1)	Nematode	Meat composition
	α -lactalbumin	Bovine	Piglet survival
	Mx, Iga, Mouse monoclonal antibody (mAb)	Murine	Disease Influenza resistance
Salmon	Growth hormone	Piscine	Growth rate
	Lysozyme	Piscine	Animal health
	wfIAFP-6	Piscine	Cold tolerance
Sheep	Growth hormone, growth hormone releasing factor,	Ovine	Growth rate
	IGF-1, wool intermediate filament keratin, CsK	Ovine, Bacterial	Wool growth
	Visna resistance	Viral	Disease resistance
	Omega-3 (Fat-1)	Nematode	Meat composition
	Prion Protein (PrP)	Knockout	Animal health
	Mouse monoclonal antibody	Murine	Disease Influenza resistance
Trout	Follistatin	Piscine	Muscle development

Table 1. Examples of genetically engineered (GE) food animals that have been produced for agricultural applications. Adapted from Lievens, et al. [7], and Laible et al. [8].

Unsurprisingly, many of the applications in Table 1 align with common animal breeding objectives targeted by conventional breeding programs. To date, the inefficiencies of producing GE animals using random integration following microinjection of an rDNA construct or viral transformation methods, along with the uncertainties and expense of the regulatory process, have impeded the commercialization of GE animals for food purposes [9]. Some GE food animals have been approved for biomedical pharmaceutical production including goats producing ATryn® (human antithrombin-III), rabbits producing Ruconest™ (Rhucin® outside the EU), and chickens producing Kanuma™ (sebelipase alfa). There have also been some trials using GE insects for pest control applications. Only a single GE animal, the fast-growing AquAdvantage Atlantic salmon, has been approved for food purposes. The founder of this GE fish line was generated in 1989 [10], and the product underwent a lengthy and unpredictable regulatory evaluation [11]. Although it was approved in December 2015 by the United States Food and Drug Administration (FDA), its future is still uncertain. Commercial sale of the fish for food is currently blocked by a pending federal bill introduced to the United States House of Representatives on March 4, 2016 requiring mandatory labeling of the product and an additional review of “the study of genetically modified salmon’s impact on wild salmon stocks carried out by the FDA” by an independent scientific organization.

Genome Editing Methods

Genome or gene editing refers to the use of site-directed nucleases (SDN) to precisely introduce a double stranded break (DSB) at a predetermined location in the genome. The cell can repair that DSB break in one of two ways – homologous recombination (HR) using a nucleic acid template that includes the sequences homologous to either side of the double-strand break, or nonhomologous end joining (NHEJ). The outcomes of these repair processes result in precision gene edits or random mutations, respectively. As the name “gene editing” suggests, HR can be employed to precisely add, delete, or replace letters in the genetic code at the location of the break by providing the appropriate template nucleic acid. In the case of NHEJ, although the location of the cut site is very precise, the exact change that occurs when the DNA is repaired is random and so a number of different outcomes representing minor sequence insertions (ins) or deletions (del), termed indels, are possible (Figure 1; [12]).

Gene editing has many potential applications. For example, it can be used to correct diseases and disorders that have a genetic basis by altering the error that resulted in the disease phenotype. It could also be used to change a less desirable allele of a gene to a more desirable allele without the need to introgress (repeatedly backcross) or bring in that allele through outcrossing with an animal that carries the desirable allele. Genome editing technologies enable breeders to efficiently turn off a gene through NHEJ or introduce the specific allelic variants [13,14], and conceptually entire genes or transgenes [15-17] as dictated by the HR template nucleic acid sequence, that they would like in their target population using SDN rather than employing “artificial” selection, cross breeding, and/or genetic engineering using random integration.

How might gene editing be used in animal breeding?

In the last 5 years, SDNs (zinc finger nucleases (ZFNs) transcription activator-like effector nucleases (TALENs), and clustered regulatory interspersed short palindromic repeats (CRISPRs)

associated system) have been used to mediate the generation of 300 gene edited pigs, cattle, sheep and goats [18]. Several recent reviews describe the potential to use these tools in food animals for agricultural purposes [8,9,18-21], and include detailed descriptions of their mechanics and relative efficiencies. Table 2 summarizes agricultural applications including product yield, animal health and welfare. Although there are some new traits being targeted using gene editing, many are in common with those listed in Table 1. This is because the goals of genetic improvement programs do not change with new tools; breeders tend to employ whichever breeding method(s) most effectively achieves progress towards their breeding objective(s). It is likely that gene editing will synergistically complement traditional breeding programs, rather than replace or radically disrupt them.

Species	Target	Publication	Trait/Goal
Cattle	Intraspecies <i>POLLED</i> allele substitution	Tan, et al. [22]; Carlson et al. [13]	No horns
	Myostatin KO	Proudfoot et al. [23] Luo et al. [24]	Increased muscle yield
	Beta-lactoglobulin KO	Yu et al. [25]	Elimination of milk allergen
	Lysostaphin transgene	Liu et al. [15]	Disease resistance
	Lysozyme transgene	Liu et al. [16]	Disease resistance
	SP110 transgene	Wu et al. [17]	Resistance to tuberculosis
Chicken	Ovalbumin KO	Park, et al. [26]	Elimination of ovalbumin in egg
	Immunoglobulin heavy chain locus	Dimitrov et al. [27]	Germline gene editing
Goat	Myostatin Prion protein KO Beta-lactoglobulin KO	Ni, et al. [28]	Increased muscle growth Elimination of prion protein Elimination of milk allergen
Pig	CD163 KO	Whitworth, et al. [29]	PRRS Virus Resistance
	RELA interspecies substitution	Lillico, et al. [14]	African Swine Fever Resistance
	Myostatin KO	Wang, et al. [30] Qian et al. [31]	Increased muscle yield
Sheep	Myostatin KO	Proudfoot et al. [23] Crispo et al., [32] Han et al. [33]	Increased muscle yield

Table 2. Examples of successful gene edited agricultural applications in food animal species. KO = knock out or inactivation of gene function.

How might gene editing intersect with conventional breeding?

To become an important driver of genetic change, gene editing methods must seamlessly integrate with conventional animal breeding programs. That means that they must reliably function to germline-edit animals that are selected to be the next generation of parents. Edits can be introduced through gene editing of somatic cells followed by somatic cell nuclear transfer (SCNT) cloning, or injection of the gene editing reagents into the cytoplasm of single cell zygotes of the next generation.

To date, SCNT has been the primary method to deliver nuclease-mediated genetic changes into livestock [18]. The advantage of SCNT is that the gene edited cell line can be genotyped and/or screened prior to transfer into the enucleated oocyte to ensure that the desired edits, and no off-target edits, have occurred. The disadvantage is that there are well-documented drawbacks associated with cloning including early embryonic losses, postnatal death, and birth defects [34].

Direct editing of zygotes is advantageous since it is applied to the next generation, but not all embryos will be edited. However, on average fewer embryos are required to gene edit a pig, for example, using this approach as compared to SCNT due to the losses associated with cloning [18]. Entire interspecies allele substitutions have been successfully knocked-in using direct editing of zygotes [14,35]. One problem associated with direct editing of zygotes is mosaicism [23,33,36], although groups are working on approaches to minimize this problem. A potential approach to incorporate genome editing into current breeding programs through the use of advanced reproductive technologies are outlined in Figure 2 [37].

Data coming out of some of the large-scale genomic and sequencing projects are revealing situations in which the sequence of one naturally-occurring allele results in superior performance to that observed when animals inherit an alternative allele of that gene. It is envisioned that it might be possible to edit an animal's genome to the superior allele, and to do that at several genomic locations, or for several different genes. Simultaneous targeting of different genes has allowed bi-allelic modification of up to three genes at the same time [38,39]. The advantage of gene editing over conventional selection to move these naturally-occurring alleles from one animal to another is that favorable alleles rarely all occur in one single individual and editing offers the opportunity to increase the frequency of desirable alleles in an individual or a breed more rapidly than could be achieved through conventional breeding, and in the absence of undesirable linkage drag.

One could potentially envision editing several alleles for different traits – such as known fertility impairing haplotypes [40], polled, and to correct known Mendelian genetic defects that affect cattle [41] – all while using conventional selection methods to keep making genetic progress towards a given selection objective. Gene editing offers an approach to translate the thousands of SNP markers discovered through livestock sequencing projects, the information obtained from numerous genome wide association studies, and the discovery of causative SNPs (Quantitative Trait Nucleotides; QTNs) into useful genetic variation for use in animal breeding programs [42]. One study reported that combining gene editing with traditional genomic selection could improve the response to selection four-fold after 20 generations [43]. In other words, editing will complement, not replace, conventional breeding programs.

It should be remembered that complex traits are typically impacted by many different genes. It is unlikely that all of the genes impacting such traits are known, nor is it typically evident which might be the desirable molecular edits for these genes (i.e. what is the sequence of the desirable allele). It is likely that editing will be focused on large effect loci and known targets to correct genetic defects or decrease disease susceptibility, and conventional selection will continue to make progress in selecting for all of the many small effect loci that impact the complex traits that contribute to the breeding objective.

Will gene editing be regulated?

Many agencies are involved with the regulation and governance of genetically engineered animals including the United States Food and Drug Administration (FDA), the European Medicine Agency (EMA), the European Food Safety Authority (EFSA), and the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO). The definition of a “genetically engineered” animal differs among these different agencies.

The Codex Alimentarius (Codex), or “Food Code”, was established by FAO and WHO to develop harmonised international food standards, which protect consumer health and promote fair practices in food trade. In 2008 the Codex developed the science-based “Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Animals (GL68-2008)” [44] which provides internationally-recognized recommendations for assessing the nutrition and safety of food from GE animals. In that document, a “Recombinant-DNA Animal” is defined as an animal in which the genetic material has been changed through *in vitro* nucleic acid techniques, including rDNA and direct injection of nucleic acid into cells or organelles. The Cartagena Protocol on Biosafety (CPB) is an international agreement which aims to ensure the safe handling, transport and use of any living modified organism. The CPB defines “Living modified organism” to mean any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology, and specifically excludes techniques used in traditional breeding and selection. Likewise, the EU definition of a genetically engineered organism included in Directive 2001/18/EC encompasses an “organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination”. And finally, the FDA defines “genetically engineered (GE) animals” as those animals modified by rDNA techniques, including the entire lineage of animals that contain the modification [45]. The rDNA construct in the GE animal is considered a new animal drug and thus is a regulated article under the new animal drug provisions of the Federal Food Drug and Cosmetics Act. All GE animals are captured under these provisions, regardless of their intended use.

It is clear that many gene editing applications will likely result in animals carrying desirable alleles or sequences that originated in other breeds or individuals from within that species. As such, there will be no rDNA construct in the animal and no genetic material that has been altered in a way that could not be achieved by mating or techniques used in traditional breeding and selection. As such, at the current time it is unclear whether gene editing will be formally regulated as is the case with animals containing rDNA constructs that are the more traditional products of genetic engineering.

Animal breeding per se is not regulated by the federal government, although it is illegal to sell an unsafe food product regardless of the breeding method that was used to produce it. Gene editing does not necessarily introduce any foreign genetic DNA or “transgenic sequences” into the genome, and many of the changes produced would be indistinguishable from naturally-occurring alleles and variations (e.g. Figure 1 SDN-1 mutations). As such, many applications will not fit the classical definition of genetic engineering. For example, many edits are likely to alter alleles of a given gene using a template nucleic acid dictated by the sequence of a naturally-occurring

allele from the same species (e.g. the hornless Holstein example described earlier used template sequence based on the polled allele from Angus) [13]. As such, there will be no novel DNA sequence present in the genome of the edited animal, and likewise no novel phenotype associated with that sequence. It is not evident what unique risks might be associated with an animal that is carrying such an allele given the exact same sequence and resulting phenotype would be observed in the closely-related breed from which the allele sequence was derived [46].

It is possible that nucleases might introduce double stranded breaks at locations other than the target locus, and thereby induce alterations elsewhere in the genome [47]. Such off-target events are analogous to spontaneous mutations and can be minimized by careful selection of the guide sequence that targets the specific DNA sequence to be cut as well as the design of the gene editing reagents [48]. There are groups working on ways to identify such off-target effects rapidly [49], and approaches to suppress off-target cleavages. Complete sequencing of polled calves derived from two independent cell lines to 20X coverage did not find any off-target introgression of the polled allele, nor any insertion-deletions (indels) ascribable to off-target DNA cleavage by the TALENs and subsequent repair by non-homologous end joining within 10 bp of any of the identified degenerate targets. These data suggest high specificity of the TALENs used to target the polled locus in this experiment [13].

Globally, governments and regulators are currently deliberating about how, or even if, gene-edited animals should be regulated. It is no coincidence that there have been a slew of recent policy papers from normally low-profile public sector breeders and academicians from around the world discussing the need for regulation of genome editing to be science-based, proportional to risk, product focused, and fit for purpose [46,50-54]. Most recently the US National Academy of Sciences [55] concluded that the distinction between conventional breeding and genetic engineering is becoming less obvious. Some emerging genetic engineering technologies have the potential to create novel varieties that are hard to distinguish genetically from varieties produced through conventional breeding or processes that occur in nature. They reasoned that conventionally bred varieties are associated with the same benefits and risks as genetically engineered varieties. They further concluded that a process-based regulatory approach is becoming less and less technically defensible as the old approaches to genetic engineering become less novel and as emerging processes — such as genome editing and synthetic biology — fail to fit current regulatory categories of genetic engineering. They recommended a tiered regulatory approach focused on any intended and unintended novel characteristics of the end product resulting from the breeding methods that may present potential hazards, rather than focusing regulation on the process or breeding method by which that genetic change was achieved.

It is likely that gene edited animals will be considered on a case-by-case basis depending upon the novelty of the edited DNA sequence and the resulting attributes or phenotypes displayed by the animal. There is a need to ensure that the extent of regulatory oversight is proportional to the unique risks, if any, associated with the novel phenotypes. This question is of course important from the point of view of technology development, innovation and international trade. Given there is currently not a single genetically engineered animal being sold for food anywhere in the world, animal breeders are perhaps the group most aware of the chilling impact that regulatory gridlock can have on the deployment of potentially valuable breeding techniques. While

regulation to ensure the safety of new technologies is necessary, in a world facing burgeoning animal protein demands, overregulation is an indulgence that global food security can ill afford.

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Simulation study of the impact genome editing could have to improve quantitative traits that are controlled by many loci through promotion of alleles by genome editing (PAGE). Response to selection after 20 generations was between one to more than four times higher with genomic selection combined with PAGE than with genomic selection alone. This study shows the potential of genome editing to accelerate the rate of genetic gain in traditional selection programs.

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This study examines 20 years of data on commercialized genetically engineered (GE) crops and discusses the field experience of the existing GE crops and those that are in development. Importantly, this report reviews all available data on the health and environmental impacts of commercialized GMO crops and determines that “the study committee found no substantiated evidence of a difference in risks to human health between current commercially available genetically engineered (GE) crops and conventionally bred crops, nor did it find conclusive cause-and-effect evidence of environmental problems from the GE crops.”

Figure Legends

Figure 1. Schematic representation of intended modification by site-directed nuclease (SDN) types. Double-stranded break repair can occur via non-homologous end-joining (NHEJ; SDN-1), or homologous recombination (HR) when a nucleic acid template is present (SDN-2; -3). Figure from [12].

Figure 2. Production of high genetic merit calves using a range of biotechnologies and showing where gene editing might fit into the process. Image modified from [37].

Figure 1

Figure 2