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Nicolas Rasmussen
Professor of History & Philosophy of Science
School of Humanities, University of New South Wales, Sydney, Australia

GENETICALLY MODIFIED ORGANISMS, ANIMALS

Animal breeders have been "genetically modifying" animals by selective breeding, or artificial selection, since animals were first domesticated more than 12,000 years ago. Using traditional breeding methods, individual animals with desirable traits were chosen to be parents of the next generation thereby ensuring that subsequent generations inherited those traits. This selection was done in the absence of any knowledge about which genes were influencing the desired attributes.

In the early 1970s, scientists began to investigate how specific segments of DNA that encode proteins of interest could be moved from one organism to another. Splicing segments of DNA together and introducing the resulting recombinant DNA (rDNA) construct into an organism's genome to give that organism new properties is called *rDNA technology*. Early experiments involved expressing rDNA in bacteria (Cohen et al. 1973). While proponents quickly recognized the potential benefits of this new technology, some experiments raised warning flags about the unknown dangers of such modifications.

In response to a letter in the academic journal *Science* voicing these concerns (Berg et al. 1974), researchers temporarily halted their rDNA work and a group of scientists, lawyers, journalists, and government officials gathered at the Asilomar Conference Center in Pacific Grove, California, in February of 1975 for the International Congress on Recombinant DNA Molecules. Now commonly referred to as the Asilomar Conference, the purpose of this gathering was to determine how to safely conduct rDNA research. The outcome was the development of safety guidelines for rDNA experiments with varying stringency based on the degree of associated risk. In 1976 the official US guidelines on recombinant DNA research were issued based on these outcomes. Asilomar has been lauded as a model of scientific responsibility, and as a catalyst for the public discussion of science policy.

Although it has been almost forty years since the Asilomar conference, and rDNA technology is now widely utilized in agriculture, pharmacology, research, and medicine, some remain concerned about unknown hazards associated with the use of this technology. This issue is particularly contentious when it comes to the use of genetically engineered plants and animals for food purposes.

METHODS OF GENETIC MODIFICATION IN ANIMALS

Animals that carry and transmit one or more copies of an rDNA construct are designated as *genetically engineered* (GE), or *transgenic*. The use of microinjection (MI) to insert foreign DNA into a single fertilized oocyte (immature egg cell) was reported in mice more than 30 years ago. The power of rDNA technology was vividly illustrated on the cover of the scientific journal *Nature* by a picture of a fast-growing transgenic mouse expressing the rat growth gene (*transgene*) alongside its noticeably smaller non-transgenic littermate (Palmiter et al. 1982). Shortly thereafter, the first transgenic livestock were produced (Hammer et al. 1985).

Although MI has been used to introduce rDNA into a number of animal species, it has several technical disadvantages. With this technique, the DNA randomly integrates into the host genome. Depending on the specific site of integration, unintended—and sometimes unwanted—results can occur; this is known as *insertional mutagenesis*. Another disadvantage of MI is that integration can occur at multiple sites, and/or multiple copies can integrate at each site, with both outcomes potentially resulting in undesirably high levels of gene expression. Finally, since the integration of the transgene is random, not all transgenic animals actually express the transgene, and the pattern of expression may be altered by the location of the integration site.

Consequently, the use of MI requires both the production of a number of different transgenic integration events, or lines, to ensure that the phenotype is due to the transgene and the selection of a line with suitable levels of transgene expression. This necessitates a large number of animals, multiple surgical procedures and, especially in livestock species that have relatively long maturation and gestation times, a significant investment of time and money. With the advent of alternative techniques for generating transgenic animals, MI is now primarily used for the production of transgenic mice, rabbits, and pigs.

Alternatives to Microinjection. Another method to develop GE animals involves the use of pluripotent embryonic stem (ES) cells. First described in mice, ES cells can be genetically modified using rDNA technology in culture and the transgenic ES cells can then be injected into blastocysts (a hollow structure of cells appearing early in an embryo's development) where they are able to enter the germ line (Robertson et al. 1986). The production of transgenic animals by ES cells has a distinct advantage in that a single copy of the transgene can be integrated into a specific location in the genome using a Nobel Prize–winning gene targeting strategy (Thomas and Capecchi 1987) (see Figure 1). ES cells have been utilized to create targeted gene knockouts, knockins, and chromosomal rearrangements. The downside to this approach is that the generation of transgenics by ES cells takes longer than MI and requires advanced skills in molecular and cell biology. This method has been successfully used in mice and rats, but efforts to obtain ES cell lines from farm animals have not been successful.

The development of nuclear transfer cloning provides an approach to efficiently produce GE animals in non-rodent species. The first experiments on nuclear transfer of embryonic animal nuclei involved transferring donor nuclei from frog blastomeres (Briggs and King 1952), closely followed by cloning frogs from tadpole stage embryos. The method became famous when nuclei from differentiated adult mammary gland cells were injected into enucleated oocytes (immature egg cells with their nucleus removed) to produce Dolly the sheep (Wilmut et al. 1997). This process of producing a clone by transferring a nucleus into enucleated oocytes is called somatic cell nuclear transfer (SCNT) cloning. Cumulina, the first live cloned mouse created by SCNT, was produced using nuclei from cumulus cells (cells that surround the oocyte) (Wakayama and Yanagimachi 1999). SCNT of GE animal cells is now the most commonly used method of transgenesis for non-rodent animals and has been used to clone a wide range of species. Nuclear transfer allows clones to be generated from cells that have been genetically modified in cell culture. Although clones resulting from SCNT of GE cells are all transgenic, SCNT cloning is an inefficient process with respect to the numbers of offspring that ultimately survive compared to the numbers of embryos that are generated and transferred to surrogate females. Large offspring syndrome and placental abnormalities have been reported in some species following the transfer of SCNT clones. Despite these potential problems, cloned animals that survive the early stages of life generally develop normally and are healthy.

Targeted Gene Editing. The absence of ES cells historically frustrated attempts to generate targeted gene modifications in non-rodent species. Many of the past issues with the genetic engineering of large animals have stemmed from concerns that unintended changes resulting from genetic engineering may affect animal and/or consumer health. Targeted gene editing, or precision genetic engineering, involves targeting specific loci and making precise changes to the DNA sequence such that only the expected genotypes and phenotypes are observed. In particular, new precision editing techniques allow for the creation of gene knockouts in large animals, an endeavor that has proven inefficient using earlier techniques.

Several methods of targeted gene editing are proving useful in mammals. Zinc finger nucleases (ZFNs), meganucleases, transcription activator-like effector nucleases (TALENs), oligonucleotide-directed mutagenesis, and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases; each of these methods offers an approach to enable the introduction of desirable alleles into somatic cells from any given animal, without the need to introduce other unwanted changes. These techniques create double-stranded breaks at a specific location in the genome, and repair can be directed by a template carrying the desired allele. ZFNs can also be used to efficiently produce homozygous knockouts, as exemplified in bi-allelic knockout pigs (Hauschild et al. 2011). TALENs have been used to generate knockouts in rodents and biallelic knockouts in Ossabaw miniature swine (Carlson et al. 2012). Finally, CRISPRs are emerging as a promising alternative to ZFNs and TALENs for targeted genomic editing.

APPLICATIONS

Biomedical Applications. Mice are the most commonly utilized animal models of human diseases due to their small size, ability to produce many offspring, and the feasibility of producing targeted gene knockouts due to the availability of ES cells. Currently, thousands of strains of transgenic mice are available for researchers investigating a variety of diseases ranging from Alzheimer's to cancer.

In the 1980s, several independent researchers genetically modified mice to carry cancer-causing genes, or oncogenes, thereby increasing their vulnerability to cancer and implying their use as promising new tools for understanding carcinogenesis. In 1988 Harvard University's OncoMouse™ became the first animal patented in the United States. Exclusive licensing rights were given to the Dupont Corporation, a company that sponsored some of the research. Although the original OncoMouse™ patent has expired, DuPont's sublicensing of the Harvard patents with restrictions and at high cost caused considerable controversy and ignited ethical and intellectual property rights debates. In spite of these concerns, OncoMouse™ continues to have a significant impact in cancer research.

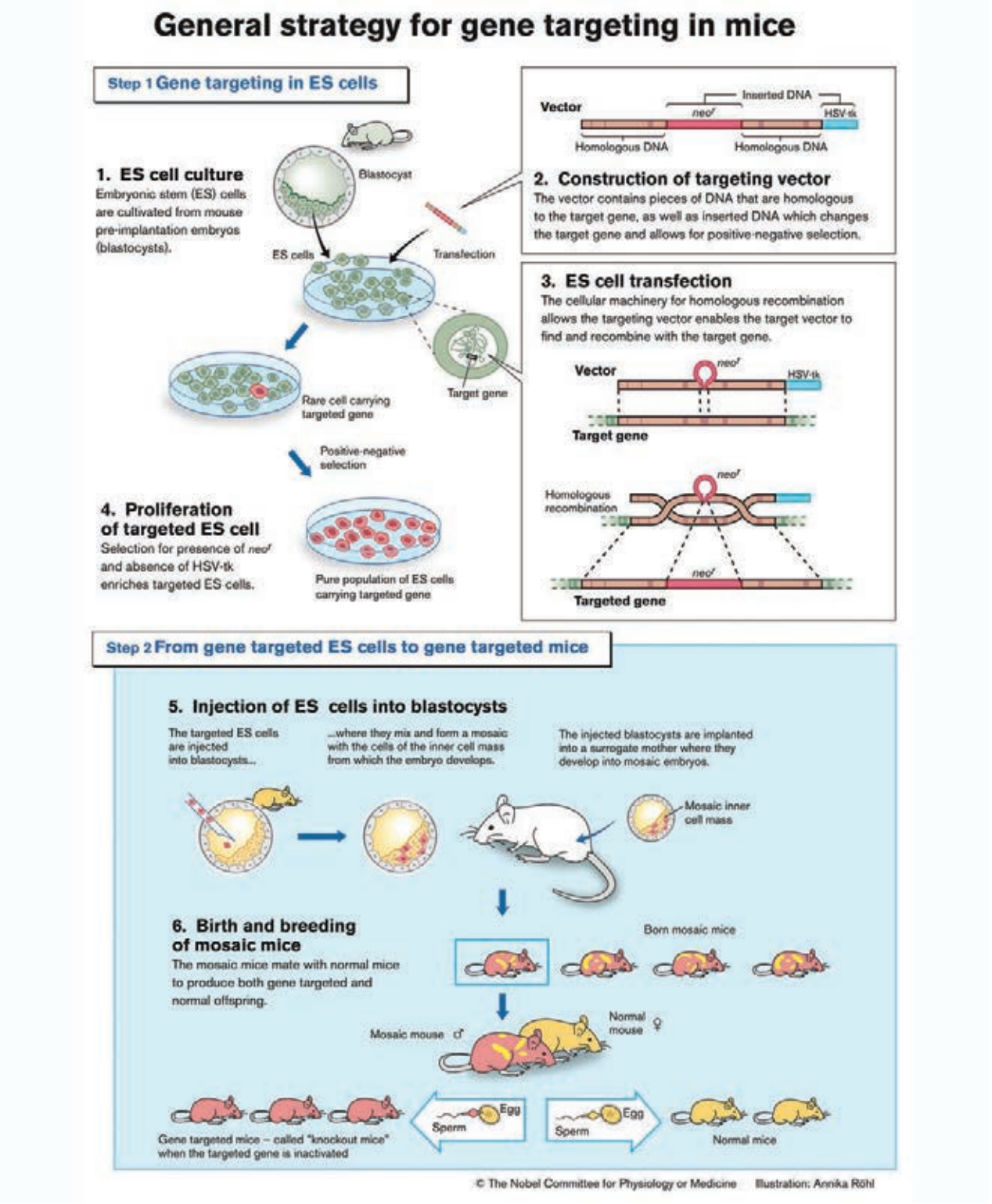

Figure 1.

Although mice are valuable disease models, there are limitations to their use. Some mouse models do not mimic human diseases closely enough, and large animal models are required to fill the gap between mouse models and certain human diseases. Non-human primates would of course most closely mimic human physiology and

likely the progression of human disease, but ethical and practical considerations frequently impede their adoption as animal models.

Due to similarities in anatomy and physiology, pigs are regarded as the best large animal models for many human diseases. Porcine models have been created for retinitis pigmentosa, Turner's syndrome, Crohn's disease, renal insufficiency, and intrauterine growth retardation. Transgenic Yucatan miniature piglets have been designed as models for muscular dystrophy, with additional GE pigs as models for cystic fibrosis, heart disease, arrhythmia, and cancer. Recently, transgenic pigs have been created as models for diabetes research (Wolf et al. 2013).

Pharming. Transgenic livestock have generated significant interest in their use as bioreactors for the production of therapeutic proteins, a technique known as "pharming." In pharming, a single gene is overexpressed to produce a functionally characterized, therapeutic protein, often in the milk of the transgenic animal. The mammary gland has been chosen due to its capacity for protein synthesis and the relative ease of collecting milk from animals. The first expression of a transgene in the mammary gland of a livestock species was in transgenic pigs. This was followed closely by the production of transgenic rabbits, goats, sheep, and cows that produce biopharmaceuticals in their milk. These early pharming efforts utilized MI techniques and thereby suffered from variability in the stability of transgenes. This issue has largely been addressed through the use of SCNT.

By early 2014, only two pharmed products had made their way into the marketplace. Antithrombin (ATryn) was the first recombinant protein derived from the milk of transgenic goats to be approved for commercialization by the Food and Drug Administration (FDA) in 2009. It is used to prevent blood clotting in patients with hereditary antithrombin deficiency. In 2014, the FDA approved a recombinant C1-esterase inhibitor product (Ruconest) produced in transgenic rabbits for the treatment of acute attacks in patients with hereditary angioedema. These are commercialized products produced by GE animals; the animals themselves are not sold publicly.

Attempts have also been made to use transgenic animals to produce antibodies. Antibody products are among the fastest-growing category of biopharmaceuticals. Successful attempts have been made to produce monoclonal antibodies in the milk of transgenic mice and goats. Transgenic cattle have been produced using an artificial chromosome vector that contains human immunoglobulin genes, a step toward the production of therapeutic human polyclonal antibodies (Kuroiwa et al. 2004). The large-scale production of human antibodies by transgenic animals has far reaching implications for human health and has the potential to transform the pharming industry.

Xenotransplantation. Organ transplantation can mean the difference between life and death for thousands of patients. Unfortunately, there are significantly more candidates on transplant waiting lists than there are available organs for transplantation. One possible solution is to utilize organs from other species for transplantation, a process known as *xenotransplantation*. Pigs are generally thought to be the most suitable species for xenotransplantation to humans based on similarities in anatomy and physiology. The primary barrier to implementation of xenotransplantation is the immunological rejection of the grafted organ.

The majority of the research on the use of transgenic pigs for xenotransplantation has focused on knocking out the enzyme α1,3-galactosyltransferase, or *GGTA1*. This protein is naturally occurring in many species (including pigs) but is not found in humans and is one of the primary xenoantigens involved in graft rejection. Transgenic pigs homozygous for the deletion of *GGTA1* (meaning that the two identical copies of the *GGTA1* gene were eliminated, thereby creating pigs with no *GGTA1*) have been successfully produced. Xenotransplantation of organs from *GGTA1* knockout pigs into baboons resulted in significant improvements in organ survival. Double knockout pigs for *GGTA1* and cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*) have been generated using ZFNs (Lutz et al. 2013). It is likely that multi-transgenic pigs, combining a number of different transgenic modifications and knockouts, will be required for the successful reduction in organ rejection.

In addition to the problem of organ rejection, another issue associated with xenotransplantation is the potential transmission of infectious agents, such as porcine endogenous retroviruses (PERV) in pigs, from animals to humans. PERV have been identified in the genome of all pig breeds but some pigs have been identified that do not harbor specific infectious PERV. Knockdown approaches have been used to create pig fibroblasts with reduced PERV expression and could be used to generate transgenic pigs that would have a reduced risk of transmitting PERV as a result of xenotransplantation.

MODIFICATION OF THE QUALITY OF LIVESTOCK/AGRICULTURAL IMPROVEMENTS

The application of genetic engineering technologies to livestock breeding is of pressing importance in the face of an ever-increasing global population and growing demands for animal products. Transgenesis presents a way for producers to improve farm animal traits including reproductive performance, fertility, growth rate, feed efficiency, carcass

characteristics, milk production and composition, disease resistance, and environmental impact.

The production of fast-growing transgenic mice expressing a rat growth hormone first suggested the ability to use transgenics to increase the efficiency of animal protein production. Since that time, improved growth rate is a trait that has been investigated in many species. Also of particular interest in the field of animal transgenesis is the potential to genetically engineer livestock with improved disease resistance. Transgenic dairy cows have been created that are resistant to bovine mastitis caused by *Staphylococcus aureus* (Wall et al. 2005), one of the most costly diseases to the dairy industry. Knocking out the prion locus in cell culture followed by SCNT resulted in cloned cattle that are resistant to bovine spongiform encephalopathy (BSE) infections or "mad cow" disease (Richt et al. 2007).

In addition to increased disease resistance, genetic engineering can be used to improve farm animal welfare in other ways as well. Concerns regarding sex selection and castration can be circumvented by the use of genetic engineering to feminize male embryos or eliminate the production of male sperm. This is particularly applicable to the dairy and egg industries, where females produce the desired products. Genetic engineering can also be used to improve animal welfare through improved health of offspring, especially in early life. Transgenic sows have been created that produce α-lactalbumin and IGF in their mammary glands, thereby increasing their milk production and contributing to healthier piglets with improved survival rates (Wheeler et al. 2001).

Transgenic technologies have been utilized to improve animal products. Overexpression of casein genes in transgenic cows led to an increase in cheese yield (Brophy et al. 2003). Expression of an omega-3 desaturase gene in GE pigs resulted in pigs that produced products with a healthier fatty acid composition (Lai et al. 2006).

Genetic engineering can also be utilized to reduce the environmental impact of farm animal production. In 2001 a line of transgenic pigs was developed with the aim of improving the sustainability of pork production by reducing the amount of phosphorus excreted. These pigs produce phytase in their salivary glands due to a gene from the common bacteria *Escherichia coli* (Golovan et al. 2001). The resulting Enviropig™ excretes 30 to 60 percent less inorganic phosphorus, which has beneficial implications for reducing surface water eutrophication and the use of phytase as a feed supplement. Despite its obvious utility, no company was willing to undertake the expense and uncertainty associated with the commercialization of the Enviropig™ pig, and the last pig of this line was euthanized in 2012.

1975	**International Congress on Recombinant DNA Molecules** (USA): International group of scientists meet at the Asilomar Conference Center to discuss the potential biohazards posed by recombinant DNA (rDNA) technology
1981	**Gordon & Ruddle** (USA): First transgenic mouse created by use of microinjection of DNA
1982	**Palmiter, Brinster, Hammer, Trumbauer, Rosenfeld, Birnberg, Evans** (USA): Fast-growing transgenic mouse expressing rat growth gene produced
1985	**Hammer, Pursel, Rexroad, Wall, Bolt, Ebert, Palmiter, Brinster** (USA): Transgenic livestock produced
1987	**Thomas & Capecchi** (USA): Transgenic animals produced via ES cells
1988	**Leder & Stewart** (USA): Patent entitled "Transgenic non-human mammals" issued for the Oncomouse
1989	**AquaBounty Technologies** (USA): AquAdvantage Salmon founder
1995	**AquaBounty Technologies** (USA): Formal AquAdvantage Salmon approval request
1997	**Wilmut, Schnieke, McWhir, Kind, Campbell** (UK): Dolly the sheep makes headlines as first animal cloned by SCNT
1999	**Wakayama and Yanagimachi** (Japan): Mouse (Cumulina) produced by SCNT from cumulus cells
2001	**Golovan, Meidinger, Ajakaiye, Cottrill, Wiederkehr, Barney, Plante, Pollard, Fan, Hayes, Laursen, Hjorth, Hacker, Phillips, Forsberg** (Canada): EnviroPig™ developed
2003	**Yorktown Technologies** (USA): GloFish® commercialized
2005	**Wall, Powell, Paape, Kerr, Bannerman, Pursel, Wells, Talbot, Hawk** (USA): Mastitis resistant dairy cows produced
2007	**Richt, Kasinathan, Hamir, Castilla, Sathiyaseelan, Vargas, Sathiyaseelan, Wu, Matsushita, Koster, Kato, Ishida, Soto, Robl, Kuroiwa** (USA): BSE-resistant cattle produced
2009	**Food and Drug Administration** (USA): ATryn & Ruconest approved in the United States.
2011	**Lutz, Li, Estrada, Sidner, Chihara, Downey, Burlak, Wang, Reyes, Ivary, Yin, Blankenship, Paris, Tector** (USA); and **Hauschild, Petersen, Santiago, Queisser, Carnwath, Lucas-Hahn, Zhang, Meng, Gregory, Schwinzer, Cost, Niemann** (South Korea): Knockout pigs produced using ZFNs
2012	**Carlson, Tan, Lillico, Stverakova, Proudfoot, Christian, Voytas, Long, Whitelaw, Fahrenkrug** (USA): Knockout pigs produced using TALENs
2013	**Tan, Carlson, Lancto, Garbe, Webster, Hackett, Fahrenkrug** (USA): TALEN and CRISPER /Cas custom endonucleases used to introduce highly precise targeted genetic changes—such as indels and SNPs—into cells from cattle, pigs, and goats
2014	**Niu, Shen, Cui, Chen, Wang, Wang, Kang, Zhao, Si, Li, Xiang, Zhou, Guo, Bi, Si, Hu, Dong, Wang, Zhou, Li, Tan, Pu, Wang, Ji, Zhou, Huang, Ji, Sha** (China): First targeted genetic modifications using CRISPER /Cas in a primate

Figure 2. *Significant advances in animal transgenics.*

REGULATORY ISSUES IN GE ANIMAL COMMERCIALIZATION

The uses of GE animals for biological and biomedical applications, and for pharming, are relatively uncontentious. The commercialization of GE animals for food, on the other hand, has moved significantly slower than for GE crops. This is partly due to the long generation time of many livestock species and the lack of a clear

regulatory path to market. Regulatory roadblocks and issues of public acceptance have made the commercialization of GE food animals an uphill battle.

In the United States, GE animals are regulated by the Food and Drug Administration's (FDA's) Center for Veterinary Medicine (CVM) under the new animal drug provisions of the federal Food, Drug and Cosmetics Act. In this context, the rDNA construct is the new drug, not the animal itself. In what is ultimately a multistep scientific review process, the FDA considers the safety of the rDNA construct with respect to the health and well-being of the animal, the safety of the products that might be generated from the transgenic animal, and any potential environmental impacts. It then evaluates the efficacy of the claims for the technology (i.e., does the product really do what the producers claim it does) before approving new animal drugs (*Food and Drug Administration* 2009). The FDA employs "enforcement discretion" on a case-by-case basis for GE animals that are deemed of very low risk, such as those routinely used for research in laboratories. Examples include transgenic rodents routinely used for biomedical research and the transgenic pet Glofish®.

The most widely cited example of the struggle between the regulatory process and GE animal products is the AquAdvantage® Salmon, a fish engineered to reach mature size faster than conventional Atlantic salmon. The company behind this product, AquaBounty Technologies, began seeking guidance for approval of this fish for human consumption in 1993, and a formal request for commercial approval was submitted in 1995. A briefing package containing all of the scientific data and an environmental assessment was made available to the FDA's Veterinary Medicine Advisory Committee (VMAC) and the public in 2010. Considerable controversy surrounded the AquAdvantage® VMAC meeting, and a protracted period of regulatory inaction ensued (Van Eenennaam and Muir 2011). As of September 2014, the FDA had not announced a decision regarding the AquAdvantage® application. The immense costs associated with the collection of regulatory data and the uncertainties of the protracted regulatory process have made companies reluctant to provide capital and pursue approval for GE animals for food purposes.

Criticisms of the current US regulatory approach to GE animals by proponents include the fact that it is process-based, meaning that the trigger for regulation is the use of rDNA technology, that the required testing is prohibitively time consuming and expensive, that regulatory timelines are unpredictable, and that the process focuses only on risks and does not adequately consider the potential benefits. Opposition groups are concerned with what they consider to be a lack of transparency, by the absence of social and ethical considerations in the process, and that the data considered in regulatory reviews are gathered by the companies that are seeking product approval (Van Eenennaam et al. 2011). This last concern is true for the regulatory evaluation of all new drugs, be they for animal or human use.

The advent of precision gene editing technologies may challenge traditional definitions of a GE animal. Gene editing techniques that utilize ZFNs or TALENs alleviate concerns associated with potential unintended outcomes resulting from insertional mutagenesis and position effects. Additionally, gene editing technologies may leave little trace in the genome other than a single base pair change; therefore, no rDNA footprint will be present in the genome to trigger regulatory oversight. In 2010, for instance, the USDA determined that a GE corn line developed using ZFNs fell outside of the agency's regulatory authority. Regulatory agencies from many countries are currently grappling with the appropriate regulatory approach for gene editing technologies, both for plants and animals (Bruce et al. 2013).

The development of GE animals for agriculture is moving forward in emerging economies such as Brazil, Argentina, and China, where government policies support the development of GE animals as one of the ways to resolve the problem of food security and improvement of living standards. In the absence of global harmonization of GE animal regulatory frameworks, developers will likely move to countries with the most favorable policy environments.

SEE ALSO *C. Elegans and Cell Fate Map; Dolly the Sheep; Drosophila Melanogaster; Genetic Engineering, Beginnings; Genetically Modified Organisms, Plant Transformation by Agrobacterium.*

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Amy E. Young
Department of Animal Science
University of California, Davis

Alison L. Van Eenennaam
Department of Animal Science
University of California, Davis

GENETICALLY MODIFIED ORGANISMS, PLANT TRANSFORMATION BY AGROBACTERIUM

The plant pathogen *Agrobacterium tumefaciens* (see Figure 1), a soil bacterium causing the crown gall disease (manifested as tumors or "galls" on the infected plants), possesses the exceptional ability to transfer a segment of its own DNA into the genome of the host plant. In fact, *Agrobacterium* represents a unique case of natural DNA transfer from a prokaryotic to a eukaryotic organism. Thus, as a natural genetic engineer, *Agrobacterium* has become the major tool to genetically transform plants for basic research as well as for commercial production of transgenic crops; it is also increasingly used for genetic transformation of fungi. Even though other, more