
The Promises and Challenges of Precision Gene Editing in Animals of Agricultural Importance

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We live in changing times—it has always been that way. But, now the times are changing more rapidly, dramatically, and unpredictably. The world must accommodate an increasing population that demands greater nutrition per person, better health, and greater energy consumption per person. As the world's population rises to a predicted level of nearly 10 billion by 2050, most want sustainable growth without harm to the environment. The inconvenient truth is that it's not possible with current agricultural, economic, and environmental operating systems (Hoekstra and Wiedmann, 2014). The green revolution has essentially run its course (Conway and Toenniessen, 2000; Pingali, 2012; Stevenson *et al.*, 2013). Even with technological gains, hunger worldwide has been increasing over the past decade. Global climate change coupled with a demand for increased nutrition in the developing world exacerbates the stress on agricultural production (Eisler and Lee, 2014). Hence, developing new methods of increasing the production of agricultural products, crops and animals, with minimal impact on the environment is essential (Godfray *et al.*, 2010). Current approaches cannot meet demands. However, gene editing (Tan *et al.*, 2012, 2013) (Figure 1)—which allows geneticists to introduce (introgress) any natural trait into any breed without the use of recombinant DNA—has the potential of improving animal genetics for meeting increasing agricultural and biomedical needs with minimal environmental impact. However, there are policy issues associated with gene-editing in livestock and in biomedical research that must be addressed for their real-world applications (Pauwels *et al.*, 2014). We discuss several types of genome editing and current deficiencies in regulatory oversight that block enthusiasm for its adoption to agriculture.

THE PROBLEM

Genetically modified (GM) animals have been around for more than four decades (Gordon and Ruddle, 1981, 1982). These animals had expression cassettes driven by constitutive promoters that are active in most cell types and were delivered by vectors that could integrate semi-randomly in genomes (Figure 1, upper panel). As a result, there were fears of unacceptable (scary) results (Rollin, 1985) that were exacerbated by the engineering of a mouse that grew like a rat (Palmiter *et al.*, 1982, 1983). The first large animals of potential commercial importance were fish (Zhu *et al.*, 1986; Hackett and Alvarez, 2000; Devlin *et al.*, 2009). Since then, several lines of transgenic livestock have been engineered for producing valuable biomedical medicines and for agriculture (Tan *et al.*, 2012). Animals engineered to become bioreactors for manufacture of enzymes and antibodies that are not for general sale or consumption have been cleared by regulatory authorities. But, in the United States, not a single animal engineered for food production has been approved by the US Food and Drug Administration (FDA), which regulates GM animals.

These demonstrations of transgenic technologies were greeted with more concern than enthusiasm by the general public and especially by several non-governmental organizations. The concerns focused on four areas:

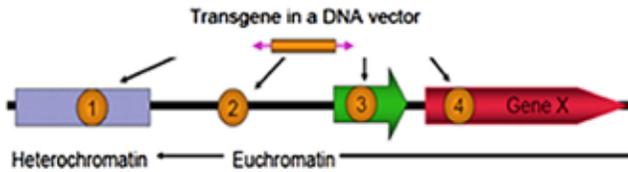
- Health effects due to the *un-naturalness* of products the modified genome might encode;
- Environmental effects due to uncontrolled release of transgenes (*i.e.* GM animals) and reduced diversity of natural genomes;
- Social concerns that huge corporations would have undue influence over diets and
- Moral concerns that were summed up by the phrase “playing God.”

These concerns were applied to animals as well as crops.

The first crops were genetically engineered in 1985, *e.g.* tobacco with a firefly luciferase gene (Lamppa *et al.*, 1985). Astonishingly, as shown in Figure 2, over the past 15 years, GM crops have been adopted increasingly year after year with respect to acreage, plant varieties and countries. In contrast, even though farm animals can be controlled to a far greater degree than crops, not a single animal has made it through full regulatory review in any country.

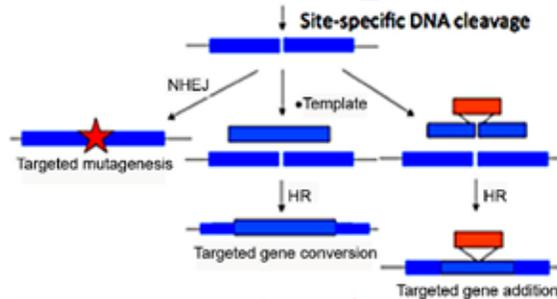
This is in part due to the different attitude taken by the FDA, which regulates GM animals and the US Department of Agriculture (USDA) and Environmental Protection Agency (EPA), which regulate GM crops. Foreign countries have in large measure deferred to FDA to formulate regulations and procedures for evaluation of GM animals. The precision of gene editing along with our greater knowledge of molecular genetics and cell biology gained over recent decades supports the belief that GM animals may become a reality.

Genetic Engineering pre-2005



Chromatin sites into which vectors carrying recombinant DNA payloads can integrate: 1) heterochromatin where transgenes cannot be expressed; 2) gene-free regions in which the transgenes can be expressed with minimal residual effects; 3) transcriptional regulatory regions that may affect transgene expression as well as expression from downstream genes; and 4) transcriptional units (>90% intronic) that may be somewhat affected by the transgenic construct. The rate of Homologous Recombination with a DNA vector with homology to a specific region of an animal genome is about 10^{-7} .

Genome Editing 2014



Genome editing in which double-strand DNA breaks are introduced with high precision. Non-homologous end-joining (NHEJ) generally introduces a mutation (red star) that can inactivate a native gene. Alternatively, appropriate choices of DNA sequence with homology to the targeted region can lead to homologous recombination (HR) with specific changes in sequence of the native DNA

Figure 1. Genetic engineering using recombinant DNA vectors compared to genome editing using site-specific DNAases: The gain in precision between the two methods is 10^7 .

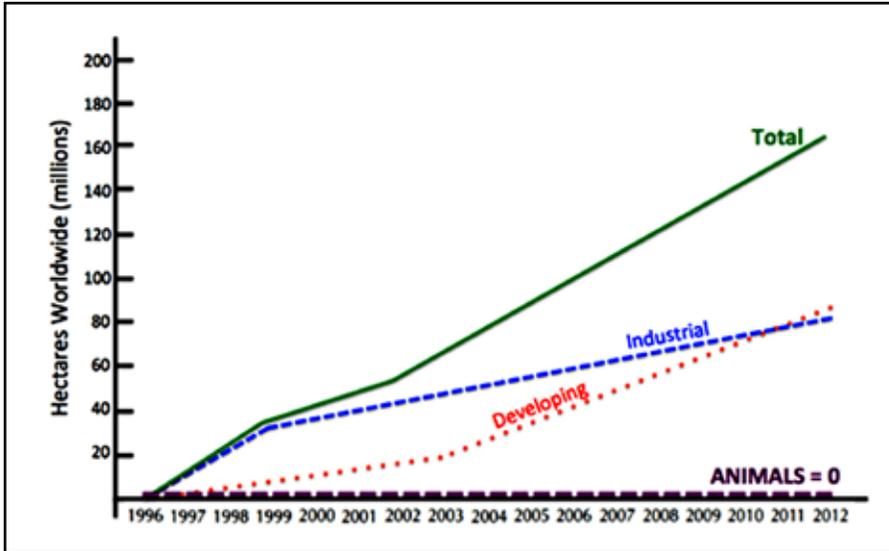


Figure 2. Crops and animals modified by molecular genetics procedures (GM) approved for agricultural purposes. No government-approved GM animal has been developed for agricultural use.

THE PROMISE OF GENE EDITING IN ANIMALS

Gene-edited animals can be fruitfully employed in three distinct areas, as shown in Figure 3. Each of these areas, illustrated in the figure, would be a game-changing event globally with respect to personal health. In 2013 in the USA, more than 121,000 individuals were on the waiting list for transplantable organs; only 29,000 organs were transplanted. However, with precision inactivation (knockout, KO) of specific genes required for organ development *in utero*, pigs could be used as bioreactors for production of donor-specific organs/tissues by a process called *blastocyst complementation* or *exogenic organ production*. This is theoretically applied by injection of induced pluripotent stem cells (iPSCs) into a KO-pig blastocyst from which the donor cells fill the vacant developmental niche, resulting in a human organ to match the donor. This would be analogous to an autograft, which will avoid immune responses when transplanted into a patient. Likewise, pigs are far closer to humans in physiology than other commonly used model animals.

Gene editing allows production of animals with specific conditions that mimic human disorders, which will allow more-accurate pre-clinical evaluation of novel drugs and advanced medical devices before human clinical trials. The most dramatic promise of gene-edited animals is in agriculture worldwide. In the Anthropocene era (Vince, 2011), characterized by global changes in climate with attending alterations in spreads of animal-disease vectors, the exchange of new genes (traits) from different breeds in

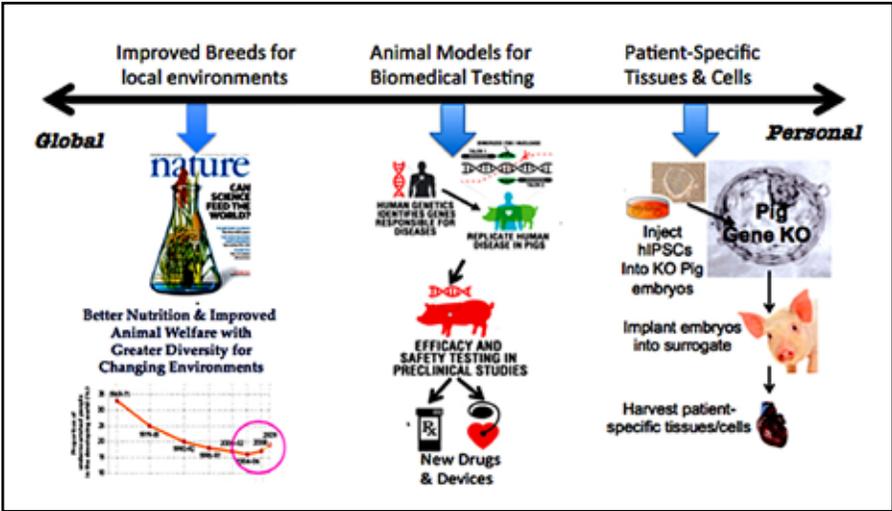


Figure 3. Promise of gene-edited animals. The major areas of application are, from left to right, 1) improved animal genomes adapted to local environmental conditions to produce food more efficiently, 2) improved animal models of human disease that provide more reliable pre-clinical information regarding safety of new drugs and medical devices, and 3) animals designed to harbor patient-specific organs, tissues and cells for transplantation that will not induce adverse immune responses.

various regions and microenvironments of the world will be vital for adaptation of animals and their abilities to provide improved nutrition while simultaneously improving their welfare. Additionally, gene editing will allow novel tweaking of genomes in order to introduce new approaches to the control of animal diseases as well as to improve animal health and efficiency.

The three major technologies for introducing site-specific double-stranded DNA breaks into genomes—zinc finger nucleases, ZFNs, transcription activator-like element nucleases, TALENs, and RNA-guided endonucleases, RGENs, of which the CRISPR-Cas9 system is best known (Gaj *et al.*, 2013; Kim and Kim, 2014)—are effective in livestock genomes (Carlson *et al.*, 2012; Tan *et al.*, 2013). The steps for creating livestock with specific genome-edits are illustrated in Figure 4. The process begins with introducing double-strand DNA breaks in the genomes of somatic cells, typically fetal fibroblasts, expanding the cells into colonies that then are screened for the desired outcomes. This process is between 10% and 70% efficient, depending on the gene, the size of the edit, the genetic locus and cell type. Counter-intuitively, introducing single-nucleotide changes is generally less efficient than introducing longer alterations (Tan *et al.*, 2013). Table I shows the approximate efficiencies of introducing defined gene edits into the genomes of pigs, cattle, goats and sheep.

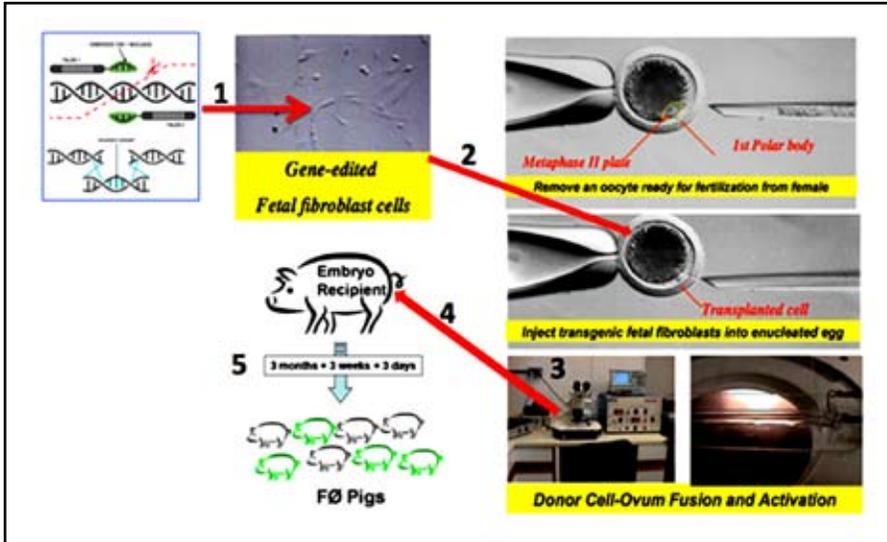


Figure 4. Route of obtaining gene-edited livestock (pigs). 1) Introduced gene-edits into fetal fibroblasts (1 month; 10–70% efficiency). 2) Transfer validated gene-edited cells into enucleated oocytes. 3) Activate embryos by electrical pulse to fuse the cell with the oocyte. 4) Transfer activated embryos into surrogate female to produce 5) F0 pigs with the desired gene-edit (4–9 months; 1–5% efficiency).

Table I. Summary of homology-directed recombination for livestock fibroblasts.

- >98% success (60+ genes/loci successfully targeted; 1 failure)
- Heterozygosity (monoallelic conversion) up to 70%
- Homozygosity (biallelic conversion) up to 40%
- Single-nucleotide polymorphism(SNP) edits with 1 bp alteration about 10% efficiency
- SNP edits much more efficient with silent mutations

The *POLLED* gene in cattle illustrates the power of gene editing in large animals. Dairy cattle, like many other mammals, naturally have horns. Early in the domestication process of dairy cattle thousands of years ago, horns were valuable for survival, whereas today horns have no intrinsic value because the animals are confined to secure enclosures. However, horns do pose a significant risk both to the animals and to humans because of inadvertent nicking and, consequently, are removed efficiently and cost-effectively, but not without suffering (Graf and Senn, 1999). Two mutations that prevent development of horns in certain breeds of cattle have been mapped on the bovine genome (Medugorac *et al.*, 2012; Seichter *et al.*, 2012) that may encode an lncRNA rather than a protein (Allais-Bonnet

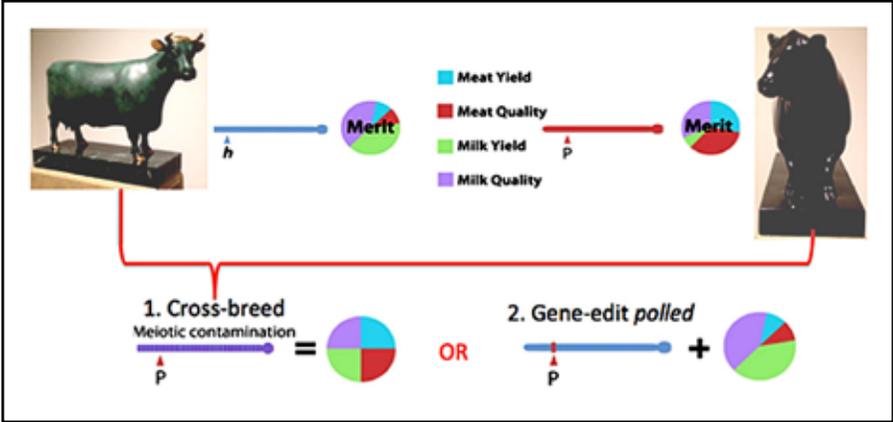


Figure 5. Introgression of the *POLLED* allele into dairy cattle. Top: Merits for traits of commercial value are shown by the circles for dairy (left) and beef (right) breeds. The *POLLED* locus is shown by the P (or h for the Hereford breed) on chromosome 1 (BTA1) for the two breeds. Bottom: Cross-breeding to introgress P into a dairy background will mix all the valued traits for both breeds. Alternatively, gene editing of the h locus to introduce the P allele maintains the dairy merits.

et al., 2013). The standard method for introducing *POLLED* in dairy breeds would be by crossing with a hornless breed (Figure 5), but that produces a hornless animal without the best combination of traits for the dairy industry. Recovering the dairy merits would take nearly a quarter century of backcrossing and selection.

Gene editing saves about eight generations of backcrossing and the entire attendant screening for alleles desirable to industry. Figure 6 shows molecular introgression of *POLLED* into the Hereford breed. The efficiency of introducing this particular site-specific mutation with this particular pair of TALENs was 1% to 5%, substantially below the average rate shown in Table I. However, because the genomic edits can be introduced into the chromosomes of fibroblasts, even this relatively low rate is sufficiently high for easy selection of appropriate genomes for transfer into embryos.

Gene editing is not limited to single changes. Because of the high efficiency of the procedure, multiple selected mutations can be simultaneously introduced into genomes. Thus, gene editing offers parallel, precise changes in genomes of animals rather than sequential changes. This can save decades of time. Moreover, as illustrated by the recent elucidation of the genetic basis of *POLLED*, as greater numbers of genomes of various breeds of livestock are sequenced, our abilities to identify further traits conferring disease-resistance, drought-tolerance, temperature-tolerances (high and low), *etc.*, will be enhanced. All findings of this sort will provide a bank of alleles for molecular introgression. The process is fast, efficient, and essentially unlimited in the combinations of traits that can be moved as needed. In the coming decades, in which significant variations in global as well as local environmental conditions are predicted, gene editing offers the ability to substantially expand the diversity of animals that will be better adapted for climatic

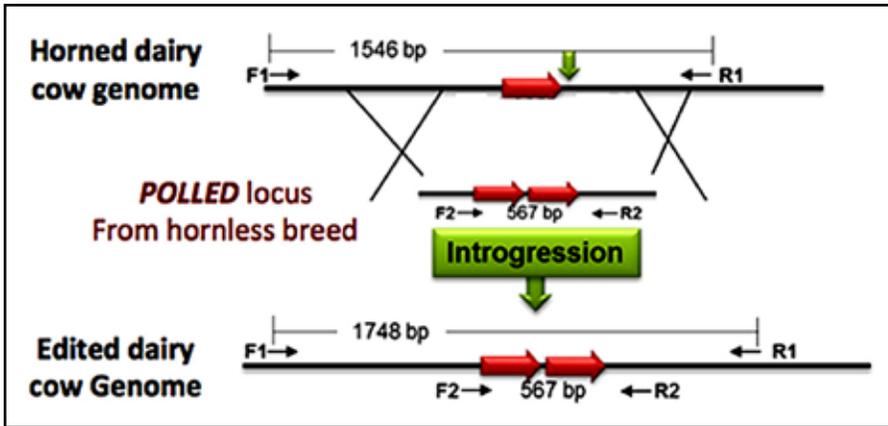


Figure 6. Molecular introgression of the *POLLED* allele into dairy cattle by gene editing. A double-stranded oligonucleotide serves as a template for homology-directed recombination (HDR) following introduction of a double-stranded DNA break (DSB) in the genome following cleavage by a specific pair of TALENs. Because gene-editing is precise, genomes can be screened by PCR for exactly the desired changes.

F1, F2, R1 and R2 are specific DNA oligonucleotide primers for PCR analysis.

changes. This will benefit the animals as well as their caretakers and the populations that depend on agricultural production. The promise of gene editing in livestock is enormous (Howe *et al.*, 2008).

CHALLENGES FOR GENE EDITING IN ANIMALS

The challenge for application of gene editing in animals is evident from Figure 2: not a single commercial animal for agricultural purposes has been approved in the United States. Regulatory reluctance to issue approval for the first proposed commercial animal, faster-growing salmon, has been stymied by regulatory delays (Figure 7) (Van Eenennaam and Muir, 2011; Maxmen, 2012b; Ledford, 2013; Editorial, 2014). There are suggestions that molecular introgression using the procedures described above will meet the same regulatory purgatory (Maxmen, 2012a). One way of appreciating the consequences of regulatory reluctance to approve any genetically modified livestock are the hundreds of citations on genetically modified animals 10–20 years ago, reviewed by Tan *et al.* (2012), compared with the paucity of citations today. If there is no method of gaining approval, there is no incentive for development.

The letters (many form) about the transgenic salmon on FDA's website¹ raised the same concerns listed above (p. 40) for transgenic organisms in general:

- *Health effects*—possibly allergic responses to a transgenic product, but more commonly general uncertainty in what eating a transgenic food might mean over many years.

¹<http://www.regulations.gov/#!docketBrowser;rpp=25;po=0;dct=PS;D=FDA-2011-N-0899>.



Figure 7. Year-after-year delays in approval of transgenic salmon by the FDA.

- *Environmental effects*—diversity may be reduced and/or transgenic animals may take over ecosystems.
- *Social effects*—transgenic products come from large corporations catering to large farms that dominate over smaller “family” farms; hence, greedy corporations benefit at the expense of consumers.
- *Moral concerns*—tampering or tinkering with nature, often referred to as “playing God.”

Regulatory agencies may take emotional reactions of citizens into consideration in decisions, but the primary driver should always be scientific understanding. In that light, what have we learned from thirty years (and more than \$100 billion) of molecular genetics and agricultural research, much of which can be understood by the large investment into the molecular, cellular and developmental biology of humans, which represent a pretty good model system for livestock?

Health Effects

Regarding health effects, if a genetic alteration is not detrimental to the animal, it is highly unlikely it would be to humans. The many cultures and societies of humans eat almost every type of life form. All food is foreign to someone, but not necessarily unhealthy. Generally it is processing of food, not the original product that can lead to adverse health effects. Regulatory pandering to public fears of food safety (DeFrancesco, 2013) must change.

Environmental Effects

Environmental effects for gene-edited animals will be no different from any other introduction of a related animal; the changes are too minor. For instance, although there are peer-reviewed studies that suggest ecological disaster from escape of even a few genetically altered fish (Muir and Howard, 2002; Devlin *et al.*, 2006) hundreds of thousands of farmed fish escape from Norwegian fisheries with little discernable effect (Glover *et al.*, 2012; Skilbrei *et al.*, 2014). There is theory and there is the real world, which often goes ignored (Hackett, 2002; Fedoroff, 2013). More importantly, as noted earlier, gene editing offers the *opportunity of introducing far greater diversity* into ecosystems because it is so economically efficient that it can be used by mega-farms and even the smallest family farm in a unique micro-ecosystem where agricultural efficiency can bring the greatest benefits.

Social Effects

The issue of huge corporations dominating the availability of genetically modified products is in large part a direct consequence of the cumbersome regulatory processes that take years for approval, if they come at all. That is, governmental policy demands expenditures of millions of dollars for regulatory approval that only large companies can afford.

Moral Concerns

Moral concerns are beyond the scope of most scientific discourses. But, an often-overlooked point on this issue is that genetic modifications with modern techniques are mere engineering, not creation.

REGULATORY ISSUES

Regulatory problems are far greater than just addressing the common concerns above. The regulatory system is designed to fail (Figure 8). It begins with the notion that the procedure for introducing genetic alteration is a greater issue than the outcome. That is completely based on fears in the 1980s about the unknowns of genetic engineering. That attitude has remained in place for thirty years, even though our understanding and our techniques have advanced beyond what was imaginable back then. A second problem is that scientists thrive on conducting experiments to address unresolved scientific questions. However, lab testing has severe cost and infrastructural constraints that restrict the numbers of animals and minimize the variations and conditions that can be considered. In contrast, the natural world is characterized by large numbers of organisms, innumerable variables, and confounding interactions that are poorly understood. Hence, singling out and testing only a few of the myriad variables inevitably leads to irrelevant results that, because they are so controlled and do provide reproducible results, are often published in premier journals. The result is that the question remains unanswered for which, ironically, the scientists are rewarded: more publications and more work. Regulators are also rewarded; they recognize that issues brought up by the scientific community remain unresolved and, thereby, no decision needs to be made. The results are evident. No genetically modified animals derived from modern genetic techniques have passed regulatory approval.

Broken Partnership

Gene editing represents the ultimate level of genetic engineering wherein precise changes can be made in genomes to achieve exact goals. The old ways of looking at genetic engineering are outdated. Regulators and their advisors must update and apply new ways of evaluating the coming tidal wave of gene-edited animals. The partnership between the regulatory agencies and the funding agencies is broken. The new tools, understanding and approaches to improve agricultural efficiency to meet needs of the 21st century have been developed, but they are not available to those who paid for the basic research. That has to change.

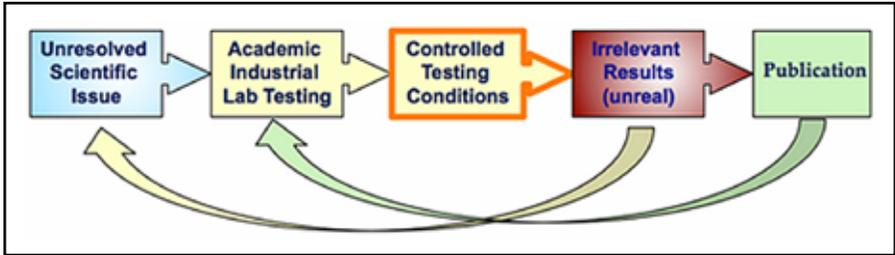


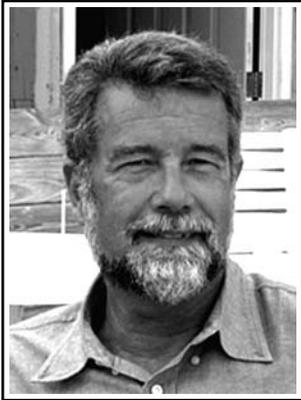
Figure 8. Regulation of genetic modification of animals is designed to fail.

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for gene editing in large animals including livestock. He has used bacteria, fish, mice and dogs as model systems for investigating various transgenic technologies in vertebrate animals. The *Sleeping Beauty* Transposon System, invented in his lab as a way of delivering genes to vertebrates, is in human clinical trials for treatment of lymphoma and other cancers.

DR. HACKETT is a cofounder of Discovery Genomics, to develop the *Sleeping Beauty* Transposon System for human gene therapy, and Recombinetics, to genetically engineer livestock for biomedical and agricultural purposes. Both companies are based on technologies developed in his and his students' labs. Currently, Dr. Hackett is the chief science officer of Recombinetics. He serves on three editorial boards and three scientific advisory boards in the areas of transgenesis, genome engineering and gene editing.