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Precision Editing of Large Animal Genomes

Wenfang (Spring) Tan[*,†], **Daniel F. Carlson**[*,†,§], **Mark W. Walton**[§], **Scott C. Fahrenkrug**[*,†,§], and **Perry B. Hackett**[*,§,‡]

[*]Center for Genome Engineering, University of Minnesota, Minneapolis, Minnesota, USA

[†]Department of Animal Science, University of Minnesota, Minneapolis, Minnesota, USA

[§]Recombinetics, Inc, St. Paul, Minnesota, USA

[‡]Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota, USA

Abstract

Transgenic animals are an important source of protein and nutrition for most humans and will play key roles in satisfying the increasing demand for food in an ever-increasing world population. The past decade has experienced a revolution in the development of methods that permit the introduction of specific alterations to complex genomes. This precision will enhance genome-based improvement of farm animals for food production. Precision genetics also will enhance the development of therapeutic biomaterials and models of human disease as resources for the development of advanced patient therapies.

1. INTRODUCTION

1.1. The Need for Genetically Modified Large Animals

Hunger worldwide is increasing; approximately 1 billion people are already chronically malnourished (Godfray et al., 2010). Contemporary efforts to meet demand are degrading an already taxed environment (Foley et al., 2011; Tilman, Balzer, Hill, & Befort, 2011). Improvements in the efficiency of production and safety are becoming even more important considerations for protection of the environment and reduction in land usage (Clark & Whitelaw, 2003). Global climate change will only exacerbate the lack of animal protein production (McMichael, 2012; Schmidhuber & Tubiello, 2007; Wolkovich et al., 2012). The *green revolution* has practically peaked according to its father, Borlaug (2000), who asserted that farm animals are critical to nutrition and that genetic engineering of foodstuffs will be required to feed the world. Both genetic- and management-based increases in sustainable productivity will be a key to satisfying global protein needs (Fahrenkrug et al., 2010).

Genetically engineered animals have a larger role than just as food (Fig. 1). They contribute to our health by serving as model systems for treatment of diseases and disorders as well as a source of biomaterials used for rebuilding tissues and organs (Kues & Niemann, 2004; Snaith & Törnell, 2002). Mice have historically been the prime medical models for finding disease-causing genes and testing drugs. Owing to their large numbers and the availability of in-bred lines that improve the reproducibility of experimental results, molecular and cellular investigations generally are first conducted in mice. Moreover, powerful selection protocols in cultured mouse embryonic stem cells allow identification and incorporation into genomes of genetic alterations that occur at very low frequencies, i.e. 10^{-5}–10^{-8} (Mansour, Thomas, & Capecchi, 1988; Smithies, Gregg, Boggs, Koralewski, & Kucherlapati, 1985). As a result, specific mutants can be made that mimic human mutations, e.g. cystic fibrosis (Snouwaert et al., 1992). However, the complete panoply of symptoms in humans does not always manifest in mice with the same genetic defects [e.g. the cystic fibrosis mouse does not have

the same range of problems that humans encounter with the same mutant genes (Rogers et al., 2008)]. Moreover, many of the advantages for academic studies are disadvantages for translation to human studies. For example, in-bred strains of mice provide highly reproducible experimental results because important alleles that control physiological pathways are homozygous at every locus and identical in every individual (Erickson, 1996), a situation that does not apply to the heterogeneous human population. Likewise, mice that have major differences in overall physiology have been selected for high-density, low-activity living, which results in abnormal metabolic characteristics that interferes with translation to humans (Martin, Ji, Maudsley, & Mattson, 2010).

Unfortunately, the selection techniques that are so powerful in conjunction with mouse embryonic stem cells have not been translated to other animals. For human applications where safety is paramount, larger animals are desirable as model systems for testing therapeutic procedures. Deleterious mutations that are similar to those in humans have been identified in certain breeds of cats and dogs because of the close relationship to their owners (Ellinwood, Vite, & Haskins, 2004; Haskins, Desnick, DiFerrante, Jezyk, & Patterson, 1984; Koeberl, Pinto, Brown, & Chen, 2009; Ponder et al., 2006; Wolfe, 2009), but the spontaneous appearance of these animals in veterinary clinics does not provide for on-demand and replicable lines for scientific studies. Generally, the range of spontaneous disease models in large animals is highly limited compared to the number of genetic disorders in humans.

That will change. Precision genetics, developed in the first decade of the twenty-first century, will be a key player for the challenges ahead. Specific genetic alterations in the genomes of the pig, which is similar in size, physiology, organ development, and disease progression (Kuzmuk & Schook, 2011; Lunney, 2007), will provide subjects that significantly accelerate the development of new medical devices, pharmaceuticals, therapeutic protocols, and tissue-based products from *humanized* transgenic lines. In this review, we summarize the game-changing genetic methods that are under development that will support unprecedented progress in adapting the genomes of farm animals to support their multiple roles in human societies. The implications of the new genetic technologies can be appreciated by acknowledging problems and issues that arose during the early years of genetic engineering.

1.2. Genetic Engineering of Animals Pre-2000

Transgenic animal technology is entering its fourth decade. The first recombinant DNAs were designed to express specific genes in bacteria (Cohen et al., 1973). Almost immediately, there was concern by some that reshaping genetic systems might be hazardous in some unknown way, which led to a self-imposed moratorium on recombinant eukaryotic genetic material (Berg et al., 1974). As a consequence, elucidation of the gene expression machinery in animals was slowed until it became evident that the fears were based on fears of the unknown rather than any scientific evidence (Berg & Singer, 1995). The moratorium served as an unfortunate precedent for ignorance and unspecified fears impeding progress in animal genetics.

1.2.1. Classical Methods for Genetic Engineering of Animals—Once anxieties of cloning eukaryotic genes were addressed, plasmid-based recombinant DNA technology supported the rapid characterization of the molecular genetic mechanisms by which genes are expressed in complex animals and plants. Introduction of genetic material into an animal's genome requires overcoming the elaborate cellular mechanisms that minimize DNA modification and keep out foreign DNA. These mechanisms have evolved to maintain the integrity of the information in genomes and to prevent the subversion or destruction of

cellular activities. In animals, transgenic DNA faces three barriers to its introduction into genomes—the cell membrane, the nuclear membrane, and the structure of chromosomes (Fig. 2).

There are two fundamental ways of delivering genetic material into an animal genome (Fig. 3). Plasmid-based gene delivery has been the most common because these vectors can be made and isolated in abundance in most laboratories using simple procedures. Plasmids nearly always contain an antibiotic resistance gene to raise the concentration of the recombinant plasmid in host *Escherichia coli* cells. However, organisms containing a transgenic antibiotic gene, often referred to as a *selection marker*, generally are not advised for release outside laboratories, even though there is not any evidence whatsoever that such transgenes will have any effect on the environment. Although plasmids can be easily produced and purified, their introduction into genomes is difficult. The astonishing integrity of the boundaries is best appreciated by realizing that the average human consumes more than 1000 trillion genes per day, all of which are kept from the chromosomes of his/her cells. Hence, chemical treatments of the cells or direct injections generally are required for delivery of plasmids to cells. Of the hundreds of plasmids that actually enter the cell, only a few are incorporated into a chromosome. The outcome of plasmid delivery is uncertain in two ways. First, the transgenic DNA can integrate into any of billions of sites in a mammalian genome and second, the actual sequence that integrates into any site can vary. Consequently, these uncontrollable features can result in undefined sequences integrating into resident genes, which can lead to unwanted genetic effects. This is called *insertional mutagenesis*. Most concerns with genetically engineered organisms derive from the potential collateral effects that are hard to predict. An important, relatively recent modification of the plasmid delivery involves the use of transposons to carry the transgene into genome. DNA transposons insert a rigorously defined sequence into a genome with much higher efficiency than occurs by random recombination. Transposons are described in more detail in Section 2.5.1.

Viruses comprise the second generic method used for gene delivery into animal cells. Their activities and properties have been studied for decades. There are several hurdles with the use of viruses (Hackett, Largaespada, & Cooper, 2010). The first is cost of manufacture and purification in amounts required for effective delivery to cells, which prohibits their use in most laboratories. Second, viruses often direct integration into and/or proximal to resident genes and thereby influence normal cellular function. Third, cells have evolved elaborate defenses against viruses. Fourth, for commercial animals, there has always been anxiety about undefined virus effects.

A major issue in genetic engineering animals is controlling expression of the new genetic material so that the protein it encodes is made at the appropriate level in the right tissues (Jaenisch, 1988). Genetic elements called *enhancers* and *promoters* regulate the expression of a gene. The combination of an appropriate promoter with a transgene is called an *expression cassette*. For an expression cassette to be useful in commercial animals, it must be reliably expressed as it is inherited from one generation to the next. Regardless of whether the transgenic material is introduced as a plasmid, transposon, or viral genome, the site of its integration may affect the spatial and temporal features of its expression.

1.2.2. Early Genetic Engineering in Mice, Chickens, and Fish—The first transgenic animals were produced more than 30 years ago (Brinster et al., 1981; Cline et al., 1980; Gordon, Scangos, Plotkin, Barbosa, & Ruddle, 1980) and stable lines of animals were produced soon after (Gordon & Ruddle, 1981, 1982). The expression cassettes for the transgenes generally had viral promoters and were delivered on plasmids that integrated fairly randomly. As a result, they lacked tissue-specific expression of the transgenes (Lacy,

Roberts, Evans, Burtenshaw, & Costantini, 1983). The dramatic demonstration of growth enhancement in mice, a phenotype with clear relevance to food animals, following delivery of transgenic growth hormone genes (Palmiter et al., 1982; Palmiter, Norstedt, Gelinas, Hammer, & Brinster, 1983), led to predictions that recombinant DNAs would be introduced into food crops and animals (Bauman, McCutcheon, Steinhour, Eppard, & Sechen, 1985; Seidel, 1985; Wagner & Murray, 1985). However, in some cases random integration led to adverse effects, including death (ref). These observations led many to appreciate the delicate balance between introducing new desirable traits without incurring unwanted genetic effects. Insertional mutagenesis also rekindled the lingering fears of genetic tampering in animals (Rollin, 1985).

Two of the earliest genetic engineering projects in agricultural animals involved chickens and fish. Chickens are a major agricultural product and their susceptibility to viral infections stimulated interest in genetically engineering resistance to diseases. Moreover, transforming chicken eggs into bioreactors for the production of therapeutic proteins of high value appeared to be significantly better than transforming mammalian mammary glands to secrete the biological milk (Ivarie, 2003). The earliest experiments in avian transgenesis utilized retroviruses. Retroviral infections of poultry can cause sarcomas (Rous, 1910) and leukemias (Beard, Sharp, Eckert, Beard, & Mommaerts, 1952). However, cells that express viral envelope (*env*) proteins are resistant to infection. This observation led investigators to engineer lines of chickens that would be immune to infection by avian viruses by using modified avian viruses as vectors to deliver *env* genes to chicken genomes (Crittenden & Salter, 1985, 1986). Transgenic lines of chickens were achieved (Bosselman et al., 1989; Mizuarai et al., 2001; Salter, Smith, Hughes, Wright, & Crittenden, 1987; Thoraval et al., 1995); however, the efficiencies using retroviral vectors were low, the cargo capacity of retroviruses was limited, and some of the transgenic birds shed replicating virus. Other viral vectors, including lentiviruses, and transposons have been used to introduce transgenes into the chicken germline (Macdonald et al., 2012; Sang, 2004), but the efficiencies remain low, expression of the transgenes may be subject to epigenetic effects (Hofmann et al., 2006), and use of viral vectors to engineer food remains unsettling to the public. No transgenic poultry have been commercialized.

Genetic engineering in fish has a very long history because fish comprise a major source of protein and produce large numbers of eggs whose nuclei are easy to genetically manipulate (Yan, 1998). A further stimulus to genetic engineering of fish is the worldwide over-exploitation of fisheries that has led to a declining marine capture since its peak in 1996 (Smith, Asche, Guttormsen, & Wiener, 2010; Worm et al., 2009). Genetic engineering in fish is as simple as it gets. Microinjection of plasmids into eggs is easy but the efficiency of actually obtaining fish that will pass on the gene in an expressible state is quite low (Hackett, 1993). Nevertheless, owing to the large numbers of eggs and the ability to inject hundreds of fertilized embryos per hour, even inefficient random recombination of transgenic DNA into genomes with subsequent, reliable expression through multiple generations can be achieved. Consequently, following the isolation of vertebrate growth hormone genes, several groups throughout the world initiated programs to engineer fish with accelerated growth and development (Hackett & Alvarez, 2000). The most visible product from these endeavors was the Aqua-Advantage salmon (*Salmo salar*), fish that contained a single expression cassette comprising a Chinook salmon (*Oncorhynchus tshawytscha*) growth hormone gene transcriptionally controlled by a promoter from the ocean pout (*Zoarces americanus*) antifreeze protein gene. A critical achievement was the specific introduction of defined eukaryotic genetic sequences without attendant genes of either bacterial origin or known antibiotic activity that are commonly used for cloning of transgenic DNA sequences. Nevertheless, the genetically engineered salmon encountered intense opposition by a variety of groups concerned with food safety, environmental impact,

and other assorted issues, despite the finding that the fish were essentially equivalent to domesticated salmon (Devlin, Sakhrani, Tymchuk, Rise, & Goh, 2009; Smith et al., 2010; Van Eenennaam & Muir, 2011).

A large number of genes encoding both markers and proteins of commercial interest have been introduced into animal germlines using plasmids, naked DNA sequences, and viruses (Tables 1–4). Several effective methods of introduction of recombinant genomes into embryos have been developed. The most common are illustrated in Figure 4—somatic cell nuclear transfer (SCNT), microinjection, and sperm-mediated gene transfer (SMGT) (Carlson, Garbe, et al., 2011; Clark & Whitelaw, 2003). The studies reported in Tables 1–4 show that all three of the applications of transgenic technologies in large animals shown in Figure 1 have been initiated— improvement of intrinsic traits, improved medical products, and creation of better models of human disease. In all of these cases, the integration sites of the DNA sequences were uncontrolled and the efficiencies of producing germ-line transgenic animals were invariably low.

From a human gene therapy perspective, it would appear that the safety issues for gene delivery to humans are more relaxed than they are to animals! Between 1989 and mid-2012, 1786 gene delivery clinical trials in humans have been approved (http://www.wiley.com/legacy/wileychi/genmed/clinical/) of which about two-thirds employed viral vectors and the rest plasmid or other forms of “naked” DNA. There are two important differences in the design of gene therapy vectors. First, selectable marker genes are permitted in vectors introduced into human cells, with some restrictions (e.g., the kanamycin-resistance gene is preferred over genes encoding resistance to other antibiotics). Second, safeguards must be taken to ensure that only somatic cells take up transgenes; germline transmission of transgenic material is strictly forbidden. For genetic engineering of large animals, the important lessons from human gene therapy trials derive from comprehensive evaluations of insertional mutagenesis by a plethora of vectors. These vectors have a variety of integration preferences that include actively transcribed genes (lentiviruses), promoters and other transcriptional motifs (some retroviruses and adeno-associated viruses), and more random patterns (*Sleeping Beauty* transposons) (Berry, Hannenhalli, Leipzig, & Bushman, 2006; Mitchell et al., 2004). The issue of transgenes abnormally affecting resident genes has led to some adverse effects and to intense scrutiny of every patient for insertional mutagenesis. The results of these studies suggest that single gene activities do not cause adverse events, rather it appears that multiple events are responsible for adverse effects (Baum, 2011; Kustikova et al., 2009). This conclusion is not surprising given that there are hundreds of active endogenous transposable elements in human genomes that do not cause problems at a significant rate (Iskow et al., 2010); clearly, animal genomes have defenses against most random integrations. The totality of data from gene therapy studies, in which genetic material has been inserted into millions of human genomes strongly suggests that germline transgenesis will cause few significant effects on the recipient animal besides those designed by the genetic engineers.

The acceptance of the introduction of transgenic DNA into humans should serve as a model for evaluating gene transfer in farm animals. Yet, by mid-2012 only two types of transgenic animals have been approved for commerce. The first type includes transgenic goats that produce a human protein product in their milk (ATryn, sold by GTC Biotherapeutics). These animals are not sold to the public; only their transgenic product is sold for medical purposes. Ironically, ATryn was approved for human therapy in an arguably more stringently regulated European market 3 years prior to approval in the USA. The second type comprises genetically modified freshwater aquarium fish, called Glofish® (Knight, 2003), which have been cleared for retail sale by pet stores in most states. In the meantime, transgenic salmon, containing an extra copy of a salmon growth hormone gene, have languished in a regulatory

morass for more than a decade (Van Eenennaam & Muir, 2011). The legacies of transgenic chicken and fish are clear—there is widespread suspicion by the public, which is reflected by governmental regulatory agencies, involving the safety of transgenic animal products. Most of these concerns over health and safety issues, environmental containment, etc. were also expressed for transgenic crops where the regulatory history has been far different.

1.2.3. Genetically Engineered Animals Preceded Genetically Modified Plants— The first genetic engineering of plants came a couple of years after transgenic animals were made (Lamppa, Nagy, & Chua, 1985). The far more rapid progress in the genetic engineering of animals in comparison to plants was the result of several causes, including (1) strong financial support by National Institutes of Health (NIH) for developing human gene therapy that required a detailed understanding of molecular genetic processes in mammals and (2) the relative ease in introducing transgenic DNA into animal cells through the plasma membrane compared to the far more difficult procedures required to traverse plant cell walls. Yet, despite the increased scientific challenges involved with genetic engineering of plants and the far greater propensity of transgenic pollen and seed to spread, thereby increasing environmental concerns, by 2011, there were 67 million hectares of transgenic crops in the USA and 89 million hectacres worldwide, accounting for more than 85% of the maize, cotton, soybean, and sugar beet crops and worth billions of dollars (Peng, 2011). Containment and other environmental concerns (Hutchison et al., 2010; Sears et al., 2001) have been overcome in transgenic crop species that are far harder to contain physically and genetically (Tabeshnik, 2010) than in animals. Transgenic crops are commonly thought to contribute to more than 80% of the items on supermarket shelves (http://www.womenshealthmag.com/health/frankenfish).

1.2.4. Lessons from the Early Genetic Engineering of Commercially Important Species—Since the birth of the first genetically engineered large farm animal in 1985 (Hammer 1985), more than 180 successful trials of transgenic large livestock production have been reported in the subsequent 27 years (Tables 1–5). In the 1980s, the focus was on enhancing animal growth performances by ectopically expressing heterogenic or extra copies of growth factor genes. Common transgenes included growth hormone genes from a variety of sources, insulin-like growth factor, growth hormone-releasing factor, and others (Table 1). These early studies demonstrated the feasibility in expression of exogenous transgenes in livestock but failed to produce any animals with value worthy of translating to agriculture. Many transgenic animals either did not transmit their transgenes and/or the transgenes failed to remain active due to epigenetic silencing (Kues et al., 2006) or the animals failed to thrive (Table 1). In retrospect, these experiments likely failed for a variety of reasons including either the use of an inappropriate transgene promoter and instability of transgenes due to repeated structure, epigenetic silencing, or position effects. During the 1990s, the attention shifted to large animals as bioreactors for the production of a variety of proteins in milk, including many hematopoietic human proteins such as Factors VIII and IX, von Willebrand factor (vWF), and alpha-1 antitrypsin (AAT) in blood clotting pathways (Table 2). For this, the casein and whey acidic protein transcriptional regulators were employed as they provided high levels of expression of the transgenic proteins in milk (Clark & Whitelaw, 2003). These systems largely restricted expression of the transgene to mammary glands; thus, expressed proteins were less likely to interfere with the welfare of transgenic animals. Despite a higher success rate in terms of producing animals with economically viable levels of protein production, the framework for their regulatory approval lagged behind scientific developments by almost two decades. Indeed, only a single product from transgenic biore-actors has reached the U.S. market, ATryn, sold by GTC Biotherapeutics. A second product, recombinant human C1 esterase inhibitor produced

in the milk of transgenic rabbits, has been approved for use in Europe but not yet in the USA (van Doorn et al., 2005).

Pigs due to similar size and physiology also became the leading candidate for production of tissues and organs for xenotransplantation to humans (Bucher, Morel, & Buhler, 2005). As our knowledge in the molecules and reactions involved in xenograft rejection following tissue and organ transplantation grew, another wave of modifications arose to *humanize* the cell surface proteins of animals to suppress animal-specific antigens that initiated strong immunological rejections by the immune systems of human recipients (Klymiuk, Aigner, Brem, & Wolf, 2010; Sachs & Galli, 2009). A primary goal was to neutralize α1,3-galactose, the primary antigen responsible for hyper-acute rejection (Cooper, 2003) from the cell surface of pigs by inactivating the α1,3-galactose transferase gene (*GGTA1*). Several other transgenic approaches were developed to combat immune rejection, including either introducing or knocking out cell surface determinant proteins such as CD55, CD46, and CD59, followed by homologous recombination and SCNT to create GGTA1 knockout animals (Tables 3 and 5). Additional transgenic animals have been created to neutralize incompatibilities between blood coagulation systems and to limit T-cell responses (Table 3). Another key target for inactivation was the porcine endogenous retrovirus (PERV) locus that might allow recombinant retroviruses to emerge from transplanted porcine chromosomes, though transmission of PERV from swine to humans has never been observed *in vivo* (Fishman & Patience, 2004).

The physiological similarities that make pigs good candidates for xeno-transplantation also made them ideal candidates for modeling of human diseases (Table 4). Some human diseases cannot be accurately modeled in rodents due to differences in size and physiology. The first such example was created nearly 15 years ago by transgenic expression of a dominant–mutant rhodopsin gene (Pro347Leu) (Petters et al., 1997) as a model of retinitis pigmentosa. The phenotype of this model has remained stable through more than nine generations of outcrossing (Sommer et al., 2011) and is used yet today. The ability to perform homologous recombination in livestock fibroblasts and creation of animals by SCNT enabled modeling human disease caused by of loss-of-function (LOF) mutations (Table 5). The cystic fibrosis pig was the first porcine model of human disease to take advantage of targeted gene knockout. In contrast to mice, pigs either knocked out or containing a common mutation of the *Cftr* gene (Δ508) accurately recapitulate many of the pathologies observed in humans (Rogers et al., 2008). The similar size and physiology of pigs and humans suggests that introducing disease-associated alleles into pig genomes will result in relevant platforms for development of human therapeutics and devices.

All of the studies in Tables 1–4 led to substantial understanding of the limitations of transgenic technology using randomly integrating expression cassettes or recombinant sequences to inactivate selective genes. But, in addition to practical modifications that were based on direct benefits to humans, there were also innovative studies designed to generate transgenic animals that would enhance sustainability, e.g. the Enviropig (Golovan et al., 2001) was created to reduce manure phosphorous emissions, and fortuitously enhanced bone strength. Improved animal welfare is a clear area for animal genomics to flourish using precision genetics.

Yet, in contrast to transgenic plants and despite U.S. government (NIH, U.S. Department of Agriculture, National Science Foundation, Financial Services Authority, Environmental Protection Agency) investments of around $100 million dollars in funding research and risk analysis on large transgenic animals, not even one line of transgenic animal has been cleared for human consumption. The stated principle concerns have been either potential harm to consumers or potential harm to the environment, yet these concerns are not supported by

scientific findings (Fedoroff, Haselkorn, & Chassy, 2011). These are exactly the same issues faced by transgenic plants that have far greater abilities to spread and where far less is known about their genetics (Schurman & Munro, 2010). The advent of precision genetic techniques promises to satisfy scientifically based concerns regarding the development of transgenic farm animals.

There are five principle concerns with current transgenic organisms wherein expression cassettes were introduced randomly into recipient genomes: (1) insertional mutagenesis—the incoming genetic regulatory motifs affect the activity of a resident gene by either inappropriately activating or suppressing its expression; (2) inability to precisely control the expression of the transgene—resident genetic regulatory motifs in the vicinity of the integrated transgene influence its expression; (3) unstable expression of the transgene due to epigenetic effects that occur over time; (4) presence of unwanted DNA sequences that are required by the vector— plasmid or viral; and (5) unknown effects on expression of the transgene in various tissues—the transgene may be designed for expression in one tissue, but its expression in other organs and cells may vary considerably.

Over the past decade, newly developed methods allow specific replacement, addition, and/or deletion of genetic sequences in animal genomes. The application of precision genetics will avoid nearly all of the substantive issues of genetically engineered organisms that have been raised in the past.

2. PRECISION GENETIC ENGINEERING

As noted above, there are two issues critical to genetic modification of food animals. *The first, only defined changes are made at specific genetic loci*. This is important to ensure that only the expected phenotype will occur in the animal without collateral changes that could lead to unintended effects on consumers' health (e.g. production of an allergen as a result of random insertion leading to gene fusion or activation of genes in unexpected ways). *The second is the efficiency and precision with which such defined genetic changes can be introduced into genomes of large animals*. Over the past decade there has been enormous progress in both areas, as predicted by Clark & Whitelaw (2003).

There are three types of modifications to genomes that will enable efficient transgenesis in animals without unanticipated consequences: (1) adding precisely defined genetic sequence that will confer a new trait to an animal; in this case, the actual location of the gene is not important. (2) Editing a gene so that it either is inactivated or is converted to a desirable allele. (3) Adding a gene to a specific site in the genome, e.g., to express a protein under the direction of a native gene or placement of a gene in a location previously defined to permit effective gene expression (e.g., a *safe harbor*).

2.1. Precision Introduction of Expression Cassettes Using Transposons

Transposons are used to accomplish the first category of precision genetic engineering. Transposons are natural mobile elements that move either by a *copy-and-paste* mechanism via an RNA intermediate (class I transposon; by far the most numerous in animal and plant genomes) or a *cut-and-paste* mechanism (class II transposons) in which a precise DNA sequence is excised from one source of DNA and inserted into another DNA. Class II transposon systems consist of two components: (1) the transposon vector that contains a transgenic expression cassette flanked by inverted terminal repeats and (2) a source for the transposase enzyme (Fig. 5). Generally, class II transposons, cloned in plasmids, are used for genetic engineering because they can direct the integration of a defined expression cassette harboring a transgene and its regulators while leaving behind the rest of the plasmid with its selection markers (Dupuy et al., 2002; Hackett, Ekker, Largaespada, & McIvor,

2005). Nearly all of the class II DNA transposons identified in vertebrate genomes appear to be inactive (Plasterk, Izsvák, & Ivics, 1999; Venter et al., 2001; Waterston et al., 2002). Hence, the first transposon used in animal cells, called *Sleeping Beauty* because it was awakened from a ca. 14-million year sleep (Ivics et al., 1997), was synthetic. One consequence of the synthetic engineering of *Sleeping Beauty* from hundreds of extinct and active transposase genes is that it has considerably higher activity than natural transposons (Grabundzija et al., 2010). A number of other transposon systems have been developed for use in vertebrate cells, mainly for gene therapy in order to avoid viruses (Ivics et al., 2009). The advantages of transposons for human gene therapy, where transposons have been used for more than a decade in animal models (Aronovich, McIvor, & Hackett, 2011), extend to genetic engineering of large animals as well (Carlson, Garbe, et al., 2011; Clark et al., 2007).

2.2. Precision Editing of Genomic Sequences Using Meganucleases and Zinc Finger Nucleases

The studies listed in Tables 1–4 depended on random introduction of new DNA sequences into animal genomes. Random integration can produce unpredictable genetic effects that are bilateral between chromosomal genes and transgenes (Voigt, Izsvák, & Ivics, 2008). Position-effect variegation wherein transgenic sequences are silenced when introduced into chromatin and transactivation by the transgene on endogenous genes that are switched off can occur. One potential method to target transposons to specific sites would use *E. coli* RecA fusion proteins to induce genomic modifications. The bacterial recombinase RecA forms a nucleic acid-protein filament on single-stranded DNA during the repair of DNA double-strand breaks that efficiently undergoes a homology search and engages in pairing with the complementary DNA sequence. The pairing activity of RecA–DNA filaments that leads to site-specific breakage of DNA strands has been explored in zebra fish but awaits extension to large animal genomes (Cui, Yang, Kaufman, Agalliu, & Hackett, 2003; Liao & Essner, in press).

Rare-cutting DNases such as the yeast meganuclease *I-SceI* (Jasin, 1996; Rouet, Smih, & Jasin, 1994; Smih, Rouet, Romanienko, & Jasin, 1995) show great promise for the alteration of chromosomal sequences at a few specific sites (Choulika, Perrin, Dujon, & Nicolas, 1995). Meganucleases are precise and effective at cleaving their cognate recognition site in the genome, but the overlap of DNA recognition domains and the enzymatic centers of these compact proteins has made reprogramming them to recognize different sites in the genome difficult, although some progress has been made (Arnould et al., 2011; Chames et al., 2005). Efforts to use these reagents have been confounded by the rarity of sites present in livestock genomes that correspond to the addresses represented in current enzyme libraries (Fahrenkrug unpublished).

A major step toward the goal of developing site-specific genetic engineering was construction of chimeric nucleases composed of a nuclease domain and a separate, designer DNA recognition domain. The first such enzymes employed zinc finger (ZF) DNA recognition domains tethered to the endonuclease domain of *FokI* (Kim, Cha, & Chandrasegaran, 1996). Because Cys_2His_2 ZFs can be designed to bind to specific sites (Desjarlais & Berg, 1993; Jamieson, Wang, & Kim, 1966), artificial zinc finger nucleases (ZFNs) became a tool to cleave specific genetic loci (Bibikova, Beumer, Trautman, & Carroll, 2003; Bibikova et al., 2001; Kim et al., 1996; Park et al., 2003; Porteus & Carroll, 2005). The human gene therapy community quickly recognized the potential of site-specific integration of therapeutic transgenes and developed the use of ZFNs in human cells (Carroll, 2011; Hockemeyer et al., 2009; Porteus & Baltimore, 2003; Urnov et al., 2005; Urnov, Rebar, Holmes, Zhang, & Gregory, 2010). Table 5 lists studies in large animals that have employed ZFNs for targeted mutagenesis.

ZFNs were revolutionary, but although their assembly appeared easy theoretically (Klug, 2010), in practice, it was not. Generally, specific ZF-binding domains recognize a three-base sequence. Unexpectedly, it turned out that the various finger domains influenced each other such that when assembled into arrays, the fingers did not bind to targeted sequences with high efficiency (Lam, van Bakel, Cote, van der Ven, & Hughes, 2011). This problem necessitated the testing and selection of multiple combinations of fingers to determine those with the highest ZFN specificity and efficiency. The Oligomerized Pool Engineering strategy permits manufacture of ZFNs that recognize sites about every 200 basepairs of *random* genomic sequence (Maeder et al., 2008; Sander et al., 2010). Alternatively, context-dependent assembly (CoDA) (Sander, Dahlborg, et al., 2011) uses an archive of validated two-finger units derived from selection that have been validated to function when positioned adjacent to each other. CoDA-based ZFNs can be constructed that recognize approximately one site in every 500 basepairs of *random* genomic sequence. Other options that claim to have a targeting range of 1 in 125 basepairs of *random* genomic sequence are available (Kim, Lee, Kim, Cho, & Kim, 2009; Ramirez et al., 2008).

2.3. Precision Editing of Genomic Sequences Using TALENs

Recently a new type of chimeric nucleases has exploded onto the genetic engineering scene due to their ease in design and greater range of sites that can be targeted (Bogdanove & Voytas, 2011; Carlson, Fahrenkrug, & Hackett, in press). Transcription activator-like (TAL) effector nucleases (TALENs), like ZFNs, consist of assembled DNA-binding motifs coupled to a *Fok*I endonuclease domain (Boch & Bonas, 2010; Boch et al., 2009; Christian et al., 2010; Li, Huang, Jiang, et al., 2011; Mahfouz et al., 2011; Moscou & Bogdanove, 2009). TAL-effector DNA-binding motifs are found in proteins secreted by plant pathogens in the bacterial genus *Xanthomonas*. Typically, TAL-effectors consist of tandem repeated 34 amino acid blocks. Residues 12 and 13 of the 34 amino acid repeats are referred to as repeat variable *diresidues* (RVDs). The RVDs define the binding to a specific base. Unlike ZFs that bind to three basepairs, each TAL-effector repeat binds to a single basepair (Boch et al., 2009; Moscou & Bogdanove, 2009) (Fig. 6). A simple cipher greatly simplifies the design of TALENs and makes their modular assembly far easier than is possible with ZFNs (Cermak et al., 2011; Li, Huang, Zhao, et al., 2011; Miller et al., 2011; Morbitzer, Elsaesser, Hausner, & Lahaye, 2011; Reyon et al., 2012; Weber, Engler, Gruetzner, Werner, & Marillonnet, 2011; Zhang et al., 2011).

Since the demonstration by Boch et al. (2009) that artificial TAL effectors could be targeted to specific DNA sites to activate transcription, sequence-specific DNA-binding proteins with predicted binding specificities have been generated economically in a matter of days using standard methods of molecular biology (Cermak et al., 2011; Li, Huang, Zhao, et al., 2011; Morbitzer et al., 2011). TALENs introduced into human cells can direct site-specific mutagenesis at rates of up to 45% of chromosomes (Hockemeyer et al., 2011; Mahfouz et al., 2011; Miller et al., 2011; Mussolino et al., 2011; Orlando et al., 2010). TALENs have been used to create site-specific modifications in zebrafish (Huang et al., 2011; Sander, Yeh, Peterson, & Joung, 2011) and rats (Tesson et al., 2011) at levels equivalent to those achieved with ZFNs. In addition to their ease of assembly, TALENs have another advantage over ZFNs—studies of native TAL-effector sequence preferences suggest a good TALEN sites occur in every 35 bp (Cermak et al., 2011). However, a recent study stretched the rules proposed by Cermak et al. (2011) and found that the true targeting range may be even better than 1 site per 35 basepairs in the genome (Reyon et al., 2012). In addition, the recent elucidation of the molecular structures of TAL-effector binding to DNA (Deng et al., 2012; Mak, Bradley, Cernadas, Bogdanove, & Stoddard, 2012) may further improve the design process and specificity.

2.4. Off-Target Cleavage Activity by ZFNs and TALENs in the Context of Natural Variation

A potential concern in the use of ZFN and TALEN site-specific nucleases is cleavage at unintended sites, referred to as *off-target activity*. This issue has been addressed over the past decade. While some potential off-target sites can be predicted, unbiased studies of ZFN off-target cleavage reveal shortcomings of *in silico* off-target predictions (Gabriel et al., 2011; Pattanayak, Ramirez, Joung, & Liu, 2011). Both Gabriel et al. (2011) and Pattanayak et al. (2011) chose to evaluate off-target cleavage of the highly characterized CCR5-224 ZFN pair, currently in clinical trials for gene therapy in humans. A total of 13 off-target sites were identified that occurred at an appreciable frequency (1:7–1:10,000 cells). In all cases, cleavage at the desired site was greater than five-fold more frequent than at other sites. The most important conclusion from these studies is that while off-target activity was present in a minority of cells, it was highly restricted to a small subset of loci, which implies that selective screening of potential off-target sites can be conducted following use of other ZFNs and TALENs.

As with ZFNs, early studies reveal that TALENs can bind degenerate sequences and have demonstrated activity at related off-target sites (Mussolino & Cathomen, 2011; Tesson et al., 2011). The specificity of TALENs has yet to be characterized in detail. Preliminary studies in cells and zebra fish reveal that cytotoxic effects of TALENs are either lower or similar to those with comparable ZFNs (Mussolino et al., 2011). Notably, TALEN pairs in these studies utilized the wild-type homodimeric *Fok1* domain, which are more prone to cleaving erroneous sites, while ZFNs used one of the three obligate heterodimer domains that increase specificity and reduce cytotoxicity (Doyon et al., 2011; Miller et al., 2007; Szczepek et al., 2007).

Regardless of the platform (ZFNs or TALENs) and *Fok1* domain (homodimer or heterodimer) used, there will be the potential of generating off-target genetic lesions. To address the implications of off-target lesions in genetically modified animals, we compared the worst-case estimate of off-target frequency with natural variation and germline mutation rate. As an example, consider a theoretical ZFN (or TALEN) with a poor on/off-target activity ratio of 1:1 that directs targeted cleavage and mutagenesis at a 25% efficiency, then one in four cells with an on-target event also would be expected to have an accompanying single off-target lesion. As a result, one in four animals derived from cloning of these cells would have a *de novo* change to its genome outside of the intended locus. In comparison, deep sequencing of two parent–child trios in the 1000 genomes project (a total of six people) revealed that each individual has 30–50 *de novo* germline mutations (Durbin et al., 2010; Marth et al., 2011). Assuming the data for humans is applicable to other large mammals, the risk of a random change to the genome by reproduction is more than 100-fold greater than any unintended mutations resulting from a site-specific nuclease employed for directed genome modification.

There is a further consideration. Most *de novo* germline mutations in humans are single-base substitutions in contrast to an indel that would result from non-homologous end-joining (NHEJ) activity during repair of an off-target site (Fig. 6B). Two-thirds of exonic indels would be expected to cause a frameshift leading to premature termination of translation, whereas only a small portion of naturally occurring single nucleotide polymorphisms (SNPs) would result in a protein truncation. Deep sequencing has found that indels are about 10-fold less frequent in the human genome than SNPs (22,000 vs. 1800 per genome compared to reference) with up to 50% of the indels being novel in any given individual (Alkan, Coe, & Eichler, 2011; Marth et al., 2011). Thus, introducing this aspect into the calculation for the worst possible scenarios, off-target NHEJ activity would occur more than 10-fold less frequently than the background indel mutation rate. Moreover, because only about 2% of the

genome encodes proteins, about 98% of off-target events would be unlikely to affect protein sequences.

Deep sequencing of hundreds of human genomes has revealed that the average human genome has approximately 250–300 LOF mutations, with 50–100 in human disease genes (Durbin et al., 2010; Pelak et al., 2010) and about 20 completely inactivated genes (MacArthur et al., 2012) as classified by the Human Gene Mutation Database (http://www.hgmd.org). Thus, *the human genome* is highly variable (Kidd et al., 2010) and recent next-generation sequencing of the cattle genome suggests similar, high degrees of variation (Bickhart et al., 2012). Indeed, sequence survey of around 100 cattle (Fahrenkrug, unpublished) and high-density genotyping (J. Taylor, personal communication) have revealed similar frequencies of both heterozygous and homozygous LOF alleles.

2.5. Precision Alterations in Livestock Genomes

2.5.1. Transposon-Modified Animal Genomes—Transposon systems have been mainly and extensively used in mice for identifying oncogenes and for developing methods for human gene therapy. Transposons have been used less frequently in large, genetically modified animals. As shown in Tables 1–4, many of these animals were accomplished through random insertion of naked linear DNA introduced by early embryo injections, SMGT, or transfection of harvested animal cells accompanied by SCNT. As noted earlier, epigenetic effects, position-effect variegation, and variations in the numbers of integrated expression cassettes hampered the efficiencies of generating modified animals with predictable levels of transgene expression. Alternatives were broadly sought to optimize such situations; recombinant viruses or the *Sleeping Beauty (SB)* transposon system (Ivics et al., 1997) bearing desired transgenes have been shown to mediate insertions more efficiently via embryo injections, transfections, and SCNT (Tables 1–4). Moreover, they are less prone to integrate in the form of concatemers, and through intricate ways, one is able to control the copy number insertions. Transposons may be preferable to viruses given public concern about even functionally impaired viral relics in the modified genomes.

2.5.2. ZFN-Modified Animal Genomes—Gestation length and maturation to reproduction age for pigs and cattle is significant. For example, generation of a homozygous knockout from heterozygous mutant cells (both sexes) by cloning and breeding requires 16 and 30 months for pigs and cattle, respectively. It is possible to reduce this burden with sequential cycles of genetic modification and SCNT (Kuroiwa et al., 2004); however, this is both technically challenging and cost prohibitive. Taking advantage of the proclivity of ZFNs to modify both alleles, Hauschild et al. (2011) recently generated bi-allelic *GGTA1* knockout pigs using commercial ZFN reagents and cloning. In this example, bi-allelic null cells could be enriched by fluorescence-activated cell sorting for the absence of the α1,3-galactose surface epitope. Unfortunately, biological enrichment for null cells using flow sorting will not be available for the majority of genes. Others have generated heterozygous knockout animals by ZFN-induced NHEJ in flbroblasts from pigs and cattle (Table 5). These studies demonstrate proof-of-principle; in about half of the examples engineered, ZFNs were relatively inefficient (i.e. only 2–4% of transfected cells were modified), which in terms of colony screening is not a significant improvement over standard homologous recombination. However, in contrast to traditional methods of homologous recombination, gene knockouts can be accomplished by introducing frame-shifts in coding regions from NHEJ without the use of selection markers.

2.5.3. TALEN-Modified Animal Genomes—At first glance, TALENs appear as somewhat of a redundant tool to ZFNs; they support the same types of precision genetic alterations (Fig. 6). However, there are two key features of TALENs that set them apart

from ZFNs for widespread adaptation by livestock biotechnologists. First, and most importantly, simple design and assembly strategies for TALENs have been developed that can be implemented in any molecular biology laboratory (Cermak et al., 2011). A second advantage of TALENs is their targeting range that is far superior to that of ZFNs. For instance, we were able to rapidly assemble 36 TALEN pairs using the Cermak assembly procedure, 64% of which were active in livestock fibroblasts with an average chromosome modification frequency of 25% (Carlson, Tan, et al., in press). We recently reported the births of 18 low-density lipoprotein receptor ± Ossabaw piglets from TALEN-induced NHEJ and SCNT (Table 5). Carlson et al. also demonstrated that several TALEN pairs were efficient at inducing indels by direct injection of mRNA encoding them into the cytoplasm of both swine (about 30%) and bovine (about 75%) embryos.

Application of TALENs to cultured cells has also shown great promise for the creation of livestock with precise modifications. For example, we developed strategies for derivation of fibroblast clones with bi-allelic modifications (up to 10%) without biological enrichment (Carlson, Tan, et al., in press). TALENS are also capable of more complex changes in livestock fibroblasts. Cotransfection of two pairs of TALENs targeting the same chromosome was capable of creating large chromosomal deletions or inversions (Carlson, Tan, et al., in press). Perhaps most compelling, cotransfection of TALENs with a donor template has allowed directed homologous recombination for efficient insertion of either a transgene or for copying small, defined change to the genome without the aid of selection markers (authors, unpublished).

3. FUTURE DIRECTIONS—APPLICATIONS OF PRECISION GENETICS IN ANIMALS

3.1. Rapid Allele Introgression for Improvement of Food Animals

There are numerous livestock breeds that have been extensively selected for a specialized set of traits, i.e. milk yield and composition, meat yield and composition, growth rate, thermotolerance, disease and parasite resistance, etc. Frequently, alleles that would benefit a particular breed are present within the species but exist only in undeveloped breeds or breeds that have historically been selected for traits that differ to those that are of priority in the target breed (e.g. meat vs. milk production). TALEN-based gene conversion may provide an opportunity for transferring beneficial alleles between animals/breeds without disrupting the improved genetic architectures achieved by long-term selection within these breeds. However, traits for which only a few loci account for a large proportion of the observed genetic variance are clearly more attractive targets for this technology (Casas et al., 1999; Grisart et al., 2002) than traits for which a large number of loci contribute only minor magnitudes of effect (Cole et al., 2009; Kemper, Visscher, & Goddard, 2012), such as those that appear to predominate for complex traits.

The example presented in Figure 7 is of particular interest. Holstein cattles have been extensively selected for high milk yield and milk quality. Unfortunately, the great majority of both male and female Holsteins develop horns. To protect the welfare of both dairy farm operators and the cattle themselves, horns are routinely manually removed from the majority of Holstein cattle. Mechanical de-horning is painful, elicits a temporary elevation in animal stress, and adds expense to animal production (Graf & Senn, 1999), and despite the intent of protecting animals from subsequent injury, the practice is viewed by some as inhumane. In contrast, several breeds (e.g., Red Angus, specialized for high quality/yield meat) are naturally horn free, a trait referred to as polled (Fig. 7). The polled trait follows a dominant inheritance pattern (Long & Gregory, 1978) and multiple groups are making progress on

identifying the causative mutation (Seichter et al., 2012; J. Taylor, personal communication).

Introgression of the polled allele into horned breeds could easily be accomplished by crossbreeding (Fig. 7B); however, the total genetic merit for milk production in the crossbred animals would dramatically suffer. Furthermore, meiotic recombination would mix alleles influencing beef and milk production traits in each crossbred animal that would require numerous generations of backcrossing and intensive genome-wide, marker-assisted selection to recover the original level of quality milk production. During the same period, continued selection for milk production alone within the purebred Holstein population would have created genetic improvement that could never be recovered in the graded-up polled Holstein population. Thus, the inability to transfer a distinct allele from one breed to another translates to significant temporal and economic losses due to the long generation intervals in livestock. However, our results demonstrate that TALEN-mediated homologous recombination can be used to direct efficient allelic introgression in livestock without contamination of untargeted sequences and/or introduction of undesirable traits (authors, unpublished). In the specific case of the polled trait, once the responsible locus is identified, TALEN-mediated homologous recombination could in theory be used to introduce just the polled allele without meiotic contamination (or allelic diffusion) (Fig. 7C). The resulting animals would both lack horns and retain their high genetic merit for milk production.

There are numerous additional examples where TALEN-mediated allelic introgression could benefit animal agriculture. As previously mentioned for humans, each genome harbors 200–300 defective/broken genes in both heterozygous (the majority) and homozygous states. The fact that putative LOF alleles are observed in homozygous states indicates that many of these loci are not lethals, possibly due to functional redundancy with other genes. However, within each individual about seven of these loci are early developmental lethal and many of the others are likely to have deleterious effects on animal productivity and these loci are excellent targets for repair using TALEN-mediated allelic correction. Often, while desired alleles are being accumulated through selection, closely linked defective alleles are perpetuated and even enriched within a population. Causative mutations for at least 62 disease loci have now been determined in cattle and are cataloged at OMIA (http://omia.angis.org.au/home/) (Table 6). Recently, several haplotypes were discovered that affect the fertility in common dairy breeds of cattle including Holstein, Brown Swiss, and Jersey (VanRaden, Olson, Null, & Hutchison, 2011). These haplotypes were identified due to their lack of occurrence in the homozygous state, despite their significant frequency in the population (4.5–25% carriers), which suggests that the homozygous haplotype results in lethality. Given the frequency of predicted LOF alleles from sequence surveys, more examples like this will emerge.

Management of known disease alleles has traditionally relied on the culling of carriers via marker-assisted elimination from genetic improvement programs. However, given the frequency of such alleles within the population, it seems likely that selection programs will be confounded by linkage disequilibrium between LOF and beneficial alleles. We propose that under these circumstances, the confounding genetic defects may be candidates for correction by TALEN-mediated gene conversion. Indeed, of the 75 mutations for the 62 cattle disease loci described in Online Mendelian Inheritance in Animals website (http://omia.angis.org.au/home/), 87% are either SNPs or small indels of less than 20 bp (Table 6), which are highly likely to be amenable to homology directed allelic correction. Such targetable loci will likely predominate as suggested by deep sequence surveys of numerous species.

Correction either of genetic lesions or the introgression of desirable alleles into livestock must be consistent with the objectives of ongoing genetic improvement programs. This could be achieved by either (1) editing the genomes of animals previously determined to be of significant genetic value or (2) editing the genomes of animals prior to determining their implicit genetic value (Fig. 8). In the case of cloning (Fig 8A), gene-editing would need to be implemented sufficiently quickly to keep pace with ongoing genetic improvement programs. The application of genomic selection is already accelerating genetic improvement by allowing the estimation of genetic merit without the requirement of performance testing. In theory, genetically superior newborn animals could immediately be identified and subjected to gene editing for the correction of an LOF allele or the introgression of desirable alleles that are not already present. This approach provides for a controlled and characterized outcome at every step of the process. Theoretically, there are no limitations in the types and numbers of edits that can be made. Alternatively, since embryo transfer is already part of the genetic improvement paradigm for some livestock (e.g., cattle), editing could be applied by the direct treatment of embryos (Fig 8B). The efficiency of such modifications would need to be sufficiently high to offset any losses in reproductive rate engendered by embryo treatment. In the case of simple gene inactivation, the frequency of success is already very high (75%), with even homozygous modification in 10–20% of embryos (Carlson, Tan, et al., in press). More sophisticated edits have yet to be tested in livestock embryos, but results with ZFNs in mice, rats, and rabbits (Carbery et al., 2010; Flisikowska et al., 2011; Meyer et al., 2010) and with TALENs in zebra fish (Huang et al., 2011; Sander, Cade, et al., 2011) and rodents (Tesson et al., 2011) suggest that even template repair can reach signifcant frequencies in treated embryos. Furthermore, the use of repair templates in association with RecA-mediated sequence searching, alignment, and strand-invasion functions may further increase the number and frequency of gene-editing events in injected embryos. Moreover, precision genome editing can also be used to introduce alleles that do not currently exist within a species by homology-driven allelic substitution. Geneticists working with non-livestock species, e.g., humans, have identified candidate alleles with potential utility in farm animals. There are now the possibilities to create livestock that can be used for disease models as well as enhance agricultural sustainability, food safety, and security. At the current rate of improvement in efficiency, gene editing will be limited only by our imagination.

3.2. Regulatory Issues

Safety to consumers is the primary concern of regulatory as well as agricultural workers and geneticists. Precision genetics clearly will reduce unexpected alterations in genomes compared to those that occurred in the first waves of transgenic animals as well as crops and in human gene therapy. However, no technology is completely free of risk. As previously mentioned, ZFNs have already advanced to human clinical trials (Cannon & June, 2011). Effective gene therapy of humans requires treatment of several million cells and re-implantation into a host. This amplifies the chance of accumulating a deleterious mutation several million fold compared to single genetically modified embryonic cells with genetically edited genomes. The current paradigm for generation and approval of genetically engineered animals either for human consumption or for biological products that will be used in humans or for treatment of human disorders emanates from a single modified cell/embryo. All subsequent animals would be generated from one or a few founder(s). This paradigm offers several opportunities to eliminate mutations that might compromise animal welfare. First, generation of animals by either SCNT or microinjection allows biological selection in culture against compromised genomes prior to delivery to an embryonic environment. Second, animal genomes can be sequenced for less than $5,000 and this cost is rapidly declining (http://www.genome.gov/sequencingcosts/). Since off-target lesions in founder animals would be clonal, their identification by sequencing will become a standard

step before the animals are proposed for commercialization. Breeding will allow segregation of any off-target lesions from the desired genetic alteration. In severe cases, afflicted animals would be culled. Fortunately, since the majority of off-target lesions occur at a very limited number of sites that do not have to be in genes, screening for off-target events will be relatively easy to apply to the paradigm described in Fig. 8B.

What are the real risks of consuming GE animals? The first question to answer is what are the feared, not necessarily legitimate, effects of off-target lesions in food animal genomes to human or animal welfare. First, an on- or off-target change could result in a LOF mutation affecting the animal's welfare (Jackson et al., 2010). In this case, the animal would be culled and not proposed for commercial sale. Second, an on- or off-target lesion could alter a protein's sequence such that a novel peptide could elicit an immunological response. Actually, nature already runs this experiment. Agricultural animals have genomes similar in size to that in humans and thus should accumulate *de novo* mutations at a similar rate as humans, i.e., about 40 mutations/ individual/generation. In the case of pigs, about 1.3 billion animals are consumed per year. The accumulated number of *consumed* mutations per year would then be about 50 billion, corresponding to about 10 changes at every position in the porcine genome per year. Third, an interaction between an untargeted alteration and other factors could produce an unspecified deleterious effect. As mentioned above, each individual genome harbors many thousands of unique SNPs, indels, and copy number variants. There is no way to quantify an unspecified interaction between genetic elements of a sort that have not been seen before. However, whatever the chances might be of a heretofore-unknown genetic interaction having an adverse effect, they are certainly less than known genetic interactions that occur by crossbreeding, which has never been considered to have a negative impact on food safety.

Although a recombinant DNA construct may be considered a drug (FFDCA, 21 U.S.C. 321 et. seq.), the question is whether animals derived through the application of precision genetics also meet the definition. While the process used in precision genetics is different from natural processes by virtue of being man caused, the outcomes obtained through precision genetics, e.g., substituting one naturally occurring allelic form of a gene for another of the same gene or inducing a mutation in an existing gene that is similar to one obtained through classical animal breeding, are the same as those that occur in nature. *All* scientific evidence suggests that precision genetics should be a method that has far fewer risks than conventional breeding and therefore should be generally regarded as safe (Waltz, 2012).

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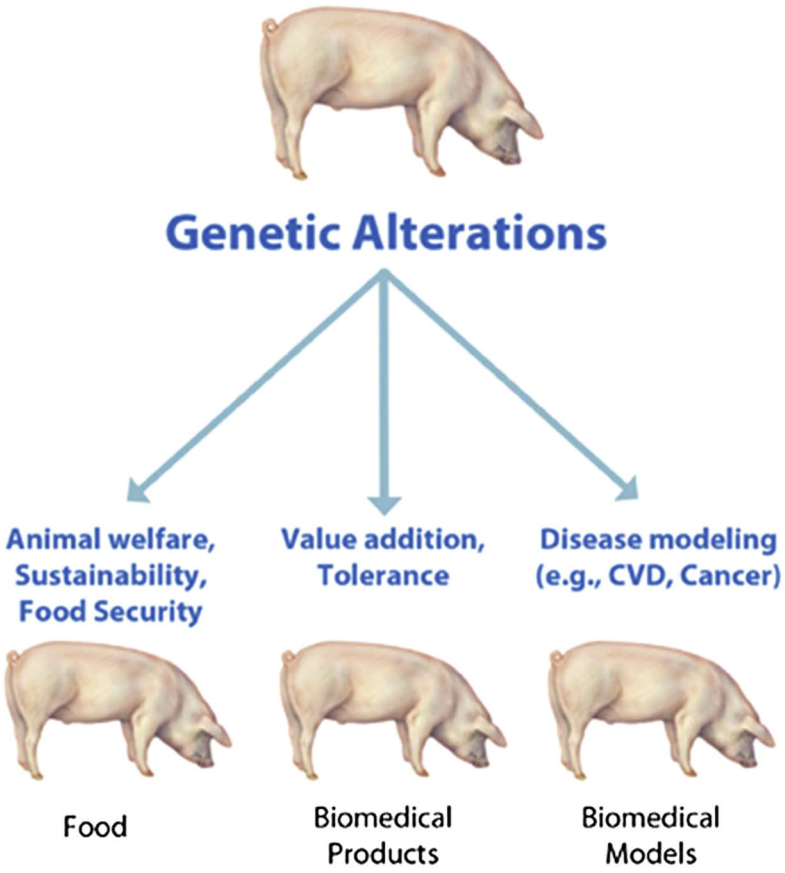

Figure 1.

The multiple applications of genetically modified large animals. The pig is shown as an example. The first application is to improve traits in the farm animal. Examples of the potential improved traits include (1) resistance to diseases, (2) improved nutrition such as introducing a gene to produce the healthier omega-3 fatty acids to replace the normal omega-6 fatty acids (Lai et al., 2006), and (3) reducing the environmental impact of major pig production facilities by reducing phosphorous in manure (Golovan et al., 2001). The second application of genetically modified pigs is for biomedical products such as organ transplantation (http://web.archive.org/web/20071210031618/http://www.fda.gov/fdac/features/596_xeno.html) or specific functional organ parts such as heart valves and subcellular structures. Examples include inactivating genes such as α-1,3-galactose that produce powerful immune responses when introduced into humans and eliminating the potential spread of porcine endogenous retroviruses. The third application of genetically modified pigs is the creation of animals that closely mimic human diseases such as cystic fibrosis (Rogers et al., 2008), cardiovascular disease, and cancer. For color version of this figure, the reader is referred to the online version of this book.

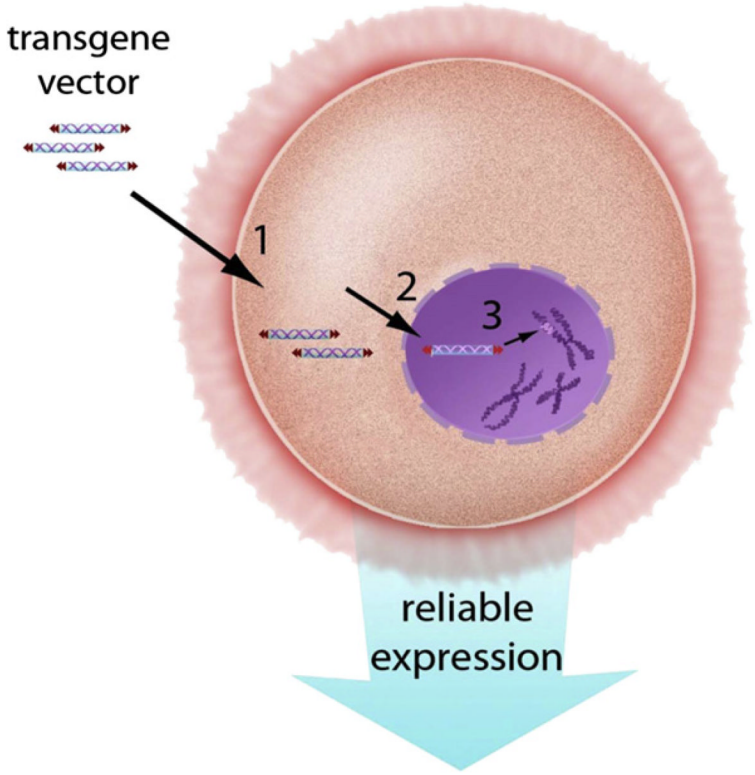

Figure 2.
The three barriers to the introduction of foreign DNA into genomes: (1) the cell membrane, (2) the nuclear membrane, and (3) the chromosomal DNA in the chromosomes. For effective transgenesis, the foreign DNA must overcome the three barriers and then be able to withstand protective measures such as methylation that are employed to reduce expression of transgenic DNA that has inserted into the chromatin. For color version of this figure, the reader is referred to the online version of this book.

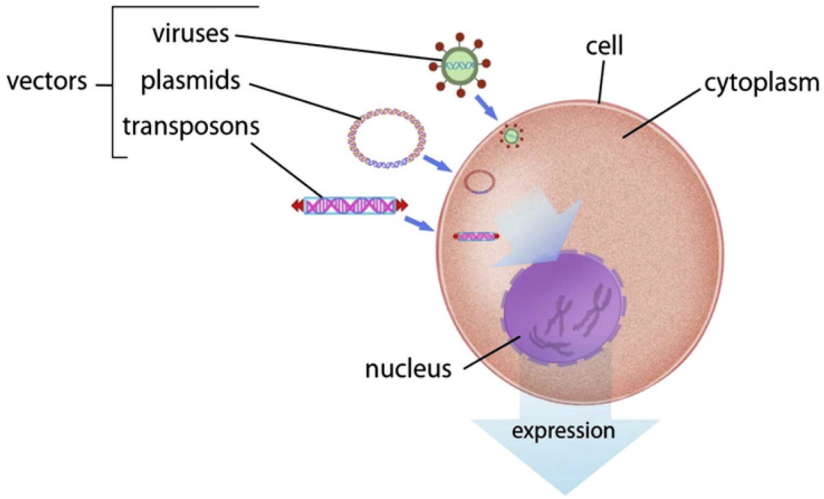

Figure 3.
The three vectors for introduction of foreign DNA into genomes: (1) plasmids, (2) viruses, and (3) transposons. For color version of this figure, the reader is referred to the online version of this book.

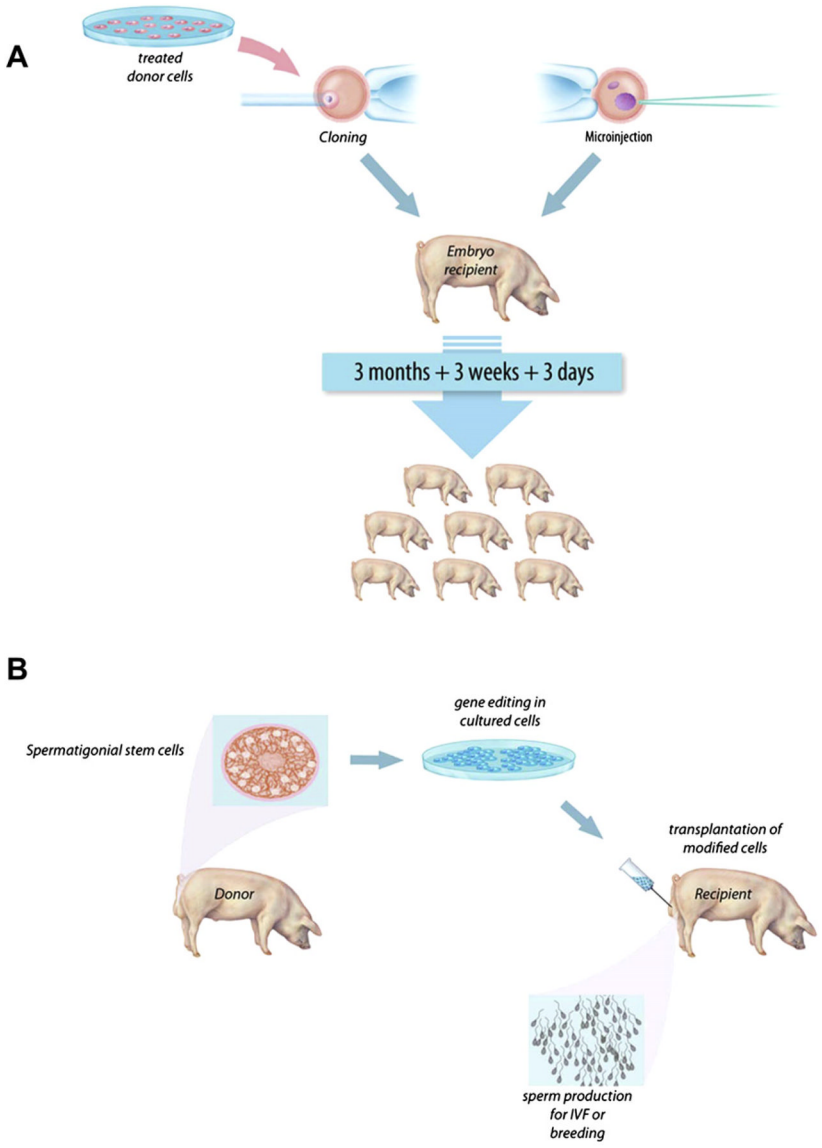

Figure 4.

Methods for genetic modification in livestock. (A) A flow diagram of the primary steps involved with the production of transgenic livestock by SCNT (cloning) and embryo microinjection. For simplicity, the illustrations show pigs only, but the general procedure applies to each of the major livestock species. Each procedure requires either surgical or *in vitro* production of oocytes or embryos. Donor cells used for SCNT (left) can be genetically modified in culture by a number of methods described in this review. Modified donor cells are injected into enucleated oocytes, which are then fused and activated prior to embryo transfer into a recipient. Embryo microinjection (right) is performed on zygotes 18–24 h after fertilization. The injection site can vary, but typically, DNA is delivered directly to the pronucleus by pronuclear injection, *SB* trans-posons plus transposase mRNA, ZFN, or TALEN mRNA can be injected into the cytoplasm, and viral particles are typically injected into the perivitelline space. Embryos manipulated in each case are implanted into a synchronized recipient female to establish pregnancy. Resulting offspring can be screened for the desired modifications and expression patterns. (B) Spermatogonial stem cells offer a second method for genetic modification of livestock. Genetic modification or gene edits can

be executed *in vitro* in spermatogonial stem cells isolated from donor testes. Modified cells are transplanted into germ cell-depleted testes of a recipient. Implanted spermatogonial stem cells produce sperm that carry the genetic modification(s) that can be used for breeding via artificial insemination or *in vitro* fertilization (IVF) to derive founder animals. For color version of this figure, the reader is referred to the online version of this book.

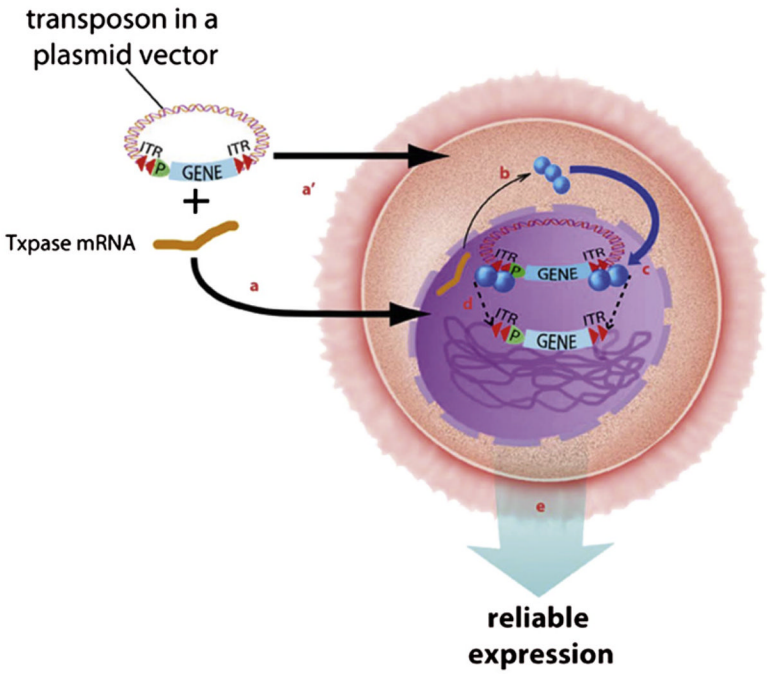

Figure 5.

DNA transposition consists of an enzymatic cut-and-paste reaction in which a transposon containing a gene of interest [shown in blue, with its promoter (P)] is cut out of a plasmid and inserted into a chromosome. The cleavage reaction occurs at the ends of the ITRs (inverted set of red double arrowheads) of the transposon. The transposons integrate only into TA-dinucleotide basepairs (about 200 million in a mammalian genome). The ITRs are the only DNA sequences required by the transposase enzyme for transposition. The transposase enzyme (Txpase, blue balls) drives the cut-and-paste reaction. Transposase activity is obtained by co-injecting transposon and an mRNA encoding the Txpase (blue squiggle) into either the nucleus (a) or cytoplasm (a′). The plasmid carrying the transposon and transposase-encoding mRNA enter a cell (large back oval) and proceed through the nuclear membrane (dashed line) (b). The transposase mRNA is translated in the cytoplasm to give an appropriate level of enzyme (c). The transposase molecules enter the nucleus and bind to the transposon, two at each end (c). Four transposase enzymes work in concert to cleave the plasmid at the termini of the transposon and paste it (dotted lines) into chromosomal DNA (green tangled lines) (e). Monomeric integration into a chromosome can confer reliable expression of the gene of interest that is contained within the transposon through multiple generations. For color version of this figure, the reader is referred to the online version of this book.

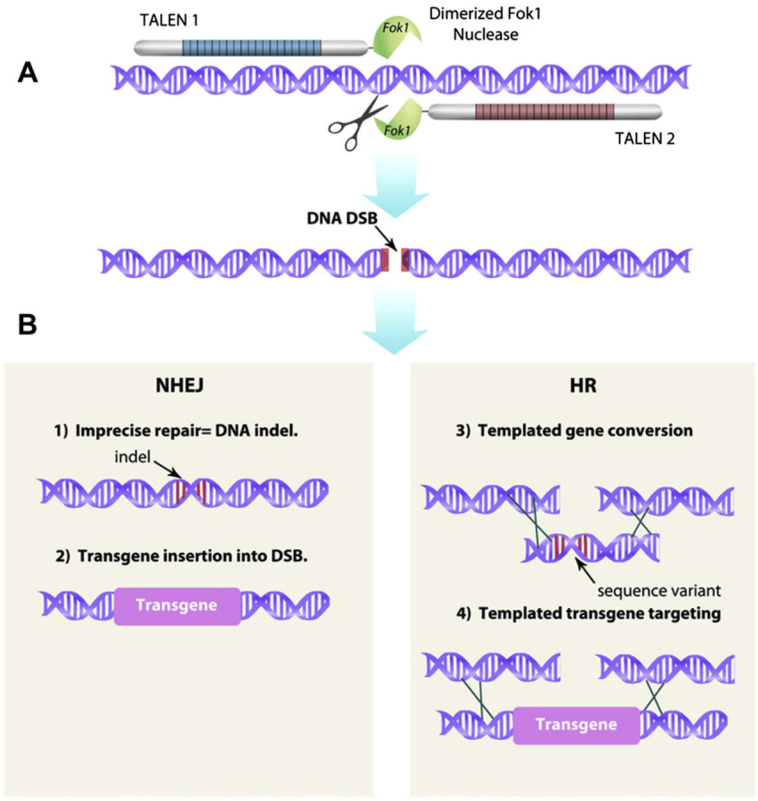

Figure 6.

Site-specific targeting of genetic changes using hybrid DNases. (A) A pair of TALEN nucleases is shown as an example of hybrid DNases designed to cleave at a unique sequence in a genome. The pair of TALENs executes a double-strand DNA break (DSB) at the targeted locus. (B) If no other DNA sequences are added, the DSB will be repaired by the process of NHEJ that will generally result in a minor insertion or deletion of a few basepairs (indels; example 1). Alternatively, because the NHEJ DNA repair enzymes that assemble at the DSB can facilitate the integration of a foreign DNA sequence, a transgene can be introduced into the site with higher than random efficiency (example 2). Alternatively, if a DNA sequence that has a high identity with the region surrounding the DSB is introduced, homologous recombination (HR) can occur (examples 3 and 4). The introduced DNA sequence may vary by only a single (or a few) basepair, which results in a defined mutation that is equivalent to a natural allele (example 3). However, if an entire expression cassette with a foreign transgene is flanked by homologous sequences at the DSB, then the transgene will have a high probability of being copied precisely into the DSB (example 4). For color version of this figure, the reader is referred to the online version of this book.

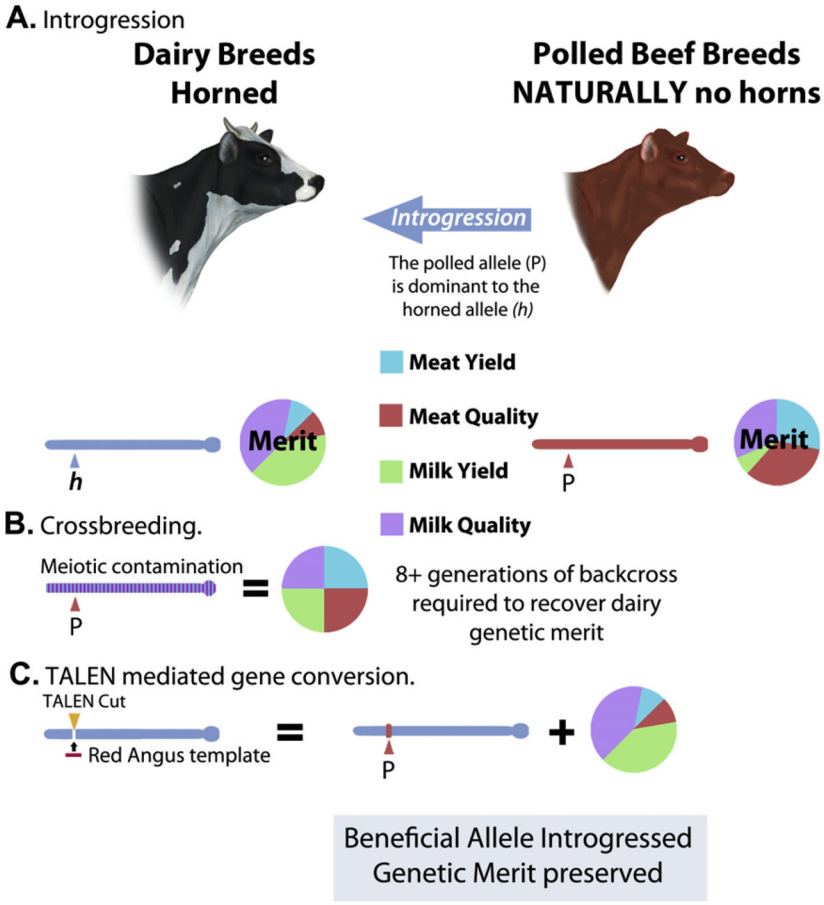

Figure 7. Rapid allele introgression in livestock

A) The diagram contrasts introgression of desired alleles (*polled allele to horned animals*) by crossbreeding (panel **B**) versus TALEN-mediated gene conversion (panel **C**). Beef and dairy breeds are selected for divergent classes of traits resulting in *genetic merit* selected for production of meat or milk, respectively. The accumulation of these traits is referred to as the genetic merit of each animal. Crossbreeding mixes these traits, which would result in animals that would not be ideal for either milk or meat production. The trait-selected genome architecture of these animals is conflicted by meiotic contamination, which would require about eight generations of selection to recover the original genetic merit. Panel C shows how TALEN-mediated gene conversion is able to transfer just a desired trait from beef cattle into dairy breeds. In this example, TALENs generate a double-strand DNA break at the *horned-polled* locus that can be repaired by a homologous template carrying the *polled* allele from a polled beef breed, e.g., Red Angus. The resulting animal will be both free of horns and maintain the original genetic architecture and merit for milk production. For color version of this figure, the reader is referred to the online version of this book.

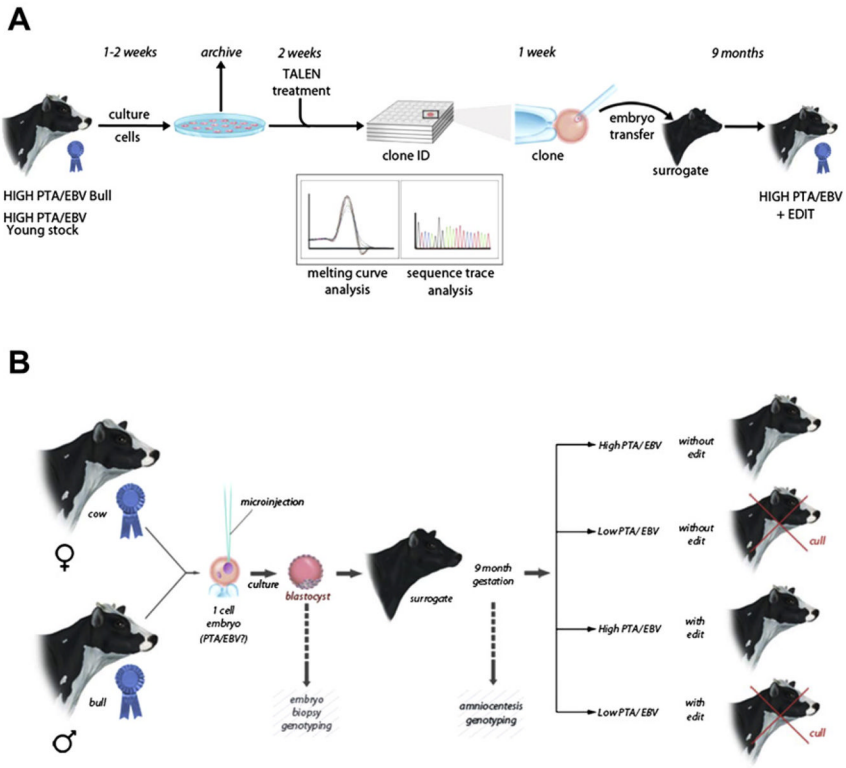

Figure 8.

Strategies for implementation of allelic introgression. The introgression of desirable alleles into livestock could follow either a vertical (panel A) or a horizontal (panel B) paradigm. (A) In the vertical paradigm, allelic introgression would be performed in cells derived from a donor individual(s) with a high predictability of transmitting *ability/estimated breeding value* (PTA/EBV, denoted by a blue ribbon). One or several genetic heterozygous or homozygous allele conversions (genetic edits) could be made and verified (e.g., by sequence analysis) prior to cloning of an individual. The resulting animal would not only carry the edits but would also maintain the original PTA/EBV of the donor animal. This animal would be entered back into the genetic improvement program and edits would be selected in subsequent generations. (B) Horizontal implementation takes advantage of the fact that embryo transfer is routine in genetic improvement programs of some livestock species, e.g., cattle. Zygotes produced from animals with high PTA/EBV could be injected with TALENs plus repair templates corresponding to the desired alleles and implanted into a surrogate for establishment or pregnancy. Resulting offspring could be scored for high PTA/EBV and either the presence or the absence of the targeted edits. Animals with high PTA/EBV would be maintained in the genetic improvement program regardless of the edit status, while animals with low PTA/EBV would be culled. Two potential improvements of this process can be envisioned. (1) An embryo biopsy at the blastocysts stage could be collected to evaluate the edit status or PTA/EBV so that only edited and/or high PTA/EBV embryos would be implanted into surrogates. (2) Fetal cells could be collected early in pregnancy by amniocentesis for evaluation of the edit status or PTA/ EBV. Low PTA/EBV or non-edited animals could be culled prior to parturition. Development of these technologies could further accelerate the rate of livestock improvement. In contrast to the vertical paradigm, allelic introgression and genetic improvement will continue to occur in the horizontal paradigm, thereby producing animals that would be one generation ahead in terms of genetic improvement. This method could be easily applied to generate numerous animals from

multiple lines such that dissemination of converted alleles (genetic edits) would be accomplished rapidly within a population with minimal risk of inbreeding. For color version of this figure, the reader is referred to the online version of this book.

Table 1

Transgenic animals for enhanced production or with marker genes

Cassette*	Delivery†	F0 Exp‡	F1 Exp‡	Reference
Animal production				
Pigs				
mMT/hGH	PNI	11/18	Yes	(Brem, 1985; Hammer et al., 1985; Miller et al., 1989; Pursel et al., 1987)
mMT/hGRF	PNI	2/7	Yes	(Pinkert, 1987; Pursel et al., 1989)
mMT/bGH	PNI	8/11	Yes	(Pursel et al., 1987)
hMT/pGH	PNI	1/6, 5/22	Yes	(Nottle, 1999; Vize et al., 1988)
MLV/rGH	PNI	1/1	ND	(Ebert et al., 1988)
mMT/hGRF	PNI	ND	NA	(Brem and Winnacker, 1988)
bPRL/bGH	PNI	2/4	ND	(Polge et al., 1989)
hALB/hGRF	PNI	3/3	ND	(Pursel et al., 1989)
mMT/hIGF-1	PNI	1/4	ND	(Miller et al., 1989; Pursel et al., 1989)
rPEPCK/bGH	PNI	5/7	Yes	(Wieghart et al., 1990)
CMV/pGH	PNI	3/31	ND	(Ebert et al., 1990)
MLV/pGH	PNI	1/1	ND	(Ebert et al., 1990)
MSV/cc-ski	PNI	10/29	ND	(Pursel et al., 1992)
oMT/oGH	PNI	6/15	ND	(Pursel et al., 1997)
ba-LA/ba-LA	PNI	ND	Yes	(Bleck et al., 1998)
cASK/hIGF-1	PNI	NA	Yes	(Pursel et al., 1999; Pursel et al., 2004)
bCsn/hGH	PNI	1/1	ND	(Hirabayashi et al., 2001)
mPSP/APPA	PNI	29/33	Yes	(Golovan et al., 2001)
maP2/FAD2	PNI	2/3	Yes	(Saeki et al., 2004)
bα-LA/hIGF-1	PNI	NA	Yes	(Monaco et al., 2005)
CAG/hfat-1	SCNT	3/6, 12/13	ND	(Lai et al., 2006; Pan et al., 2010)
bCsn/hLz	SCNT	1/2	Yes	(Tong et al., 2011)
Cattle				
MMTV/bGH	PNI	ND	ND	(Roshlau and Zackel, 1989)
cASK/hER	PNI	≤1/1	ND	(Hill, 1992; Massey, 1990)
bCsn/hLF	PNI	ND	ND	(Krimpenfort et al., 1991)
cASK/hlGF-1	PNI	ND	Yes	(Hill, 1992)
MMTV/hlGF-1	PNI	ND	ND	(Hill, 1992)
MSV/cc-ski	PNI	1/1	ND	(Bowen et al., 1994)
bβCsn/bβCsn & bκ-Csn	SCNT	9/11	ND	(Brophy et al., 2003)
bCsn/hGH	SCNT	1/15	Yes	(Salamone et al., 2006)
hα-LA/hα-LA	SCNT	3/3	Yes	(Wang et al., 2008)
hLF/hLF	SCNT	2/2	ND	(Yang et al., 2008)
bCsn/hLz	SCNT	17/30	ND	(Yang et al., 2011)
mTF/bGH	PNI	NA	NA	Bondioli, Hammer (unpubl.)

Cassette*	Delivery†	F0 Exp‡	F1 Exp‡	Reference
EF1α/anti-GDF8 shRNA	LV-MI	5/5	ND	(Tessanne et al., 2012)
		Goats		
bCsn/hLz	PNI	Yes	Yes	(Maga et al., 2003)
oCsn/hGH	PNI	NA	NA	(Lee et al., 2006)
oCsn/hLF	PNI	NA	Yes	(Zhang et al., 2008)
		Sheep		
mMT/hGH	PNI	ND, 0/1	ND	(Hammer et al., 1985; Pursel et al., 1987)
mMT/bGH	PNI, MI	2/2, 2/2	ND, No	(Pursel et al., 1987; Rexroad et al., 1989)
oMT/oGH	PNI	3/3	ND	(Murray et al., 1989)
mMT/hGRF	MI	1/7	No	(Rexroad et al., 1989)
RSV/CE, CK, oMT/CE, CK	PNI	NA	NA	(Rogers, 1990; Ward, 1991)
mTF/bGH, mAlb/hGRF	PNI	3/11	NA	(Rexroad et al., 1991)
mKER/oIGF-I	PNI	2/5	Yes	(Damak et al., 1996a)
		Marker genes		
		Pigs		
CMV/EGFP	RV, SCNT, EIAV, SCNT, SMGT, LV	1/2, 1/1, 34/37, 4/4, 4/4, 6/7, ND	Yes	(Cabot et al., 2001; Garcia-Vazquez et al., 2010; Lai et al., 2002b; Liu et al., 2008; Whitelaw et al., 2004; Whyte et al., 2011; Zhang et al., 2012)
SV40/hSEAP	SMGT	35/57	Yes	(Chang et al., 2002)
K14/GFP, PGK/GFP	LV, SCNT	32/34, 10/10	ND	(Hofmann et al., 2003; Kurome et al., 2008)
CMV/EBFP, EGFP, DsRed2	SMGT	7/7 triple TG	ND	(Webster et al., 2005)
pCMV/huKO	RV-WCI	18/18	ND	(Matsunari et al., 2008)
CAG/EGFP	SCNT	9/9	Yes	(Whitworth et al., 2009)
mOCT4/EGFP, hOCT4/EGFP	SCNT	6/11	Yes, no	(Nowak-Imialek et al., 2011)
CAG/VenusFP	SB-CPI	2/5	Yes	(Garrels et al., 2011)
CAG/YFP, CAG/TFP	SCNT	7/7	ND	(Deng et al., 2011)
Ub/GFP	SB-SCNT	4/5	ND	(Jakobsen et al., 2011)
PGK/YFP	SB-SCNT	6/6	ND	(Carlson, Garbe, et al., 2011)
mStra8/EYFP-mito	SCNT	ND	ND	(Sommer et al., 2012)
		Cattle		
RV/Neo	RV-MI	NA	No	(Haskell and Bowen, 1995)
CMV/βGEO	SCNT	3/3	ND	(Cibelli et al., 1998)
PGK/EGFP	LV	4/4	ND	(Hofmann et al., 2004)
		Sheep		
mKER/CAT	PNI	1/4	Yes	(Damak et al., 1996b)
PGK/GFP	LV-MI	3/9	No	(Ritchie et al., 2009)

Species of origin are given by lower case letters: m, mouse; b, bovine; c, chicken; h, human; o, ovine; p, porcine; r, rat.

[*] Transgenic expression cassettes show the transcriptional regulatory motifs/transgene. Promoters: ALB, albumin; aP2, adipocyte lipid-binding protein P2; ASK, α-skeletal actin; BLG, β-lactoglobulin; CAG (also called CAGG/CAGGS), human CMV early enhancer fused to β-actin promoter; CMV, cyto-megalovirus; Csn, casein; EF1α, Elongation Factor 1α; H1, pol III-dependent RNA promoter, human RNase P; H-2Kb, major histocompatibility complex H-2Kb; ICAM2, intercellular adhesion molecule 2; IgSV, immunoglobulin heavy chain enhancer; INV, suprabasal keratinocyte-specific involucrin; K14, keratin K14; KER, keratin; LA, lactalbumin; mAb, mouse monoclonal antibody; MCP, membrane cofactor protein; mIgA, mouse immunoglobulin A; MLV, mouse leukemia virus LTR; MMTV, mouse mammary tumor virus LTR; MSV, mouse sarcoma virus LTR; MT, metallothionein; MTla, Metallothionein la; MX, interferon-induced GTP-binding protein Mx1; NSE, neuron-specific enolase; NTA-RCA, auto-regulative tetracycline-responsive bicistronic expression cassette regulator of complement activation; OCT4, Octamer-binding Transcription factor 4; PEPCK, phosphoenolpyr-uvate carboxykinase; PGK, phophoglycerol kinase; PRL, prolactin; PSP, parotid secretory protein; RHO, human Rhodopsin; Rho, rhodopsin; Rho4.4, Rhodopsin promoter 4.4; RSV, Rous sarcoma virus LTR; β-Lac, β-Lactoglobulin; Stra8, Stimulated by Retinoic Acid 8; SV40, simian virus 40; TF, transferin; Tie2, Tyrosine kinase with immunoglobulin-like and EGF-like domains 1; Ub, ubiquitin; Visna virus LTR, Visna virus LTR; WAP, whey acidic protein; κP, kappa protein. Transgenes: α1AT, α1 antitrypsin; α-1,3GT, α-1,3 = GGTA1, galactosyltransferase; A20, tumor necrosis factor-α-induced protein 3 (TNFaip3); anti-GDF8 shRNA, anti-Myostatin short hairpin RNA; anti-PERV shRNA, anti-porcine endogenous retrovirus short hairpin RNA; anti-PrP shRNA, anti-major prion protein or CD230 short hairpin RNA; ApoBEC3G, apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G; APPA, *E. coli* Phytase gene; AT, antithrombin III; BChE, Butyr-ylcholinesterase; bi-scFV r28M, bispecific single-chain variable fragment (bi-scFV) molecule with anti-human CD28 anti-human melanoma specificity; BLVenv, Bovine Leukemia Virus Envelope; BSSL, bile salt-stimulated lipase; CAT, Chloramphenicol Acetyl Transferase; CD46, CD46 complement regulatory protein or Membrane Cofactor Protein; CD55, Decay-accelerating Factor; CD59, Pro-tectin, a complement regulatory protein; CE, *E. coli* cysE; CFTR, Cystic fibrosis transmembrane conductance regulator; CK, *E. coli* cysK; COL, Collagen; COL1A1, α1(I) procollagen; Cre, Cre recombinase; CTLA4-Ig, fusion gene between Cytotoxic T-Lymphocyte Antigen 4 and human IgG1; Cκ, immunoglobulin light chain; ELOVL4-5bpdel, elongation of very long chain fatty acids-4 with 5 bp deletions; ELOVL4-Y270ter, elongation of very long chain fatty acids-4 with 270 stop mutation; eNOS, nitric oxide synthase; EPO, Erythropoietin; ER, Estrogen Receptor; EYFP-mito, mito-chondria localized EYFP; FAD2, spinach Delta-12 fatty acid desaturase; FIX, coagulation Factor IX; FVIII, coagulation factor VIII; G-CSF, granulocyte colony stimulating factor; GH, growth hormone; GnT-III, N-Acetylglucosaminyltransferase III; GRF, growth-regulating factor; HbsAg, hepatitis B surface antigen; hfat-1, humanized (codon optimized) fat-1; HHT CAG, Huntington disease gene with CAG repeats; hITG b1, α2, integrins b1, a2; HT, H-transferase; hv-HA-ras, Harvey rat sarcoma viral oncogene; hα1 + hβA, hemoglobin α1 and βA; IGF, insulin-like growth factor; IgH, immu-noglobulin heavy chain; IGHM, immunoglobulin-μ; Igλ, Imunnoglobulin light chain; JH, immu-noglobulin heavy chain joining region; BLG-hAAT, COL1A1 knock-in vector containing bovine β-lactoglobulin promoter driving human α1-antitrypsin; LDLR, low-density lipoprotein receptor. LF, lactoferrin; LP2, two LoxP sites; Lz, lysozyme; mAb, mouse monoclonal antibody; MCP, membrane cofactor protein; MX, interferon-induced GTP-binding protein Mx1; PPARγ, peroxisome proliferator-activated receptor γ; PrP = PRNP, major prion protein; RHO-h23H, human Rhodopsin with Pro23His mutation; Rho-Pro374Leu, rhodopsin gene with Pro374Leu mutation; SV40, Simian vacuolating virus 40; TK, thymidine kinase; TM, thrombomodulin; TPA, tissue plasminogen activator; Visna-env, Visna Virus envelope; vWF, Von Willebrand factor; Marker transgenes: BFP, blue fluorescent protein; CAT, chloramphenicol acetyl transferase; DsRed2/RFP, red fluorescent protein; E, enhanced; -GEO, -galactosidase-GFP fusion gene; GFP, green fluorescent protein; huKO, humanized Kusabira-Orange; neo, neomycin phosphotransferase II; SEAP, secreted alkaline phosphatase; TFP, tomato fluorescent protein; VenusFP, Venus fluorescent protein; YFP, yellow fluorescent protein; SB, Sleeping Beauty Transposon system. Viruses used for transduction: AAV, adeno-associated virus; EIAV, equine infectious anemia virus; LV, lentivirus; RV, retrovirus.

[†] Methods of transgene delivery: CPI, cytoplasmic injection; ICSI, intracytoplasmic sperm injection; MI, microinjection; PNI, pronuclear injection; SCNT, somatic cell nuclear transfer; SMGT, sperm-mediated gene transfer; WCI, whole-cell injection cloning.

[‡] Transgene expression detected in F0 or F1 animals with numbers where available. NA, not available; ND, not done.

Table 2

Transgenic animals as bioreactors and sources of bioproducts

Cassette*	Delivery†	F0 Exp‡	F1 Exp‡	Reference
		Pigs		
mWAP/mWAP	PNI	3/3	Yes	(Shamay et al., 1991; Wall et al., 1991)
mWAP/hFVIII	PNI	1/1	4/4	(Paleyanda et al., 1997)
mWAP/hFibrinogen	PNI	3/4	ND	(Butler et al., 1997)
mWAP/hFIX	PNI	2/3	Yes	(Van Cott et al., 1999)
ba-LA/hFIX	PNI	NA	Yes	(Wu et al., 1999)
mWAP/hProtein C	PNI	6/8	Yes	(Van Cott et al., 2001)
CAG/hAlb	ICSI	1/1	ND	(Naruse et al., 2005)
mWAP/hEPO	PNI	NA	Yes	(Park et al., 2006)
bCsn/hvWF	PNI	2/2	Yes	(Lee et al., 2009)
gCsn/hEPO	SCNT	ND	Yes	(Cho et al., 2009)
		Cattle		
bCsn/hEPO	PNI	NA	ND	(Hyttinen et al., 1994)
hIgH and Igλ	HAC, SCNT	6/6	Yes	(Kuroiwa et al., 2002)
oBLG/hBSSL	SCNT	ND	ND	(Chen et al., 2002)
mκP/bi-scFV r28M	SCNT	9/9	ND	(Grosse-Hovest et al., 2004)
		Goats		
mWAP/hTPA	PNI	ND	Yes	(Ebert et al., 1991)
oCsn/hAT	SCNT	1/1	ND	(Baguisi et al., 1999)
oCsn/hG-CSF	PNI	1/2, 2/2	No, Yes	(Freitas et al., 2012)
mWAP/spider silk	PNI	ND	Yes	(Baldassarre et al., 2003)
oCsn/hBChE	PNI	NA	Yes	(Baldassarre et al., 2004)
		Sheep		
oBLG-hα1AT	MI, PNI	3/5, 2/3	Yes	(McClenaghan et al., 1991)
oBLG/hFIX	PNI, SCNT	2/2, ND	Yes, ND	(Schnieke et al., 1997)
oBLG/hFibrinogen	PNI	3/3	ND	(Butler et al., 1997)
mWAP/hFVIII	PNI	ND	ND	(Halter et al., 1993)
mWAP/mWAP	MI	2/2	Yes	(Wall et al., 1996)
oβ-Lac/hFVIII	PNI	ND	ND, Yes	(Niemann et al., 1999)

*,†,‡See Table 1 for standard abbreviations

HAC, human artificial chromosome

Table 3

Transgenic pigs for xenotransplantation

Cassette*	Delivery†	F0 Exp‡	F1 Exp‡	Reference
hβ-globin/hα1 and βA	PNI	3/3	ND	(Swanson et al., 1992)
mH-2Kb/hCD59	PNI	1/3	ND	(Fodor et al., 1994)
pMCP/hCD55	PNI	1/5	Yes	(Murakami et al., 2000)
hICAM2/hHT	PNI	8/185	ND	(Nottle et al., 2001)
mH-2Kb/hCD55 + hHT	PNI	4/20	ND	(Nottle, 2001)
mH-2Kb/hCD55 + hCD59 + hHT	PNI	11/16	ND	(Nottle et al., 2001)
hICAM2/hCD46 + hCD55 + hCD59	PNI	2/94	ND	(Nottle, 2001)
CAG/hGnT-III	PNI	NA	Yes	(Miyagawa et al., 2001)
RSV/hCD55	SMGT	34/53	Yes	(Lavitrano et al., 2002)
pAlb/TK	SCNT	1/3	ND	(Beschorner, 2003)
ba-LA/pLF, ba-LA/ hFIX	WCI	4/4	ND	(Lee et al., 2003)
hCD59/hCD59 + hMCP/hMCP + hCD59	PNI	1/1	ND	(Zhou, 2004)
rNSE/hCTLA4-Ig	PNI	2/8	Yes	(Martin et al., 2005)
NTA-RCA/hCD55, NTA-RCA/hCD59	PNI	9/10	Yes	(Kues et al., 2006)
hH1/anti-PERV shRNA	LV, SCNT	2/2, 12/12	ND, Yes	(Dieckhoff et al., 2008; Ramsoondar et al., 2009)
CAG/pCTLA4-Ig	SCNT	15/15	ND	(Phelps et al., 2009)
CMV/hTM	SCNT	7/7	ND	(Petersen et al., 2009)
CAG/hA20	SCNT	2/2	ND	(Oropeza et al., 2009)
PGK/hApoBEC3G	SB-SCNT	10/10	ND	(Carlson, Geurts, et al., 2011)
PGK,Ub,CAG/LP2-hApoBEC3G	SB-SCNT	3/3, 4/4, 0/1	ND	(Carlson, Geurts, et al., 2011)

See Table 1 for standard abbreviations.

Table 4

Transgenic animals for human or animal diseases

Cassette*	Delivery†	F0 Exp‡	F1 Exp‡	Reference
Human disease models				
Pigs				
MMTV/hv-Ha-ras	PNI	1/1	Yes	(Yamakawa et al., 1999)
pRho/pRho-Pro347Leu	PNI	3/3	ND	(Petters et al., 1997)
rNSE/pHTT CAGs	PNI	NA	ND	(Uchida et al., 2001)
mTie2/peNOS	SCNT	4/4	ND	(Hao et al., 2006)
pMX/Cre	SCNT	1/10	ND	(Chen et al., 2010)
CAG/hHTT CAGs	SCNT	Yes	ND	(Yang et al., 2010)
Rho4.4/hELOVL4-5bpdel,-Y270ter	PNI, SCNT	NA	Yes	(Sommer et al., 2011)
hRHO/hRHO-hP23H	SCNT	6/10	Yes	(Ross et al., 2012)
CMV, INV/hITG b1, a2	SB-HMC	6/6	ND	(Staunstrup et al., 2012)
hCOL-BAC, hALB-BAC	SMGT, ICSI	6/8	ND	(Watanabe et al., 2012)
PGK/YFP-Cre	SB-SCNT	6/6	ND	(Carlson, Geurts, et al., 2011)
Animal disease resistance				
Pigs				
mAb/mAb	PNI	1/1	ND	(Weidle et al., 1991)
mIgA/mIgA	PNI	2/2	Yes	(Lo et al., 1991)
hMT, SV40, mMX/mMX	PNI	2/9	Yes	(Brem, 1993; Muller et al., 1992)
mMX-SV40	PNI	1/6	NA	(Pinkert et al., 2001)
Cattle				
RSV/HbsAg	RV, PNI	1/1	ND	(Chan et al., 1998)
oBLG/lysostaphin	SCNT	3/3	Yes	(Wall et al., 2005)
Goats				
mIgA	PNI	0	ND	(Lo et al., 1991)
hH1/anti-PrP shRNA	LV-SCNT	0	No	(Golding et al., 2006)
Sheep				
oVisna-LTR/ oVisna-env	MI	3/3	ND	(Clements et al., 1994)

See Table 1 for standard abbreviations.

BAC, bacterial artificial chromosome.

Table 5

Gene targeting in livestock through homologous recombination (HR) and NHEJ

Gene(s)*	Success†	Agent	Efficiency = genotyping+/ total colonies (%)	F1‡	Reference
HR					
Xenotransplantation transgenics					
Pigs					
α-1,3GT	+/−	Naked DNA	1.54	Yes	(Dai et al., 2002)
α-1,3GT	+/−	Naked DNA	13.84	Yes	(Lai et al., 2002a)
α-1,3GT	+/−	Naked DNA	1.19	Yes	(Ramsoondar et al., 2003)
α-1,3GT	−/T to G	Spontaneous mutation	NA	Yes	(Phelps et al., 2003)
α-1,3GT	+/−	Naked DNA	0.32	ND	(Takahagi et al., 2005)
Cattle					
α-1,3GT	−/−	Naked DNA	0.52, 1.57	No[a]	(Sendai et al., 2006)
Sheep					
α-1,3GT	+/−	Naked DNA	1.1	NA	(Denning et al., 2001)
Bioreactor transgenics					
Pigs					
Cκ	+/−	Naked DNA	0.75	−/−[b]	(Ramsoondar et al., 2011)
JH	+/−	Naked DNA	0.64	−/−[b]	(Mendicino et al., 2011)
Disease transgenics					
Pigs					
CFTR	+/−, +/Δ	F508 AAV	0.053–8.20	Yes	(Rogers et al., 2008)
Cattle					

Gene(s)*	Success†	Agent	Efficiency = genotyping+/ total colonies (%)	F1‡	Reference
IGHM, PrP[c]	−/−, −/−	Naked DNA	0.45–6.4	Yes	(Kuroiwa et al., 2004)
PrP	−/−	Naked DNA	3.30	ND	(Richt et al., 2007)
			Goat		
PrP	+/−	Naked DNA	1.53	−/−[b]	(Yu et al., 2009; Yu et al., 2006)
			Sheep		
COL1A1	+/−, +/oBLG-hAAT[d]	Naked DNA	34.0	ND, ND	(McCreath et al., 2000)
PrP	+/−	Naked DNA	10.3	ND	(Denning et al., 2001)
			NHEJ		
			Pigs		
EGFP	+/−	ZFN	~2%	ND	(Whyte et al., 2011)
PPARγ	+/−	ZFN	~4.2%	ND	(Yang et al., 2011)
α-1,3GT	−/−	ZFN	~2%	ND	(Hauschild et al., 2011)
LDLR	+/−	TALEN	22%; 18/18 pigs	ND	(Carlson, Tan, et al., in press)
			Cattle		
BLG	+/−	ZFN	19.4%	ND	(Yu et al., 2011)

Refer to Table 1 for standard abbreviations.

*Genes are defined in the legend to Table 1; in some cases, more than one gene was inactivated.

**+/−Heterozygote knockout; −/−, homozygote knockout; −/ T to G, heterozygote knockout with a T to G mutation in the other allele; +/Δ F508, the human mutation Δ F508 knocked into one of the two alleles; +/oBLG-hAAT, oBLG-hAAT expression cassette knocked into one of the two alleles.

‡In some cases, where there is update information on transgenic offspring, the results are labeled (Y or N):

[a]One piglet resulted from sequential targeting but died shortly after birth;

[b]Homozygous KO F1 obtained by breeding of heterozygous KO F0;

[c]Sequential targeting to KO both alleles for both genes in the same cells;

[d]Knocked in oBLG-hAAT construct into one of the alleles and detected hAAT expression right after the lamb perished.

Table 6

Identified mutations causing disease in cattle

OMIA entry	Phenotype	Gene	Mutation type	Deviation
OMIA 000001 - 9913	Abortion	APAF1	SNP	Nonsense
OMIA 001565 - 9913	Abortion and stillbirth	MIMT1	~110 kB deletion	
OMIA 000593 - 9913	Acrodermatitis enteropathica	SLC39A4	SNP	Splice site
OMIA 000543 - 9913	Anhidrotic ectodermal dysplasia	EDA	SNP	Nonsense
OMIA 001541 - 9913	Arachnomelia BTA23	MOCS1	2 nt deletion	Frameshift
OMIA 000059 - 9913	Arachnomelia BTA5	SUOX	1 nt INS	Frameshift
OMIA 001465 - 9913	Arthrogryposis multiplex congenita	ISG15	~233 kB deletion	
OMIA 001106 - 9913	Axonopathy	MFN2	SNP	Splice site
OMIA 001437 - 9913	Beta-lactoglobulin aberrant low expression	PAEP	SNP	Enhancer
OMIA 000151 - 9913	Brachyspina	FANCI	3.3 kB Deletion	
OMIA 000161 - 9913	Cardiomyopathy and woolly haircoat syndrome	PPP1R13L	7 bp duplication	Frameshift
OMIA 000162 - 9913	Cardiomyopathy dilated	OPA3	SNP	Nonsense
OMIA 000185 - 9913	Chediak–Higashi syndrome	LYST	SNP	Nonsense
OMIA 000187 - 9913	Chondrodysplasia	EVC2	SNP and 1 bp deletion	Splice site and frameshift
OMIA 000194 - 9913	Citrullinaemia	ASS1	SNP	Nonsense
OMIA 001340 - 9913	Complex vertebral malformation	SLC35A3	SNP	Missense
OMIA 001450 - 9913	Congenital muscular dystonia 1	ATP2A1	SNP	Missense
OMIA 001451 - 9913	Congenital muscular dystonia 2	SLC6A5	SNP	Missense
OMIA 000262 - 9913	Deficiency of uridine monophosphate synthase	UMPS	SNP	Nonsense
OMIA 001680 - 9913	Dominant white with bilateral deafness	MITF	SNP	Missense
OMIA 001485 - 9913	Dwarfism Angus	PRKG2	SNP	Nonsense
OMIA 001271 - 9913	Dwarfism Dexter	ACAN	4 bp INS or SNP	Frameshift
OMIA 001473 - 9913	Dwarfism growth hormone deficiency	GH1	SNP	Missense
OMIA 001686 - 9913	Dwarfism proportionate with inflammatory lesions	RNF11	SNP	Splice site
OMIA 000327 - 9913	Ehlers–Danlos syndrome	EPYC	SNP	Missense
OMIA 000328 - 9913	Ehlers–Danlos syndrome type VII (dermatosparaxis)	ADAMTS2	17 bp deletion	
OMIA 000340 - 9913	Epidermolysis bullosa	KRT5	SNP	Missense
OMIA 000363 - 9913	Factor XI deficiency	F11	76 bp insertion	
OMIA 000419 - 9913	Glycogen storage disease II	GAA	SNPs	Nonsense and missense

OMIA entry	Phenotype	Gene	Mutation type	Deviation
OMIA 001139 - 9913	Glycogen storage disease V	PYGM	SNP	Missense
OMIA 000424 - 9913	Goitre familial	TG	SNP	Nonsense
OMIA 000437 - 9913	Haemophilia A	F8	SNP	Missense
OMIA 000540 - 9913	Hypotrichosis	HEPHL1	SNP	Nonsense
OMIA 001544 - 9913	Hypotrichosis with coat-color dilution	PMEL	3 bp deletion	
OMIA 000547 - 9913	Ichthyosis congenita	ABCA12	SNP	Missense
OMIA 000595 - 9913	Leukocyte adhesion deficiency type I	ITGB2	SNP	Missense
OMIA 000625 - 9913	Mannosidosis alpha	MAN2B1	SNPs	Missense
OMIA 000626 - 9913	Mannosidosis beta	MANBA	SNP	Nonsense
OMIA 000627 - 9913	Maple syrup urine disease	BCKDHA	SNPs	Nonsense
OMIA 000628 - 9913	Marfan syndrome	FBN1	SNPs	Missense and splice site
OMIA 001342 - 9913	Mucopolysaccharidosis IIIB	NAGLU	SNP	Missense
OMIA 000733 - 9913	Multiple ocular defects	WFDC1	1 bp INS	Frameshift
OMIA 000683 - 9913	Muscular hypertrophy (double muscling)	MSTN	Numerous SNPs, 11 bp deletion, 10 bp INS	
OMIA 000685 - 9913	Myasthenic syndrome congenital	CHRNE	20 bp deletion	
OMIA 000689 - 9913	Myoclonus	GLRA1	SNP	Nonsense
OMIA 001319 - 9913	Myopathy of the diaphragmatic muscles	HSPA1A	11 kb deletion	
OMIA 001482 - 9913	Neuronal ceroid lipofuscinosis 5	CLN5	1 bp duplication	Frameshift
OMIA 000755 - 9913	Osteopetrosis	SLC4A2	2.8 kb deletion	
OMIA 000836 - 9913	Protoporphyria	FECH	SNP	Stoploss
OMIA 001464 - 9913	Pseudomyotonia congenital	ATP2A1	SNP	Missense
OMIA 001135 - 9913	Renal dysplasia	CLDN16	37 kb or 56 kb deletion	
OMIA 001593 - 9913	Scurs type 2	TWIST1	10 bp duplication	
OMIA 001230 - 9913	Sex reversal: XY female	SRY	Large Deletion	
OMIA 001228 - 9913	Spherocytosis	SLC4A1	SNP	Nonsense
OMIA 001247 - 9913	Spinal dysmyelination	SPAST	SNP	Missense
OMIA 000939 - 9913	Spinal muscular atrophy	KDSR	SNP	Missense
OMIA 000963 - 9913	Syndactyly (mule foot)	LRP4	SNP or 2 bp replacement	Splice site or missense
OMIA 001452 - 9913	Tail crooked	MRC2	2 bp deletion or SNP	Nonsense or missense
OMIA 001003 - 9913	Thrombopathia	RASGRP2	SNP	Missense
OMIA 001009 - 9913	Tibial hemimelia	ALX4	45.7 kb deletion	

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OMIA entry	Phenotype	Gene	Mutation type	Deviation
OMIA 001360 - 9913	Trimethylaminuria	FMO3	SNP	Nonsense
OMIA 001079 - 9913	Yellow fat	BCO2	SNP	Nonsense