GENOME ENGINEERING IN LARGE ANIMALS FOR AGRICULTURAL AND BIOMEDICAL APPLICATIONS

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A thesis by Wenfang Tan

Abstract

Precision genetics will enhance genome-based improvement of livestock for agriculture and biomedicine. This thesis aimed to modify large animal genomes with precision; as the technologies progressed, our capability expanded from random insertional transgenesis to nucleotide-level precision.

It began with *Sleeping Beauty (SB)* transposon mediated rapid integration of dominant negative Myostatin alleles. All piglets generated from treated cells harbored the transgenes; however, we were unable to study phenotypes due to death of the founder animals. We then sought to introgress a SNP into porcine Myostatin through recombinant Adeno-associated Virus (rAAV) mediated gene targeting. We achieved a $2x10^{-4}$ targeting frequency but only one-half of the targeted colonies harbored the SNP. Similarly, we succeeded in porcine *LDLR* gene knockout; however, targeted clones were often confounded by "bystander" cells with only random insertions of the targeting vector.

We turned to develop TALENs for efficient targeting of important genes. TALENs demonstrated high activity in both cultured primary fibroblasts and early stage embryos. A simple *SB* transposon based co-selection strategy enabled enrichment for TALEN modified cells and efficient isolation of modified clones: single gene mono- and bi-allelic modification was induced in up to 54% and 17% of colonies respectively. It also enabled isolation of colonies harboring large chromosomal deletions (10% of colonies) and inversions (4%) after treatment with two TALEN pairs. We derived miniature swine models of familial hypercholesterolemia from *LDLR* mono- and bi-allelic TALEN-knockout fibroblasts.

We next utilized TALEN and CRISPR/Cas9 stimulated homology-directed repair (HDR) to edit genes with oligonucleotide, plasmid, and rAAV templates without any drug selection. We first introgressed a bovine *POLLED* allele into horned dairy bull fibroblasts to circumvent manual dehorning. We also introduced single-nucleotide alterations or small indels into 14 additional genes in pig, cattle and sheep, into 10-50% of cells from fibroblast populations treated with TALEN mRNA and oligonucleotides. Up to 67% of propagated colonies harbored the intended edits and over one-half were homozygous. Some edits were naturally occurring SNP alleles, equivalent to non-meiotic inter- or intra-species introgression of valuable alleles. We created pig models for infertility and colon cancer from colonies with TALEN-HDR knockout alleles in *DAZL* and *APC*.

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Chapter 1 Precision Editing of Large Animal Genomes

Precision Editing of Large Animal Genomes[1]

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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.

I. Introduction

A. The need for genetically modified large animals

Hunger worldwide is increasing; approximately one 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 et al., 2011). Improvements in the efficiency of production and safety are becoming ever more important considerations for protection of the environment and reduction in land usage (Clark and Whitelaw, 2003). Global climate change will only exacerbate the lack of animal-protein production (McMichael, 2012; Schmidhuber and Tubiello, 2007; Wolkovich et al., 2012). The *green revolution* has practically peaked according to its father, Norman Borlaug, who asserted that farm animals are critical to nutrition, and that genetic engineering of foodstuffs will be required to feed the world (Borlaug, 2000). Both genetic and management-based increases in sustainable productivity will be 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 and Niemann, 2004; Snaith and 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 et al., 1988; Smithies et al., 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 do 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 et al., 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 et al., 2004; Haskins et al., 1984; Koeberl et al., 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 21st 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 and 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.

B. 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 and Singer, 1995). The moratorium served as an unfortunate precedent for ignorance and unspecified fears impeding progress in animal genetics.

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 *E. 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 actually 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 II-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 et al., 2010). The first is cost of manufacture and purification in amounts

required for effective delivery to cells, which prohibits their use in most labs. Second, viruses often direct integration into and 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.

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 et al., 1980) and stable lines of animals were produced soon after (Gordon and 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 et al., 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 et al., 1983), led to predictions that recombinant DNAs would be introduced into food crops and animals (Bauman et al., 1985; Seidel, 1985; Wagner and Murray, 1985). However, the observation that random integration of recombinant DNA cassettes into genomes could lead to adverse effects, including death (Wagner et al., 1983), caused 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 in milk (Ivarie, 2003). The earliest experiments in avian transgenesis utilized retroviruses. Retroviral

infections of poultry can cause sarcomas (Rous, 1910) and leukemias (Beard et al., 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 and Salter, 1985, 1986). Transgenic lines of chickens were achieved (Bosselman et al., 1989; Mizuarai et al., 2001; Salter et al., 1987; Thoraval et al., 1995); however, the efficiencies using retroviral vectors was 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.; 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 et al., 2010; Worm et al., 2009). Genetically engineering 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 and Alvarez, 2000). The most visible product from these endeavors was the AquaAdvantage 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 et al., 2009; Smith et al., 2010; Van Eenennaam and 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 I - IV. Several effective methods of introduction of recombinant genomes into embryos have been developed. The most common are illustrated in Fig. 4 - somatic cell nuclear transfer (SCNT), microinjection, and sperm-mediated gene transfer (SMGT) (Carlson et al., 2011a; Clark and Whitelaw, 2003). The studies reported in Tables I and II show that all three of the applications of transgenic technologies in large animals shown in Fig. 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 germline 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 et al., 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 three years prior to approval in the US. The second type comprises genetically modified freshwater aquarium fish, called Glofish® (Knight, 2003), that 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 and 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.

***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 et al., 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 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 United States and 89 million hectacres world-wide, 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).

***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 I - V). 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 (GH) genes from a variety of sources, insulin-like growth factor (IGF), growth hormone releasing factor (GRF) and others (Table I). 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 I). 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 II). For this, the casein and whey acidic protein (WAP) transcriptional regulators were employed as they provided high levels of expression of the transgenic proteins in milk (Clark and 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 bioreactors 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 United States (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 et al., 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 et al., 2010; Sachs and Galli, 2009). A primary goal was to neutralize α1,3-galactose, the primary antigen responsible for hyperacute rejection (Cooper, 2003) from the cell surface of pigs by inactivating the 1,3-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 III and V). Additional transgenic animals have been created to neutralize incompatibilities between blood coagulation systems and to limit T-cell responses (Table III). 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 and Patience, 2004).

The physiological similarities that make pigs good candidates for xenotransplantation also made them ideal candidates for modeling of human diseases (Table IV). 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 V). 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 (508) 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 Table I- IV 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 Enviro-pig (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 US government (NIH, USDA, NSF, FSA, EPA) investments of around $100 million dollars in funding research and risk analysis on large transgenic animals, not 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 et al., 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 and 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.

II. 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 and 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*).

A. 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 (ITRs) 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 et al., 2005). Nearly all of the Class II DNA transposons identified in vertebrate genomes appear to be inactive (Plasterk et al., 1999; Venter and al, 2001; Waterston and 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

et al., 2011), extend to genetic engineering of large animals as well (Carlson et al., 2011a; Clark et al., 2007).

B. Precision editing of genomic sequences using Meganucleases and ZFNs

The studies listed in Tables I - IV 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 et al., 2008). Position-effect variegation wherein transgenic sequences are silenced when introduced into chromatin and trans-activation 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 zebrafish but awaits extension to large animal genomes (Cui et al., 2003; Liao and Essner, 2011).

Rare-cutting DNases such as the yeast Meganuclease *I-SceI* (Jasin, 1996; Rouet et al., 1994; Smih et al., 1995) show great promise for the alteration of chromosomal sequences at a few specific sites (Choulika et al., 1995) Meganucleases are precise and effective at cleaving their cognate recognition site in the genome, but the overlap of DNA-recognition domains but 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 towards 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 et al., 1996). Because Cys_2His_2 zinc-fingers can be designed to bind to specific sites (Desjarlais and Berg, 1993; Jamieson et al., 1966), artificial zinc finger nucleases (ZFNs) became a tool to cleave specific genetic loci, (Bibikova et al., 2003; Bibikova et al., 2001; Kim et al., 1996; Park et al., 2003; Porteus and Carroll, 2005). The human gene therapy community quickly recognized the potential of site-

specific integration of therapeutic transgenes and developed the use ZFNs in human cells (Carroll, 2011; Hockemeyer et al., 2009; Porteus and Baltimore, 2003; Urnov et al., 2005; Urnov et al., 2010). Table V 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 et al., 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 (OPEN) 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 et al., 2011b) 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 et al., 2009; Ramirez et al., 2008).

C. 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 and Voytas, 2011; Carlson et al., 2012). Transcription Activator-Like (TAL) Effector Nucleases (TALENs), like ZFNs, consist of assembled DNA-binding motifs coupled to a *Fok*I endonuclease domain (Boch and Bonas, 2010; Boch et al., 2009; Christian et al., 2010; Li et al., 2011a; Mahfouz et al., 2011; Moscou and 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 and 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 et al., 2011b; Miller et al., 2011; Morbitzer et al., 2011; Reyon et al., 2012; Weber et al., 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 et al., 2011b; Morbitzer et al., 2011). TALENs introduced into human cells can direct site-specific mutagenesis at rates of up to 45 percent 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 et al., 2011c) 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 every 35 basepairs (Cermak et al., 2011). However, a recent study stretched the rules proposed by Cermak et al. 2011 and found 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 et al., 2012) may further improve the design process and specificity.

D. 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 et al., 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 to 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 and Cathomen, 2011; Tesson et al.,

2011). The specificity of TALENs has yet to be characterized in detail. Preliminary studies in cells and zebrafish 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 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 GM 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 NHEJ activity during repair of an off-target site (Fig. 5B). Two-thirds of exonic indels would be expected to cause a frame-shift leading to premature termination of translation whereas only a small portion of naturally occurring SNPs would result in a protein truncation. Deep sequencing has found that indels are about 10-fold less frequent in the human genome than single nucleotide polymorphisms (22,000 vs 1,800 per genome compared to reference) with up to 50% of the indels being novel in any given individual (Alkan et al., 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 to 300 LOF mutations, with 50 to 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, per. comm.) has revealed similar frequencies of both heterozygous and homozygous LOF alleles.

E. Precision alterations in livestock genomes

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. As shown in Tables I-IV, 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 I - IV). 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. *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. recently generated bi-allelic *GGTA1* KO pigs using commercial ZFN reagents and cloning (Hauschild et al., 2011). 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 fibroblasts from pigs and cattle (Table V). 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 (KO) can be accomplished by introducing frame-shifts in coding regions from NHEJ without the use of selection-markers.

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 lab (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 et al., In press). We recently reported the births of 18 low-density lipoprotein receptor (LDLR) +/- Ossabaw piglets from TALEN-induced NHEJ and SCNT (Table V). 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 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 et al., in press). TALENS are also capable of more complex changes in livestock fibroblasts. Co-transfection of two pairs of TALENs targeting the same chromosome was capable of creating large chromosomal deletions or inversions (Carlson et al., in press). Perhaps most compelling, co-transfection 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).

III. Future Directions – Applications of Precision Genetics in Animals

***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 et al., 2012), such as those that appear to predominate for complex traits.

The example presented in Figure 7 is of particular interest. Holstein cattle 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, adds expense to animal production (Graf and 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 and Gregory, 1978) and multiple groups are making progress on identifying the causative mutation (Seichter et al., 2012; J. Taylor, pers. comm.).

Introgression of the polled allele into horned breeds could easily be accomplished by crossbreeding (Fig. 7A), 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 can never be recovered in the now 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. 7B). 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 loss of function 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 VI). Recently, several haplotypes were discovered that affect the fertility in common dairy breeds of cattle including Holstein, Brown Swiss and Jersey (VanRaden et al., 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 loss-of-function 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 VI), 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 a 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 et al., In press). More sophisticated edits have yet to be been 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 zebrafish (Huang et al., 2011; Sander et al., 2011a) and rodents (Tesson et al., 2011) suggest that even template repair can reach significant 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.

2. *Regulatory issues*

Safety to consumers is the primary concern of regulatory agencies. 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 and 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 GE 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 SNCT 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.), at 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).

IV. References

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Table I 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.)
EF1α/anti-GDF8 shRNA	LV-MI	5/5	ND	(Tessanne et al., 2012)
Goat				
bCsn/hLz	PNI	Yes	Yes	(Maga et al., 2003)
oCsn/hGH	PNI	NA	NA	(Lee, 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 et al., 2011a)
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: 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, cytomegalovirus; 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; KER, ultra-high-sulfur Keratin; LA, lacalbumin; 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, metalothionien; 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, phosphoenolpyruvate 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; ß-Lac, ß-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: 1AT, 1 antitrypsin; α-1,3GT, α-1,3=GGTA1, galactosyltransferase; A20, tumor necrosis factor-α induced protein 3 (TNFaip3); ALB, Albumin; 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, Butyrylcholinesterase; 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, Protectin, 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 5bp 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, mitochondria localized EYFP; FAD2, spinach Delta-12 fatty acid desaturase; FIX, 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, Immunoglobulin Heavy chain; IGHM, immunoglobulin-μ; Igλ, Imunnoglobulin light chain; JH, immunoglobulin heavy chain joining region; BLG-hAAT, COL1A1 knock-in vector containing bovine β- lactoglobulin promoter driving human α1-antitrypsin; LA, lacalbumin; LDLR, low density lipoprotein receptor. LF, lactoferrin; LP2, two LoxP sites; Lz, lysozyme; mAb, mouse monoclonal antibody; MCP, membrane cofactor protein; mIgA, mouse Immunoglobulin A; 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 II Transgenic Animals as Bioreactors and Sources of Bioproducts

	Cassette*	Delivery**	F0 Exp***	F1 Exp***	Reference
Pig					
	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[a], 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)
Goat					
	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)
	oß-Lac/hFVIII	PNI	ND	ND, Yes	(Niemann et al., 1999)

, **, ** See Table I for standard abbreviations; a.HAC, Human Artificial Chromosome.

Table III Transgenic Pigs for Xenotransplantation

Cassette*	Delivery**	F0 Exp***	F1 Exp***	References
hβ-globin / hα1 and βA	PNI	3/3	ND	(Swanson et al., 1992)
mH-2K^{b}/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-2K^{b}/hCD55+hHT	PNI	4/20	ND	Nottle, 2001 #1472}
mH-2K^{b}/hCD55+hCD59+hHT	PNI	11/16	ND	(Nottle et al., 2001)
hICAM2/hCD46+hCD55+hCD59	PNI	2/94	ND	Nottle, 2001 #1472}
CAG/hGnT-III	PNI	NA	Yes	(Miyagawa et al., 2001)
RSV/hCD55	SMGT	34/53	Yes	(Lavitrano et al., 2002)
p Alb/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-I g	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 et al., 2011b)
PGK,Ub,CAG/LP2-hApoBEC3G	SB-SCNT	3/3,4/4,0/1	ND	(Carlson et al., 2011b)

See Table I for standard abbreviations.

Table IV 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[a], hALB-BAC	SMGT,ICSI	6/8	ND	(Watanabe et al., 2012)
PGK/YFP-Cre	SB-SCNT	6/6	ND	(Carlson et al., 2011b)
		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)
Goat				
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 I for standard abbreviations;
[a].BAC, Bacterial Artificial Chromosome.

Table V Gene Targeting in Livestock through HR and NHEJ

Gene(s)*	Success**	Agent	Efficiency= Genotyping+ /total colonies (%)	F1***	Reference
			HR		
Pig					
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	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		
Pig					
Cκ	+/−	Naked DNA	0.75	−/−[b]	(Ramsoondar et al., 2011)
JH	+/−	Naked DNA	0.64	−/− [b]	(Mendicino et al., 2011)
			Disease Transgenics		
Pig					
CFTR	+/–, +/ Δ	AAV	0.053-8.20	Yes	(Rogers et al., 2008)
Cattle					
IGHM,	−/−; −/−	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.,
Sheep					
COL1A1	+/−,	Naked DNA	34.0	ND, ND	(McCreath et al., 2000)
PrP	+/−	Naked DNA	10.3	ND	(Denning et al., 2001)
			NHEJ		
Pig					
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, 2012 In press]
Cattle					
BLG	+/−	ZFN	19.4%	ND	(Yu et al., 2011)

Refer to Table I for standard abbreviations;

*Genes are defined in the legend to Table I; in some cases more than one gene was inactivated**;**
** +/− : Heterozygote knock out; −/−: Homozygote knock out; −/ 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 VI. 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 DEL	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 & 1 bp DEL	Splice Site & 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	Frameshifts
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 DEL	
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 & missense
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-colour dilution	PMEL	3 bp DEL	
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 & 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 DEL, 10 bp INS	
OMIA 000685- 9913	Myasthenic syndrome congenital	CHRNE	20 bp DEL	
OMIA 000689- 9913	Myoclonus	GLRA1	SNP	nonsense
OMIA 001319- 9913	Myopathy of the diaphragmatic muscles	HSPA1A	11 kb DEL	
OMIA 001482- 9913	Neuronal ceroid lipofuscinosis 5	CLN5	1 bp duplication	frameshift
OMIA 000755- 9913	Osteopetrosis	SLC4A2	2.8 kb DEL	
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 DEL	
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 DEL or SNP	nonsense or missense
OMIA 001003- 9913	Thrombopathia	RASGRP2	SNP	missense
OMIA 001009- 9913	Tibial hemimelia	ALX4	45.7 kb DEL	
OMIA 001360- 9913	Trimethylaminuria	FMO3	SNP	nonsense
OMIA 001079- 9913	Yellow fat	BCO2	SNP	nonsense

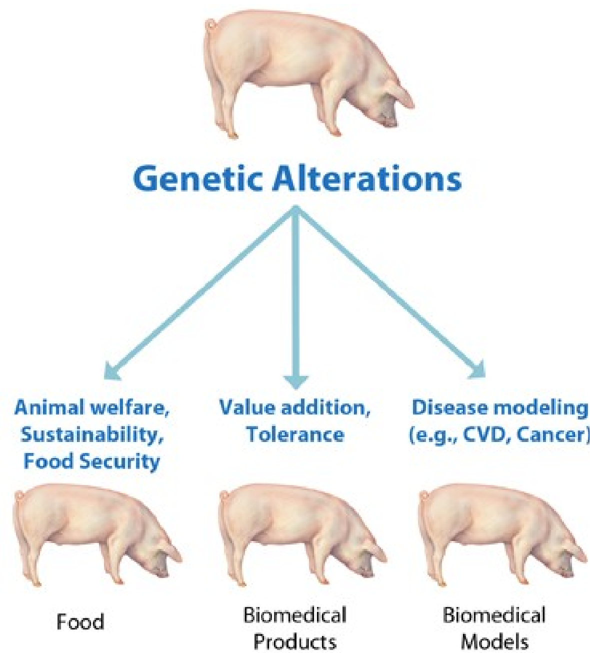


Figure 1: The multiple applications of genetically modified large animals. The pig is shown as an example. The first application is to improve traits in the farm animal. Examples of potential improved traits include 1) resistance to diseases, 2) improved nutrition such as introducing a gene to produce the healthier omega-3 fatty acids to replace the normal omega-6 fatty acids (Lai L, 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 sub-cellular 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.

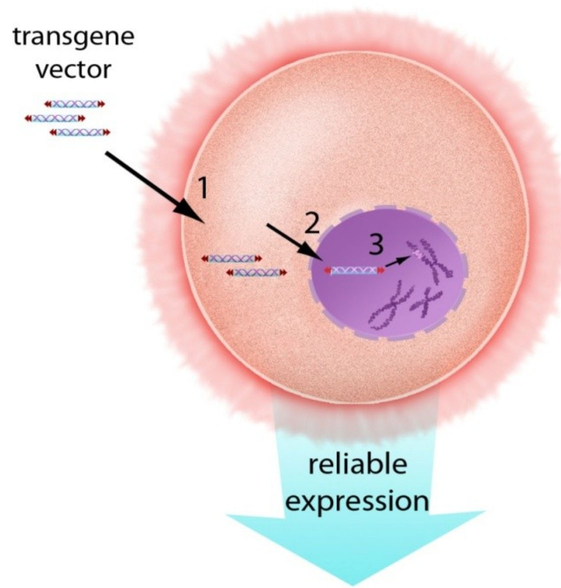


Figure 2: The three barriers to the introduction of foreign DNA into genomes: 1) the cell membrane, 2) the nuclear membrane, and 3) the chromosomal DNA in chromosomes. For effective transgenesis, the foreign DNA must overcome the three barriers and then be able to withstand protective measures such as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

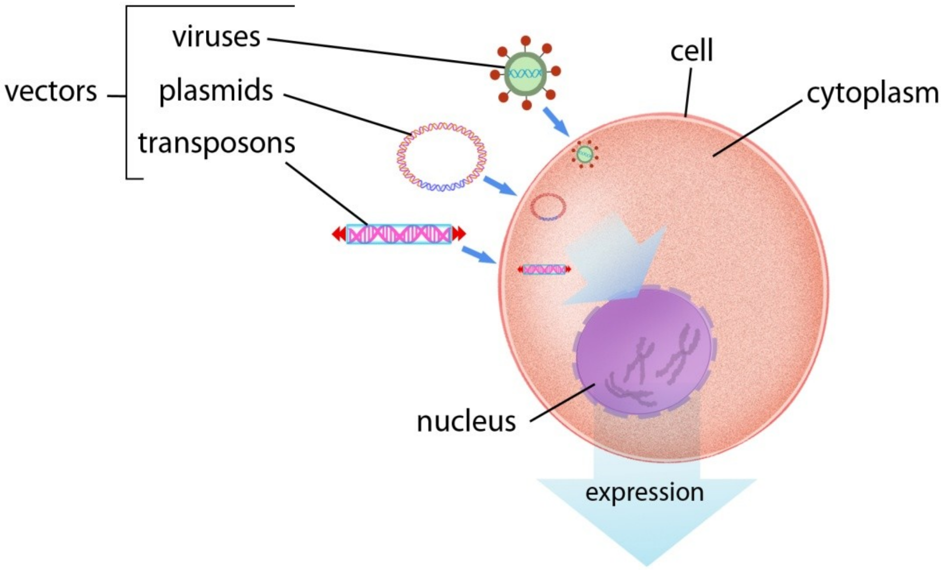
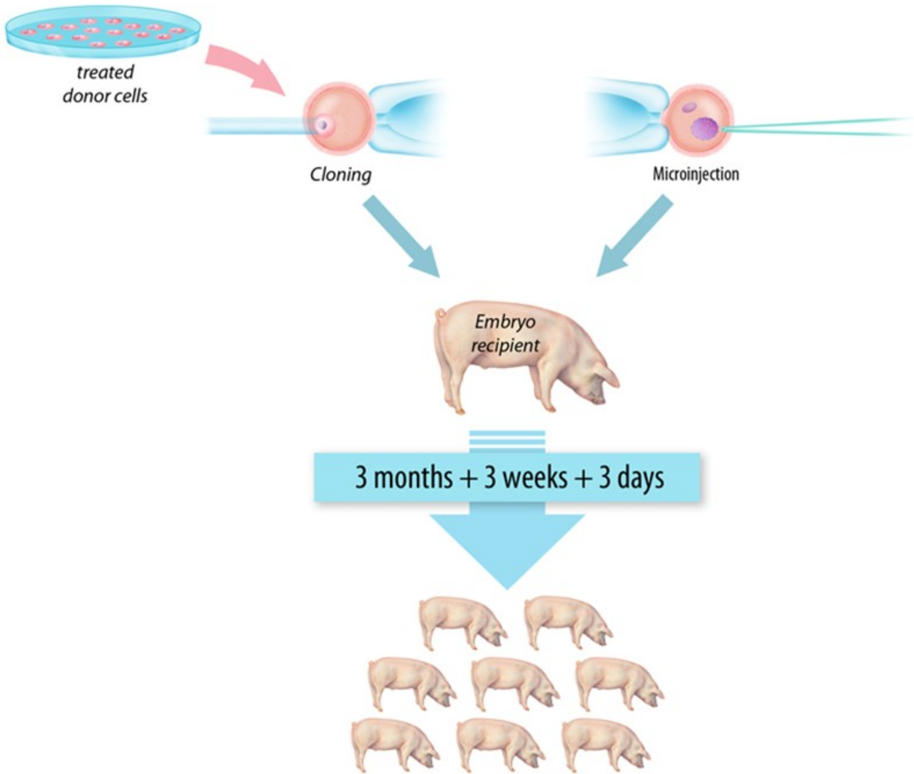


Figure 3: The three vectors for introduction of foreign DNA into genomes: 1) plasmids, 2) viruses, and 3) transposons.

A.



B.

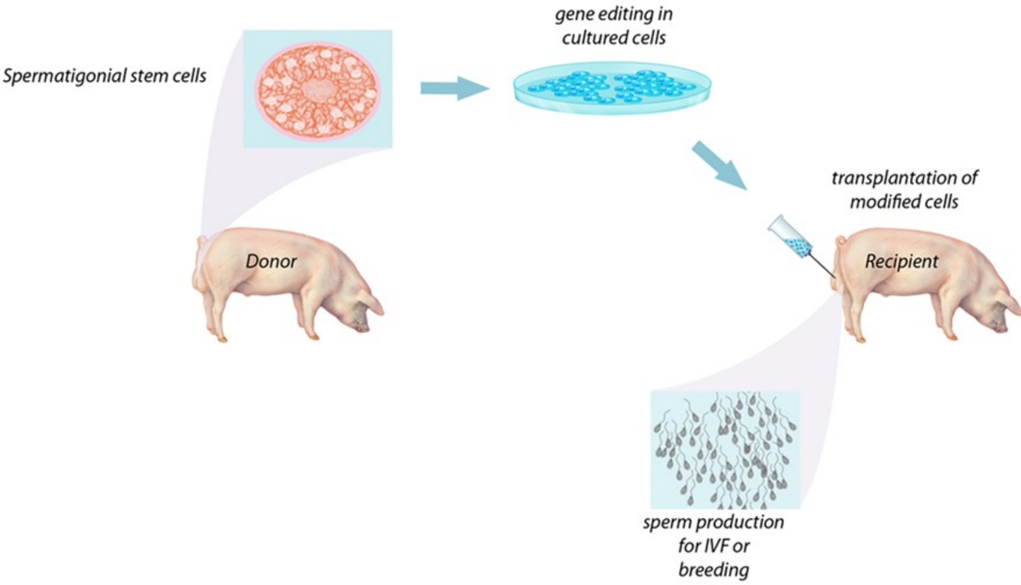


Figure 4: Methods for genetic modification in livestock. A) A flow diagram of the primary steps involved with the production of transgenic livestock by SCNT (cloning) and embryo microinjection. For simplicity, the illustrations show pigs only, but the general procedure applies to each of the major livestock 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 hour post fertilization. The injection site can vary, but typically DNA is delivered directly to the pronucleus, pronuclear injection (PNI), *SB* transposons plus transposase mRNA, ZFN or TALEN mRNA can be injected into the cytoplasm, and viral particles are typically injected into the peri-vitelline 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 spermatogonal 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 (AI) or *in vitro* fertilization (IVF) to derive founder animals.

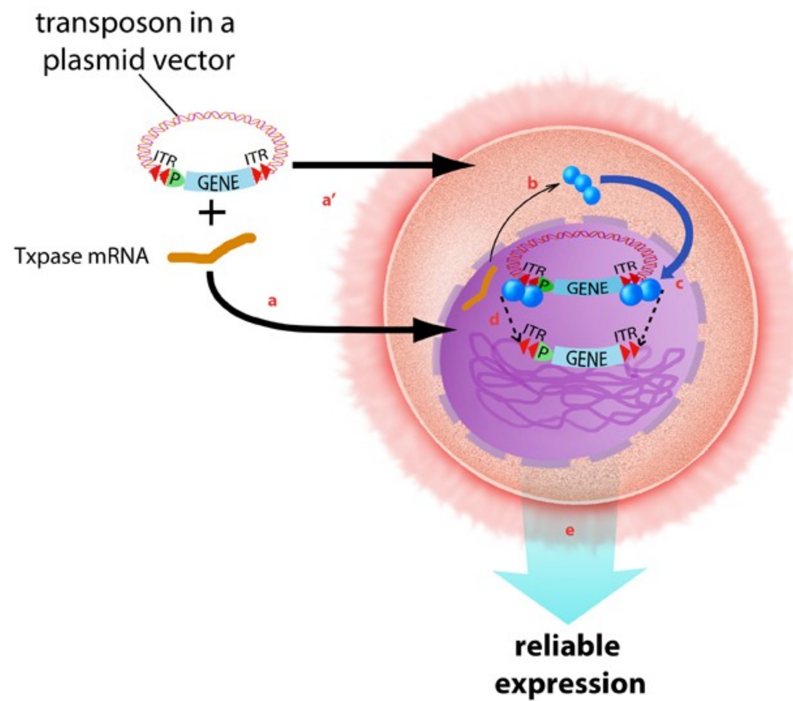


Figure 5: DNA transposition. DNA transposition consists of an enzymatic cut-and-paste reaction in which a transposon containing a gene of interest (GOI, shown in blue, with its promoter (P)) is cut out of a plasmid and inserted into a chromosome. The cleavage reaction occurs at the ends of the inverted terminal repeats (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. ***d)*** 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.

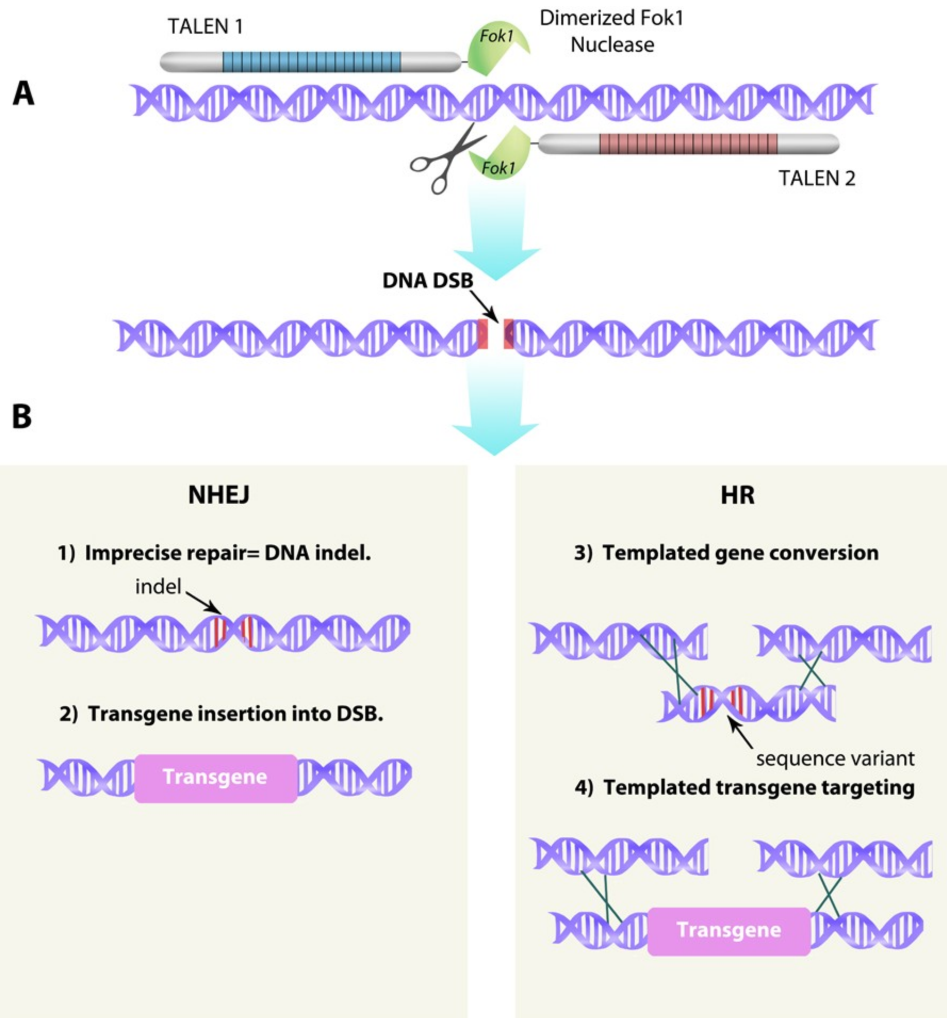


Figure 6: Site-specific targeting of genetic changes using hybrid DNases. A) A pair of TALEN nucleases is shown as an example of hybrid DNases designed to cleave at a unique sequence in a genome. The pair of TALENs executes a double-strand DNA break (DSB) at the targeted locus. **B)** If no other DNA sequences are added, the DSB will be repaired by the process of non-homologous end-joining (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).

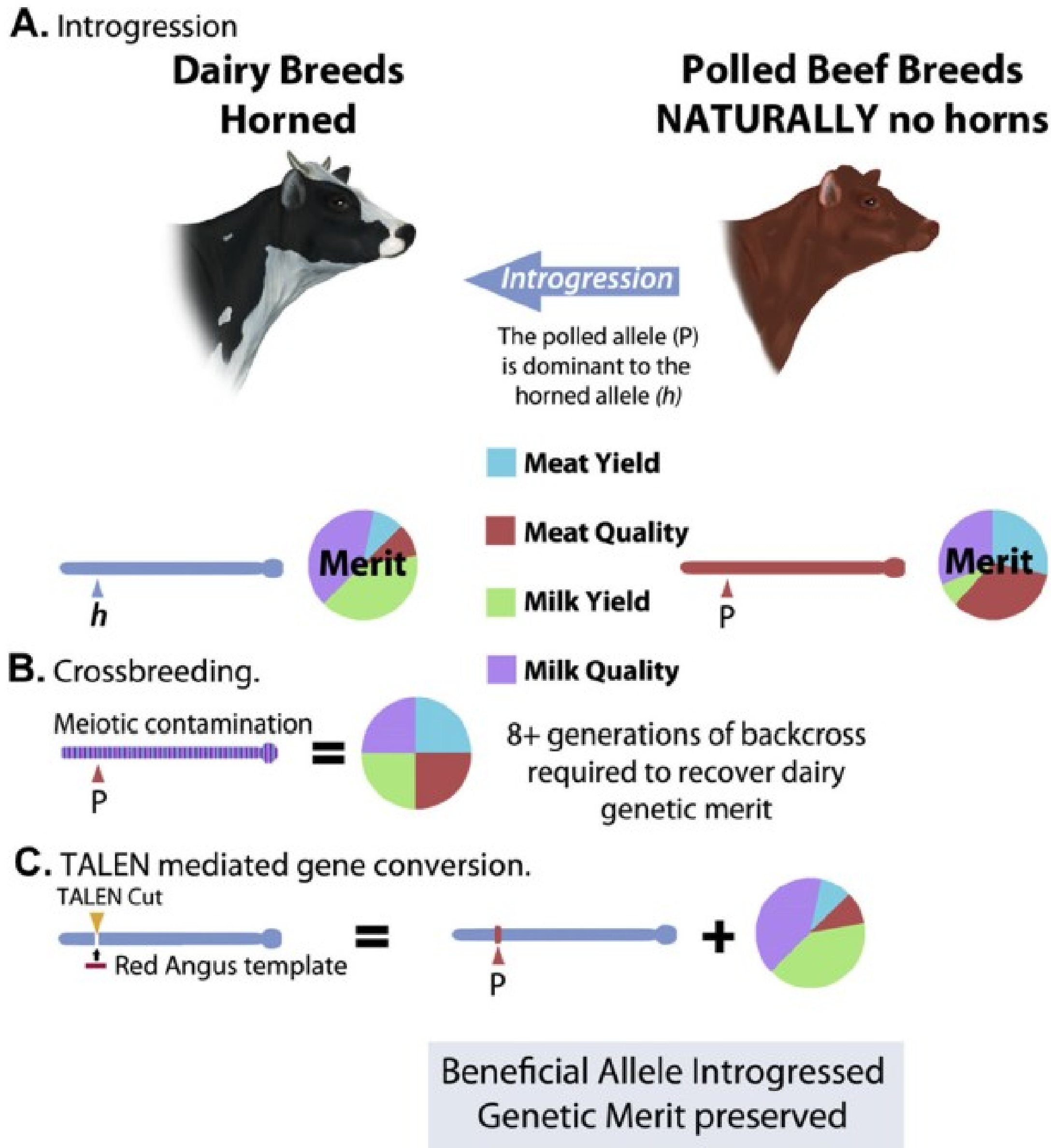


Figure 7: Rapid allele introgression in livestock. A) The diagram contrasts introgression of desired alleles (polled allele to horned animals) by crossbreeding (panel **B**) versus TALEN-mediated gene conversion (panel **C**). Beef and dairy breeds are selected for divergent classes of traits resulting 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.

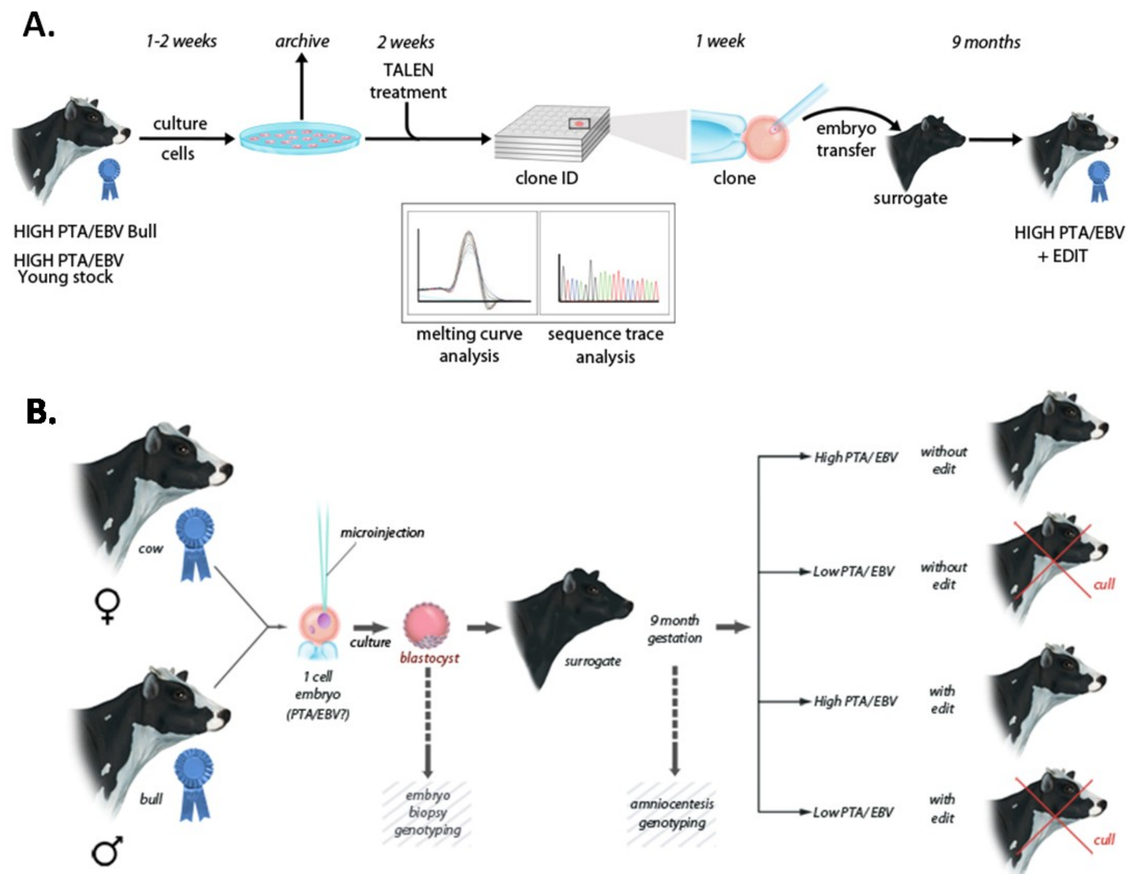


Figure 8: Strategies for implementation of allelic introgression. The introgression of desirable alleles into livestock could follow either a vertical (panel **A**) or horizontal (panel **B**) paradigm. **A)** In the vertical paradigm, allelic introgression would be performed in cells derived from a 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 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 absence of the targeted edits. Animals with high PTA/EBV would be maintained in the genetic improvement program regardless of 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 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.

Chapter 2 *Sleeping Beauty* Transposon Mediated Selection Marker-free Expression of Dominant Negative Myostatin in the Pig

Sleeping Beauty Transposon Mediated Selection Marker-free Expression of Dominant Negative Myostatin in the Pig

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Author contributions: WT constructed the Myostatin expression plasmids, conducted all the experiments and wrote the manuscript; WT, DFC, and SCF designed the experiments; DFC and KJC provided the pKT2P-PTK and pKC-SB100X plasmids; SCF supervised the study.

This project focused on rapid manipulation of porcine Myostatin (*GDF8*), a negative regulator of muscle development and growth, to clarify the strategic feasibility of Myostatin disruption in promoting the efficiency and quality of pork production. It also intended to facilitate understanding of Myostatin biology in swine myogenesis and reproduction. We proposed to disrupt Myostatin function in pigs through selection-marker free introduction of dominant negative Myostatin alleles (GVDG and D75A), using the *Sleeping Beauty (SB)* transposon mediated co-transposition co-selection.

Porcine fibroblasts were nucleofected with *SB* transposon plasmids bearing one of these alleles and a PuroΔTK selection marker *in trans*, at a controlled molar ratio to achieve an average of 3~ 5:1 transgene to marker copy number ratio in the target genome. An average of 89% of Puromycin resistant cells harbored the Myostatin transgenes delivered by the transposon. Immunoblotting on samples from these cells detected ectopic expression of the transgenes *in vitro* when promoted by a constitutive CAG promoter but not muscle specific HSA promoter.

A mixture of four cell populations bearing one of the two dominant negative transgenes driven by either promoter was used as nucleus donors to generate transgenic animals through somatic cell nuclear transfer. Six live animals out of three pregnancies were born two weeks after abortion of the other two pregnancies. Genotypic analysis identified eight out of eight aborted fetuses or founder animals as transgenic; seven of them were driven by the HSA promoter. However, the majority of founder animals died shortly after birth due to viral infection that led to sharp termination of this project.

I. Introduction

Myostatin is a muscle specific form of the Transforming Growth Factor beta family (TGFβ) members primarily synthesized and processed in skeletal muscle (1, 2). Originally known as *GDF8*, Myostatin is highly conserved among species and functions as a negative regulator of muscle development and growth (1, 3). It was first discovered in the two cattle breeds *Belgian Blue* and *Piedmontese* (**Fig. 1**) (1, 3, 4), which are renowned for their "double muscling" phenotype or hypermuscularity due to almost doubled total muscle fibers compared to wild type counterparts. Such naturally occurring hypermuscularity is also present in several other cattle breeds (5-7), dogs (8), sheep (9), and humans (10), all caused by mutations in the Myostatin gene (**Fig. 2**). Such phenotype was recapitulated in the bi-allelic Myostatin knockout mouse "Mighty" that displayed significant increase in skeletal muscle mass due to both hyperplasia (increased muscle fiber number) and hypertrophy (increased muscle fiber girth) (2), further confirming the suppressive role Myostatin plays in muscle development.

Myostatin inhibits muscle development and growth by suppressing proliferation and differentiation of muscle precursor cells during embryonic and fetal myogenesis (11-14). It was also shown to suppress activation and proliferation of satellite cells during postnatal muscle growth or regeneration (15). This protein stalls cell cycle progression and myoblast differentiation through initiation of the *SMAD* signaling cascade that leads to up-regulation of cyclin-dependent kinase inhibitors (CKI, e.g. *P21*) but reduction of cycling dependent kinase-2(*Cdk2*) (11, 16) and myogenic regulatory factors (MRFs, e.g. myogenin and *MyoD*) (12, 13, 17) (**Fig. 3**). Recent findings suggest that Myostatin prevents muscle growth also by inhibiting protein synthesis through suppressing the *Akt/mTOR* pathway (18). To ensure normal muscle development and growth, the expression and function of Myostatin itself is tightly regulated, by multiple factors including *MyoD* and other MRF factors (19, 20), follistatin (21, 22), and others(23-25).

A. Myostatin disruption for pork production and muscle disease treatments

1. The influences of Myostatin on meet quantity and quality

The natural mutations listed in **Figure 2** are responsible for observed hypermuscularity in the cattle, dog, sheep and human. *Belgian Blue* cattle (**Fig. 1**) homozygous for the 11 bp deletion at position 821 (nt821del11) produce about 20% more beef than wild type counterparts due to faster growth rates; similar patterns exist in the *Piedmontese* cattle (1). Such hypermuscularity was replicated in mice (2, 26-28), zebrafish (29, 30), and trout (22) by artificial reduction or

inhibition of Myostatin function. For instance, Mighty mouse was significantly larger and individual muscles harvested from it weighed 2-3 times more than those from wild type littermates (2). Another transgenic mice strain had a 20-35% increase in muscle mass due to elimination of the cleavage site separating the C-terminal mature protein from its propeptide (**Fig. 2**), such that mature Myostatin cannot be released from its latent form to become functional(26).

In addition to increased meat yield, disrupted Myostatin function might be beneficial to enhancing meat quality in terms of tenderness and leanness. Skeletal muscles isolated from double muscling cattle are more tender than those from ordinary animals (31, 32). It was determined that hyperplasia contributes to meat tenderness and hypertrophy resulting from boosted protein synthesis has no negative effect on meat tenderness (33). However, there is an inverse relationship between muscle fiber number and size within a certain muscle; animals with a greater number of muscle fibers and moderate muscle size yield meat with higher quantity and quality (34). Thus, Myostatin deficiency likely has failed to competently suppress proliferation of myogenic cells during embryogenesis (hyperplasia) as well as protein synthesis in the fibers and satellite activation during postnatal growth (hypertrophy).

Lower levels of Myostatin were also found to decrease body fat composition in several species including the double muscling cattle breeds (31, 35, 36). Probably a portion of the reduced adiposity is simply due to the calories withdrawn by enhanced musculature (37). Similarly, increased muscle development in Myostatin knockout mice was associated with reduced adipogenesis and leptin secretion (38), and such mice accumulated fat more slowly than wild type animals as age increased (39). Furthermore, when Myostatin deficient mouse was crossed with two models of obesity, offspring from both crossbreeding demonstrated a partial suppression of fat accumulation and of abnormal glucose metabolism (39).

Thus, we hypothesized that by manipulating Myostatin function in the pig, we might be able to promote both pork yield per head and meat quality in terms of tenderness and leanness. However, Myostatin induced hypermuscularity is complicated by reproduction problems in cattle. The double muscling *Belgian Blue* and *Piedmontese* cattle have long been suffering from reduced fertility, dystocia (calving difficulties) due to oversized fetuses (35, 40), and high neonatal mortality rates (41, 42). The broad conservation of Myostatin among species indicates that its disruption in pig might have similar benefits for pork production, but its influences on product quality, carcass composition, and reproductive performance are not predictable *a priori*. Besides, few publications are available regarding interactions between Myostatin and other growth related factors e.g. *IGF-2* (43), *RYR1* (44) and *MC4R* (45).

***2.** Myostatin in muscle-related diseases*

Knowledge in Myostatin biology could have implications for biomedical research in a wide range of muscle-related diseases as well. Postnatal blockade of Myostatin has been shown to improve muscle function by facilitating muscle regeneration thus alleviating the atrophic progression in Muscular Dystrophy, age-related muscle atrophy, or Sarcopenia patients (46-50). Several studies conducted *in vitro* and in mice indicated that the reduction or absence of functional Myostatin also enhances muscle regeneration post to injuries (15, 51). In addition, reducing Myostatin activity mitigated cancer-related or heart failure induced cachexia in murine models (52, 53). The knowledge in Myostatin biology to be gained from disrupted pigs will help understand mechanisms of the diseases and such pigs can be useful pre-clinic models to test therapeutic treatments.

B. Functionality of dominant negative alleles of Myostatin

Like other members of the TGFβ family, the Myostatin precursor undergoes three proteolytic processing after synthesis and dimerizes to form biologically active molecule (2) (**Fig. 4A**). The first cleavage removes a 24 amino acid (aa) N-terminal secretion signal and the second occurs at the RSRR site (Arg-Ser-Arg-Arg, aa 240 to 243), separating the propeptide from the C-terminus, functional part of the protein (referred to as Myostatin, 132 aa). However, the C-terminal dimer remains latent due to covalent binding by the propeptide until it is digested by BMP-1/tolloid proteinases at aa D76 (Aspartate), freeing the C-terminal dimer to gain receptor-binding activity (21, 54). Although further evidence is needed to determine the 3D structure of Myostatin, it probably folds into a Cystine knot and stabilizes itself through a disulfide bond between the monomers, based on its high similarity to other members of the TGFβ family (55, 56).

Mutations interrupting the processing of the precursor have been proven to promote muscle growth. This strategy was first shown to be effective on other TGFβ members (57, 58); when applied to Myostatin, it led to more than doubled muscularity in mice (26, 54). By deactivating the proteolytic sites required for the separation of the functional peptide from the propeptide (RSRR to GLDG) (26) or the degradation of the propeptide (D76 to A) (54), these mutated precursors failed to produce mature Myostatin. Moreover, they also functioned as dominant negative by depleting the pool of normal Myostatin precursors via dimerization to normal Myostatin monomers and sequestering the latter (**Fig. 4B**). Besides, merely by over-expressing the propeptide was one able to generate mice with hypermuscularity (21, 27), further supporting the efficacy of propeptide manipulation in controlling Myostatin functionality.

To test the hypothesis that the disruption of Myostatin in pig enhances pork yield and quality, we planned to ectopically express the above dominant negative alleles of Myostatin in the animal. We made Myostatin cDNA expression constructs bearing mutations corresponding to the GLDG and D76A tested in the mice (26, 54), GVDG (Gly-Val-Asp-Gly) and D75A (Asp75Ala, A224C) respectively **(Fig. 4B)**. We chose to deliver the expression cassettes into the pig genome by *SB* transposon mediated transpositional transgenesis (TnT). We expected to obtain fast and stable integration of the cassettes in pig fetal fibroblasts that would be used as nucleus donors to generate pigs through somatic cell nuclear transfer (SCNT).

C. *Sleeping Beauty* transposon mediated transpositional transgenesis

DNA “cut" and "paste” transposons are a group of transposable DNA elements capable of “jumping” out of a donor sequence and inserting into near random target sites. A natural transposon consists of transposase expressing DNA flanked by inverted terminal repeats (IR) that harbor direct repeats (IR/DR). During transposition, the transposase binds to the IR/DRs, excises the transposon from its original location, and integrates it into a target sequence (**Fig. 5**). In an engineered transposon however, the transposase-coding DNA is replaced by cargo or transgene; when supplemented with transposase, the cargo gains mobility. Since the revival of *SB* transposon (59, 60), several others have been recovered including *piggybac* (61, 62), *Passport* (63), and *Tol2* (64). Activities of these transposons have been tested in pig and cellular transgenesis was boosted to 28-fold above background by TnT (65).

Engineered *SB* transposons have been shown to mediate quick and stable transgenesis in various vertebrates including model organisms (66, 67), humans (68, 69), pigs (65, 70, 71) and others. Microinjection of *SB* transposon vectors and *SB* transposase mRNA into mouse and zebrafish embryos achieved efficient transgenic expression and germ line transmission (66, 72). Currently, *SB* transposons are being developed for several therapeutic applications highlighted by human clinical trials to cure B-Lymphoma (73-76). Transgenic pigs created by the hyperactive *SB100X* system (60) showed stable expression of human APOBEC3G and germ line transmission of the transgene (70). Thus *SB* mediated random insertions should be competent in rapid integration of dominant negative Myostatin alleles.

To enable elimination of selection markers associated with transgene enrichment, we adopted the co-transposition co-selection (*CoCo*) strategy illustrated in **Figure 6** (70). It harnesses the *SB100X* transposon system to deliver the transgene and drug selection marker *in trans* on separate transposons. Since transposon plasmids bearing the transgenes were represented

five times as often as those with the selection marker during transfection (**Table 1**), the majority of drug resistant cells were expected to possess an average three to five copies of the transgene per selection marker (**Fig. 6**) (70). Founder animals resulting from SCNT would be examined to determine the integration loci and expression levels of the transgenes as well as phenotypic changes in muscle growth and pork quality. To eliminate the drug selection marker and propagate Myostatin transgenes with proper levels of expression, a crossbreeding program would be established.

With *SB* transposition, we were able to deliver the Myostatin transgenes into the majority of cells treated with the *CoCo* components. The integrated transgenes were robustly expressed when promoted by a ubiquitous CAG promoter and the cells led to production of animals through SCNT. Genotypic analysis revealed that all animals were transgenic but there was a strong bias towards transgenes with tissue specific expression pattern, indicating a fetus survival disadvantage associated with over-expression of the transgenes. Due to the death of founder animals, we were not able to continue with this project; nevertheless, supported by the data already collected, *SB* transposons have been shown to mediate rapid integration of transgenes in the pig.

II. Results

We nucleofected Landrace pig fetal fibroblasts (PFFs) with *SB* transposon plasmids (**Table 1**) bearing one of the transgenes (GVDG or D75A), driven by either a constitutive CAG promoter or HSA promoter that was proven to be muscle specific in mice (77) (**Fig. 7**). Fibroblasts from this first round transfections reached confluency on both 96-well plates and 10 cm dishes after two weeks of 1.2 μg/ml Puromycin selection as expected. They displayed good morphology and maintained robust proliferation. To assess retention rates of the Myostatin cDNA in the nucleofected cells, co-retention PCR specifically amplify Myostatin cDNA instead of endogenous genomic Myostatin sequence was conducted on each colony sample. We found that 78% to 100% of the colonies from transfected populations of both sexes harbored the expression cassettes with a grand average of 89% out of 119 colonies assayed (**Table 3**).

We then performed Western blotting to evaluate transgenic expression. By anti-Flag immunoblotting, we detected transgenic protein expression around 50 kD in size, in the crude protein extract from fibroblasts transfected with transposon plasmids bearing the GVDG allele driven by CAG (pKT2C-Myostatin GVDG-F; lane 3, **Fig. 8**), whereas samples from non-transfected wild type fibroblasts showed no visible bands (lane 7) . Protein extract from the

pKT2C-Myostatin D75A-F transfection was confirmed of transgenic Myostatin expression later by a separate immunoblotting also using anti-Flag antibody (image not shown).

Supported by the satisfactory co-retention rates and positive Western blot results, all male populations transfected with *SB* transposons bearing one of the cDNA expression cassettes (**Fig. 7**) were mixed with equal representation and shipped to Minitube America for SCNT. Unfortunately, no pregnancy was detected one month after transfer of reconstructed embryos into three surrogate sows, so a second SCNT was scheduled. Even though the failure was not necessarily due to the quality of the transgenic fibroblast populations, a second round of transfections was initiated soon after, also to generate low passage and freshly prepared transgenic cells for SCNT.

Round two transfections were conducted in a similar manner as the first round but a Landrace male cell line previously yielded piglets through SCNT was used instead (unpublished data). Surprisingly, after the completion of Puromycin selection, the co-retention efficiency was only about 30% per transfection, way below former results. It was noticed that the number of colonies obtained from each 96-well plate was doubled this round than previous, so we suspected that the Puromycin selection with a concentration of 1.0 μg/ml was insufficient. Even though it was sufficient to eliminate WT cells descended from the same cell line in another independent experiment (unpublished data), it might have failed to kill all the WT cells here.

Indeed, PCR using PuroΔTK specific primers only detected PuroΔTK integration in 16% of the colonies, indicating that the majority of "drug-resistant" colonies were "fake" due to the low Puromycin concentration. Fortunately, nearly 100% of colonies with PuroΔTK integration also harbored the Myostatin transgene. Thus to remedy the situation, passage zero nucleofected cells were expanded and cultured for five more days with 1.2 μg/ml Puromycin until no more dead cells were present. We planned to repeat the colony formation using the populations treated with the higher drug dosage and conduct co-retention PCR to re-evaluate co-retention rate.

Western blotting was then conducted on protein extracts from these final populations and results are shown in **Figure 8**. By anti-Flag immunoblotting, we detected abundant transgenic protein from these Round two fibroblasts transfected with *SB* plasmids bearing Myostatin cDNA driven by CAG (lane 1 and 2). On the contrary, little signal was visible when the same transgenes were placed downstream of the muscle specific HSA promoter (lane 5 and 6). These four fibroblast populations were then mixed and shipped right away to meet the schedule for the second cloning attempt.

Three pregnancies out of two embryo transfers were detected one month after the transfer. Unfortunately, two pregnancies aborted about two weeks before due date; three fetuses were collected from one abortion and one fetus from the other (**Fig. 9A**). The remaining sow farrowed on time and gave birth to six live piglets. However, piglet 1496 (**Fig. 9B**) died from possible crushing by the sow one week later. By the end of the fourth week, all piglets died except one, which remained alive as of July 19, 2013. People at the pig facility suspected that a viral outbreak was responsible for the abortions and the death of the live-born piglets.

Upon receiving tail clips of the four aborted fetuses and four of the six live-born pigs, two sets of PCR were conducted to determine whether they were transgenic with Myostatin cDNA integration. As shown in **Figure 9**, all four aborted fetuses (**A**) and all live-born piglets tested (**B**) yielded PCR products (purple arrow) that could only be amplified from Myostatin cDNA, indicating that they were all transgenic. However, PCR using HSA promoter specific primers revealed that seven out of the eight examined fetuses or piglets contained the cDNA expression cassettes driven by tissue specific HSA promoter instead of CAG (**Fig. 9C, D**), except for aborted fetus 847-2 (lane 3, **Fig. 9C**). By deduction, fetus 847-2 was determined to contain the CAG promoter since it was transgenic but negative for the HSA promoter; nonetheless, PCR specific to the CAG promoter was not conducted to confirm the deduction.

Unfortunately, tissue samples were not collected in time from most of the dead fetuses or piglets so I was not able to conduct any phenotypic analysis to determine the expression levels and patterns of these transgenes. This project suffered from sharp termination since the majority of founder pigs died and no further analysis on the pig samples was conducted beyond this point.

III. Discussion

Plasmid based *SB* transpositional transgenesis (TnT) is indeed very efficient and economical in delivering transgenes into porcine genomes (70, 71). Particularly, the *CoCo* strategy achieved up to 100% transgene penetration in transfected fibroblasts (**Table 3**) and pigs produced from them (**Fig. 9**). *CoCo* not only enables selection marker free production of transgenic animals but also expands transgenic capacity of the transposons by separating the transgene and the selection marker. However, if the size of the gene of interest (GOI) is substantially larger than what was delivered here, the molar ratio between *SB* transposons carrying the GOI and the selection marker might need adjustment to ensure high co-retention since transposition efficiency of transposons decreases as the cargo size increases (68).

SB mediated TnT led to robust transgenic protein expression in porcine primary fibroblasts as immunoblotting detected strong transgenic Myostatin signal (**Fig. 8**). One major drawback of insertional transgenesis is the risk of positional effects that might over-express, suppress or even silence the transgene. However, *SB* mediated TnT offers the opportunity to selectively propagate transgene copies with optimal level of expression since multiple copies are usually integrated in one genome. Out of curiosity, the most prominent bands detected by Western blotting (**Fig. 8**) were around 50 kD in size, in the range of the Myostatin precursor protein (42-45 kD); this raises the question whether fibroblasts are unable to process the precursor after translation or the inability to detect smaller processed peptides was simply due to the big pore size (0.45 μm) on the blotting membrane.

We accommodated the possibility that over-expression of the dominant negative Myostatin might cause unexpected health problems and render the ability to obtain live transgenic pigs. In addition to CAG promoted constitutive expression, we also placed the transgenes downstream of muscle specific HSA promoter tested in the mouse (77). Judging from the Western blotting results (**Fig. 8**), we did not observe transgenic expression in fibroblasts when the HSA promoter was recruited, suggesting that the promoter is also likely to be muscle specific in the pig. The fact that seven out of eight fetuses or live-born pigs harbored the HSA expression cassette instead of CAG, even though both of the cassettes were equally represented during SCNT, lends support to the notion that tissue specific expression of the transgenes possessed a survival advantage whereas CAG driven high level and ubiquitous expression might have affected the survival of reconstructed embryos.

If the founder animals lived until sex maturation, we would have mapped the transgene insertions, assessed transcription levels and patterns from individual transgenes, and evaluated germ line transmission. With such information, we could then design plans to breed selected pigs to eliminate the selection marker and propagate transgenes with proper levels of expression. After genotypic and gene expression analysis, offspring from these selected founder animals would be closely monitored on daily feed intake and weight gain. Muscularity in terms of skeletal muscle volume and weight, fiber numbers and girth, and fat content should be measured. To evaluate reproduction performances, traits including litter size, birth weight and weaning weight of F1 piglets should be recorded for statistical analysis.

The *SB* transposons had been one of the most efficient systems to introduce novel genes into large animal genomes. Before the onset of the site-specific nucleases described in Chapters 1, 4 and 5, gene targeting efficiency was as low as 10^{-6}, which made it very tedious to achieve in

large animal research. The unavailability of embryonic stem cells, scarcity of embryos, and high cost of SCNT further hampered gene targeting in livestock. To generate livestock animals with transgenic protein expression, most of the past research had to rely on insertional transgenesis (Chapter 1), on this matter *SB* transposons are obviously very efficient.

IV. Methods

Construction of expression plasmids. RACE-ready cDNA preparation by Template-Switching reverse transcription using total RNA isolated from porcine embryonic myoblasts[2] as templates: a 5 µl reaction mixture containing 250 ng RNA, 0.5 µl 20µM RT2, 0.5 µl 20µM 5R-TS primers, and Rnase free H_2O was incubated at 70 °C for 5 minutes and placed in ice-water bath immediately for 2 minutes. The following reagents were then mixed with the previous reaction: 2 µl 5X 1st Strand Buffer (Invitrogen), 1 µl 0.1M DTT (Invitrogen), 0.5 µl 10mM dNTPs, 0.5 µl Rnase free H_2O, 0.25 µl RnaseOUT (Ambion), and 0.25 µl Superscript III (Invitrogen). The reaction was incubated in a thermal cycler running the following program: 6 cycles of (50 °C for 10 seconds, 42 °C for 15 minutes), 42 °C for 10 seconds, 42 °C to 37 °C gradual cooling in 2 minutes, 37 °C for 1.5 minutes, 70 °C for 10 minutes, and 4 °C hold. The reaction was then diluted with 100 µl TE (10mM Tris-Cl, 1mMEDTA pH8.0) for next step. 5R-TS: CCAAGTTCGTACAAAAAAGCAGGCTCGGGG; RT-2: ACCACTTCCTACAACAAAGCTGGGTTTTTTTTTTTTTTTTTTTTV (V= not a T).

Amplification of Myostatin cDNA: PCR using high fidelity Pfx50 polymerase (Invitrogen) was conducted to amplify the cDNA in the following reaction: 2.5 µl diluted RACE-ready cDNA, 5 µl 10X Pfx50 PCR Mix (Invitrogen), 1.5 µl 10mM dNTPs, 1 µl 5' 20µM primer, 1 µl 3' 20µM primer, 38 µl ddH_2O, and 1µl Pfx50 polymerase. The reaction is incubated in a thermal cycler running the following program: 94 °C for 2 minutes, 35 cycles of (94 °C for 15 seconds, 60 °C for 30 seconds, 68 °C for 1.5 minutes), 68 °C for 5 minutes, 4 °C hold. Myostatin specific primers used for this PCR: 5' ACCATGCAAAAACTGCAAAT; 3' TCATGAGCACCCACAGCGATC. The PCR reaction was gel purified using the QIAEX II gel extraction kit (Qiagen) and the purified DNA was TOPO cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). TOPO plasmids with the cDNA insert were confirmed by restriction digests and sequencing.

PCR site-directed mutagenesis and molecular cloning to derive transgenic Myostatin constructs: two PCR were conducted to introduce the Kozak sequence, the Flag tag and the GVDG mutation with primer sets 5' CAG-GVDG F +R for a 5' segment and 3' CAG-GVDG F+ R for a 3' segment (Table 2). For both PCR, 1 µl TOPO cDNA plasmid (1 ng/µl), 0.5 µl forward primer (20 µM), 0.5 µl reverse primer (20µM), 2.5 µl 10X Pfx50 PCR mix, 0.5 µl dNTPs (10mM), 19.5 µl ddH2O, 0.5 µl Pfx50 polymerase were mixed and the reaction was incubated in a thermal cycler running the above program. The PCR reactions were gel purified; the 5' segment was digested with XhoI and HincII and the 3' segment by HincII and

[2] Mary S. Pampusch from Professor William Dayton's Lab shared the total RNA.

BglII. Both reactions were cleaned up with the QIAquick PCR Purification Kit (Qiagen) and the digested inserts were cloned into plasmid pKT2C-mCherry to make the final pKT2C-Myostatin GVDG-F plasmid (Fig. 7) using T4 ligase (NEB). To make pKT2HSA-Myostatin GVDG-F, PCR as above was conducted using the pKT2C-Myostatin GVDG-F as template and HSA F +R as primers (Table 2). After gel purification, digestion with NCOI and EcoRI and PCR cleanup, the PCR product was cloned into plasmid pKT2HSA-YFC to produce pKT2HSA-Myostatin GVDG-F (Fig. 7).

To make the pKT2C-Myostatin D75A-F and pKT2HSA-Myostatin D75A-F plasmids, 5' and 3' segments bearing the D75A mutation were generated as above but with primer sets 5' CAG-D75A F+ R and 3' CAG-D75A F +R (Table 2). After gel purification, these segments were used as templates in a fusion PCR to generate the full-length cDNA with the D75A mutation. The PCR reaction mix was as above but contained primer set 5' T2C-D75A F + 3'T2C-D75A R and incubated with a re-annealing temperature of 55 °C. After gel purification, digestion with XhoI plus BglII and PCR cleanup, the full-length cDNA with D75A was cloned into pKT2-mCherry to generate pKT2C-Myostatin D75A-F. pKT2HSA-Myostatin D75A-F was made the same way as pKT2HSA-Myostatin GVDG-F. All the four final plasmids were confirmed by restriction digests and sequencing. Concentrated plasmid stocks ready for transfection were prepared using the PureLink HiPure Midiprep kit (Invitrogen).

Transfection. Pre-thawed passage 0-1 female (♀) and male (♂) pig fetal fibroblasts (PFF) reaching 80% confluency were trypsinized and nucleofected with cocktails shown in **Table 1**, using Amaxa Basic Nucleofection Kit for Primary Mammalian Fibroblasts (Lonza). Program U-012 was used and the total volume was controlled to be less than 105 µl per nucleofection per 500,000 cells. Other directions elaborated in the manufacturer's manual were carefully followed. Cells post nucleofection were recovered for 24 hours in 6-well plates before downstream work.

Tissue culture and colony formation. The PFFs were incubated at 37 °C in 5% CO_2 and maintained in standard media composed of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 I.U./ml penicillin and streptomycin, and 2 mM L-Glutamine (all purchased from GIBCO). During drug selection, transfected cells plated out as shown in **Figure 6** were selected by adding 1.2 µg/ml Puromycin to culture media until confluence; media change was performed every three days for around two weeks. To isolate clones, 1 or 3 transfected cells/well were seeded on 96-well plates supplemented with WT cells to reach a final density of 500 cell/well.Colonies from 96-well plates were counted and lysed for co-retention PCR analysis whereas drug resistant populations from 10 cm dishes were split for potential SCNT and crude protein extraction.

Crude protein extraction. When cells on 10 cm dishes became 100% confluent after passaging, cells were washed by PBS before applying the extraction solution to one plate per nucleofection. From this point, everything was kept on ice to remain cold including cells, pipette tips, scrapers, extract solution etc. 1 ml of solution was then added to each plate and evenly distributed on the surface. After 10-minute incubation on ice, remaining cells were scraped off the plate and the whole extract was transferred to a 1.5 ml

microcentrifuge tube and stored at -80 °C. The extraction solution was prepared by dissolving one cOmplete protease inhibitor cocktail tablet (Roche) in ddH2O.

Genomic DNA extraction. To extract genomic DNA from Puromycin resistant colonies on 96-well dishes, 40 µl of 1X PCR compatible cell lysis buffer was added to each well post PBS wash and the lysates were transferred to 96-well PCR plates after 20-minute incubation at room temperature. The lysates were further treated in a thermal cycler running the program: 55 °C for 50 minutes, 95 °C for 15 minutes, 4 °C hold. 1X PCR compatible lysis buffer was prepared by following the recipe: 10 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.45% Tryton X-100(vol/vol), 0.45% Tween-20(vol/vol) in ddH$_2$O freshly supplemented with 200 µg/ml Proteinase K.

To isolate genomic DNA from piglet tail clips, 600 µl 1X lysis buffer was added to each clip (~05mm X 4mm X 3mm in size) in a 1.5 ml microcentrifuge tube and incubated in 55 °C waterbath with rock motion overnight or until the tail clip was completely dissolved. Tubes were then spun in microfuge at maximum speed for 5 minutes; supernatant was transferred to a new 1.5 ml tube. 700 µl Iso-propanol was added to the supernatant and the tube was inverted 8 times to mix and let sit for 5 minutes. The mixture was spun in microfuge at maximum speed for 10 minutes and supernatant was removed carefully to leave the DNA pellet intact. 700 µl ice-cold 70% Ethanol was added to the tube to wash the pellet and the tube was spun again at full speed for 10 minutes before the supernatant was removed. The pellet was let air dry until no liquid was visible and resuspended in 100-200 µl TE buffer in 55 °C water bath with flicking everything 30 minutes or so to ensure complete resuspension. 1X lysis buffer was composed of 0.1M Tris pH 8.0, 0.2M NaCl, 5mM EDTA, 0.5% SDS in ddH$_2$O with freshly supplemented 200 µg/ml Proteinase K and 100 µg/ml RNase A.

Co-retention PCR. PCR was conducted to detect integration of transgenic Myostatin cDNA in Puromycin resistant colonies using primers F: CTGCTATAAGACAACTTTTGCCCAAAGCTCCTC and R: GTCTTGACGGGTCTCAGATATATCCACAG; which bind to Myostatin Exon 1 and Exon 2 sequences respectively and allow for enrichment of amplification from cDNA over genomic DNA. PCR reaction mix composed of 2 µl colony lysate, 0.5 µl F +R primer mix (20µM), 1 µl MgSO$_4$ (50mM), 2.5 µl 10X APEX PCR buffer, 0.5 µl dNTPs (10mM), 18 µl ddH$_2$O, and 0.5 µl polymerase (APEX) was incubated in thermal cycler running program 95 °C for 2 minutes, 30 cycles of (95 °C for 20 seconds, 57 °C for 20 seconds, 72 °C for 30 seconds), 72 °C for 5 minutes, 4 °C hold. The PCR reactions were then resolved on a 3% agarose gel. Similar PCR as internal control was ran on the same lysates with primer set F: TTCCAGTGCAACAGCTCCACCT and R: TTCTCCTCGTCAGACTTGTCCTTGC and 35 cycles of (95 °C for 20 seconds, 60 °C for 20 seconds, 72 °C for 30 seconds) instead. Co-retention efficiency was calculated as C.R.E. =100% × No. MSTN$^+$ colonies / No. Internal control$^+$. To detect PuroΔTK integration in colony genomes, PCR with PuroΔTK specific primers was done instead.

Western Blotting. To detect *in vitro* transgenic expression prior to SCNT, 30 µl of crude protein extract from each nucleofection was boiled with 30 µl 2x Laemmli Sample Buffer (*BIO-RAD*) plus 3 µl Beta-

Mercaptoethanol for 10 minutes before chilling on ice. The chilled sample was spun in microfuge at maximum speed for 5 minutes and 25 µl supernatant was dissolved on a 7.5% SDS-PAGE minigel (*BIO-RAD*). Proteins were electro-transferred to PVDF membrane (0.45µm pore size) pre-activated in Methanol. The membrane was incubated in 1:750 diluted anti-Myostatin (M3448, Sigma-Aldrich) or 1:2500 diluted anti-Flag (F7425, Sigma-Aldrich) antibody solution overnight at 4 °C with gentle agitation after non-fat milk blocking. It was then incubated in 1:2000 diluted goat polyclonal to rabbit IgG - H&L (HRP) (ab6721, abcam) solution for 1 hour at room temperature with gentle agitation before signal detection with the Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and image was developed on Kodak Scientific Imaging film. For internal control, 1:1000 anti-beta actin (ab13772, abcam) was used as primary antibody instead. For detailed procedure and buffer recipes please refer to the Cell signaling Western Immunoblotting Protocol (http://www.cellsignal.com/support/protocols/Western.html).

Transgenic animal production. Fibroblast populations with qualities examined by co-retention PCR and Western blotting were mixed in equivalent representation and shipped to Minitube of America for embryo reconstruction. Pigs born from SCNT were maintained in Minitube pig facility under its Animal Welfare Assurance number #A4520/01.

Genotypic analysis of cloned piglets. Genomic DNA isolated from each aborted or liveborn piglet was used as template in two independent PCR reactions to detect integration of Myostatin cDNA and determine the promoter driving the cDNA. In PCR intended to detect cDNA in piglet genomes, the same primers designed for cDNA detection in co-retention PCR were used. PCR reaction mixes with 1 µl genomic DNA (1 ng/µl), 0.5 µl primer mix (20µM each), 12.5 µl 2X Mytaq Redmix (Bioline), and 11 µl ddH_2O were incubated in thermal cycler running program: 95 °C for 2 minutes, 35 cycles of (95 °C for 20 seconds, 56 °C for 20 seconds, 72 °C for 30 seconds), 72 °C for 5 minutes, 4 °C hold. The PCR reactions were then resolved on a 2% agarose gel. In PCR conducted to detect HSA sequence in piglet genomes, HSA specific primers were used instead in the reaction and the same incubation program was used. The positive and negative controls were made by mixing 10 ng WT genomic DNA and 3.33 X10^{-2} pg (+3 ctrl.), or 1.11 X 10^{-2} pg (+1ctrl.), or 0pg (WT ctrl.) of pKT2HSA-GVDG-F plasmid per µl of TE.

V. References

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Table 1. Cocktail recipes for all four independent nucleofections

	T2C-GVDG-F	T2C-D75A-F	T2HSA-GVDG-F	T2HSA-D75A-F	T2P-PTK	C-SB100X
1	5 µg	0	0	0	1 µg	2.5 µg
2	0	5 µg	0	0	1 µg	2.5 µg
3	0	0	6.4 µg	0	1 µg	2.5 µg
4	0	0	0	6.4 µg	1 µg	2.5 µg

To produce cell populations with ectopic expression of dominant negative Myostatin, pig fetal fibroblasts are transfected with one of the four cocktails of plasmids listed above; including one of the transposon plasmids encoding Flag tagged dominant negative Myostatin (column 2 to 5), transposon plasmid delivering PuroΔTK selection marker regulated by a PGK promoter (T2P-PTK), and plasmids for transient *SB* transposase expression (C-SB100X).

Abbreviations:
T2C-GVDG-F: pKT2C-MSTN-GVDG-Flag;
T2C-D75A-F: pKT2C-MSTN-D75A-Flag;
T2HSA-GVDG-F: pKT2HSA-MSTN-GVDG-Flag;
T2HSA-D75A-F: pKT2HSA-MSTN-D75A-Flag;
T2P-PTK: pKT2P-PTK;
C-SB100X: pKC-SB100X;
T2: Sleeping Beauty Transposon;
C: CAG, Chicken beta-actin promoter;
HSA: Human skeletal alpha-actin promoter was proven to be working in mice without leakage (77)but has not been tested in the pig in our knowledge.

Table 2. Primers used for expression cassette construction

5' CAG-GVDG	F	5' AATCTCGAGCCACCATGCAAAAACTGCAAATCTAT 3'
	R	5' AATGTCGACACCTTTTGGTGTGTCTGTTACCT 3'
3' CAG-GVDG	F	5' AAGTCGACGGTGATTTTGGACTCGACTGTGATG 3'
	R	5' TATTAGATCTCACTTATCGTCGTCATCCTTGTAATCTGAGCACCCACAGCGA 3'
5' CAG-D75A	F	5' AATCTCGAGCCACCATGCAAAAACTGCAAATCTAT 3'
	R	5' GGGCAAAAGTTGTCTTATAGCAGCTTTGCTAATGTTAGG 3'
3' CAG-D75A	F	5' CTGCTATAAGACAACTTTTGCCCAAAGCTCCTC 3'
	R	5' TATTAGATCTCACTTATCGTCGTCATCCTTGTAATCTGAGCACCCACAGCGA 3'
T2HSA	F	5' ATCCATGGCCACCATGCAAAAACTGCAAATCTAT 3'
	R	5' TATAGAATTCTCACTTATCGTCGTCATCCTTGTAATCTGAGCACCCACAGCGA 3'

Note: underline sequences are restriction sited added to facilitate cloning; sequences highlighted in grey are Kozak sequences added to ensure high level transcription; circled sequences encode Flag tag at the C-terminus;

Table 3. Co-retention PCR results on colonies from first round transfections

Expression cassette	Sex	Puro^R	Internal control$^+$	MSTN cDNA$^+$	C.R.E.
T2C-GVDG-F	♂	21	15	12	80%
T2C-D75A-F	♀	20	16	14	88%
	♂	36	31	24	78%
T2HSA-GVDG-F	♀	22	17	17	100%
	♂	47	40	39	98%
Total		146	119	106	89%
T2HSA-D75A-F	♀	4	3	3	100%
	♂	10	9	9	100%

The nucleofections and selections were not necessarily conducted simultaneously or using the same cell lines. Only samples with positive internal control signals were eligible for MSTN cDNA detection. The low numbers of colonies derived from nucleofections with pKT2HSA-D75A-F HSA-D75A were due to fungus contamination that killed a majority of colonies on the 96-well plates. However, the corresponding female and male populations were closely monitored and determined to be contamination free.

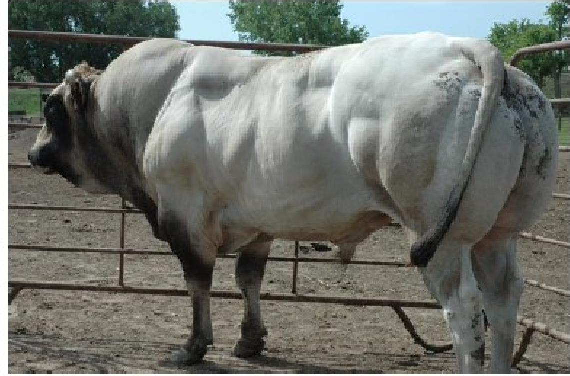

Figure 1. Double muscling cattle. A. Belgian Blue bull displaying the double muscling phenotype due the nt821del11 mutation in both alleles. Photo courtesy of Grobet et. al., Nat Genet. 1997 Sep;17(1):71-4. **B.** A full blood Piedmontese bull homozygous for the G938A or C313Y mutation has 30% more muscle than WT on average. Photo courtesy of www.piedmontese.org.

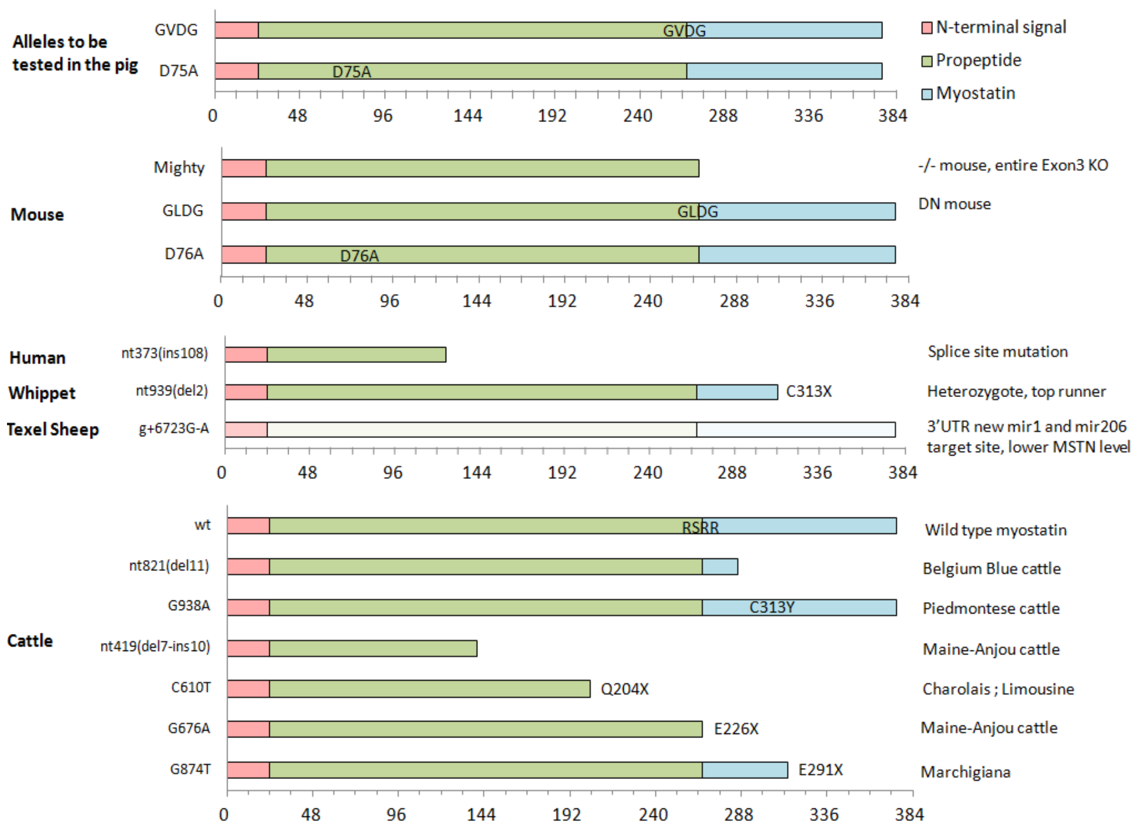

Figure 2. A diagram showing natural mutations in the Myostatin gene identified in human, Whippet dog, Texel sheep, and cattle with enhanced muscle growth; also shown are artificial disruptive mutations successfully demonstrated in mice, two of which will be tested in the pig (top of the chart). The horizontal axis of each cluster specifies the size of the peptides (N terminal signal in pink, propeptide in green and Myostatin monomer in blue) whereas the mutations responsible for hypermuscularity align along the left vertical axis. Specifically, Belgian Blue beef cattle homozygous for the 11 bp deletion at location nt821 in the coding sequence bear an out-of-frame early termination, losing 102 amino acids out of 109 of the C terminus (1, 3, 4). An nt938 G-A transition (G938A) is widely spread out in Piedmontese cattle which results in a Cystine to Tyrosine switch, disrupting the Cystine knot crucial for dimerization and receptor binding (1, 78). The other four mutations identified in Maine-Anjou (nt419 (del7-ins10), G676T), Charolais and Limousine (C610T), and Marchigiana (G874T) all end up with in frame stop codons yielding non-functional truncated proteins(5-7). In dog, Bully Whippets possess an allele with a 2 bp deletion at nt939, turning the Cystine at position 313 into a stop codon (8). Dogs homozygous for this mutation display an extremely muscular phenotype whereas heterozygotes often top in racing competitions. The hypertrophic Texel sheep is characterized by a G to A transition in the 3' UTR that creates a target site for mir1 and mir206, microRNAs (miRNAs) highly expressed in skeletal muscle and inhibitory to Myostatin translation (9). A newborn German boy with exceptional muscular appearance was diagnosed with splicing site mutation in intron 1 of both his Myostatin alleles (10). As a result, a 108 bp segment from intron 1failed to be spliced away, generating an early stop codon in the processed mRNA whose protein product loses the C-terminus completely. The three mouse models with disrupted Myostatin result either from bi-allelic knockout of the gene or deactivation of proteolytic sites (RSRR to GLDG or D76A; note that mice have one extra Met at the N terminus compared to pig or cattle). The latter two function as dominant negatives as well and are to be tested in the pig, RSRR to GVDG and D75A (GVDG was chosen over GLDG to create an endonuclease restriction to aid cloning and detection of transgenesis).

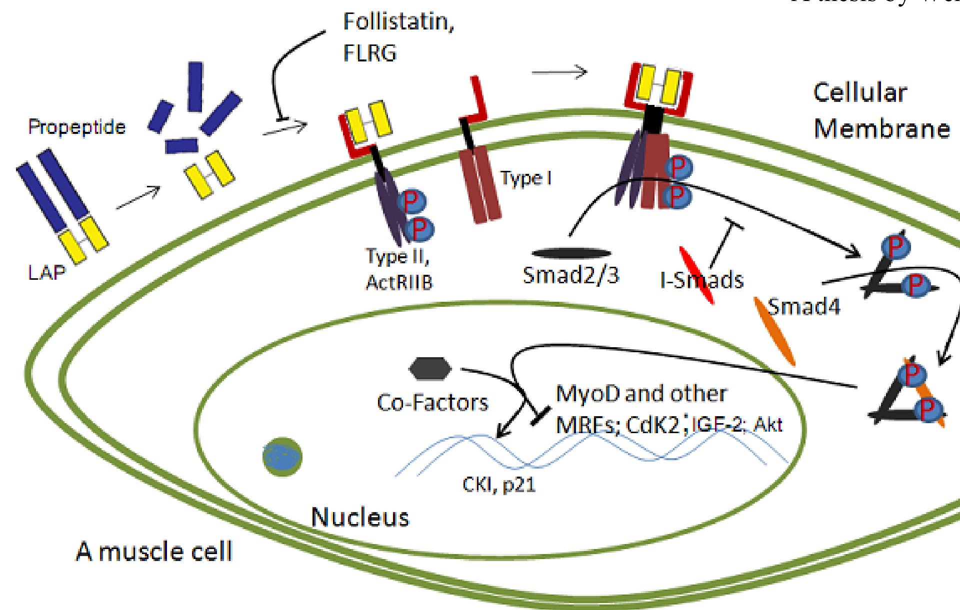

Figure 3. The Myostatin pathway regulates muscle development and growth. A typical TGFβ pathway involves the ligand (in this case, the Myostatin dimer in yellow), the Type I and Type II receptors, receptor-regulated SMAD proteins (R-Smad), Smad1-5, Smad8/9, inhibitory SMADs I-SMAD6-7, the nuclear co-factors, and the genes to be regulated. The binding of Myostatin to its Type II receptor ActRIIB occurs on the cellular membrane and bridges the Type II and Type I receptors, which dimerize to form heterotetramers (21, 79). Meanwhile, the kinase on the Type II receptor is activated upon binding and phosphorylates the Type I receptor, activating its kinase activity. Consequently, Myostatin specific R-smads in lieu of the receptor heterodimer, SMAD2 and SMAD3 are phosphorylated by the Type I receptor kinase. The phosphorylated SMAD2/3 complex then translocates to the nucleus bound by Co-SMAD or SMAD4. At the end of this pathway, gene specific co-factors in the nucleus bind to the heterotrimer complex and enable its recognition of genetic targets including CKI, p21, MyoD, CdK2, IGF-2, Akt and others, which are up- or down-regulated. This process is negatively regulated by the inhibitory I-SMAD7 who competes with SMAD2/3 for binding to the inner cellular domain of the Type I receptors.

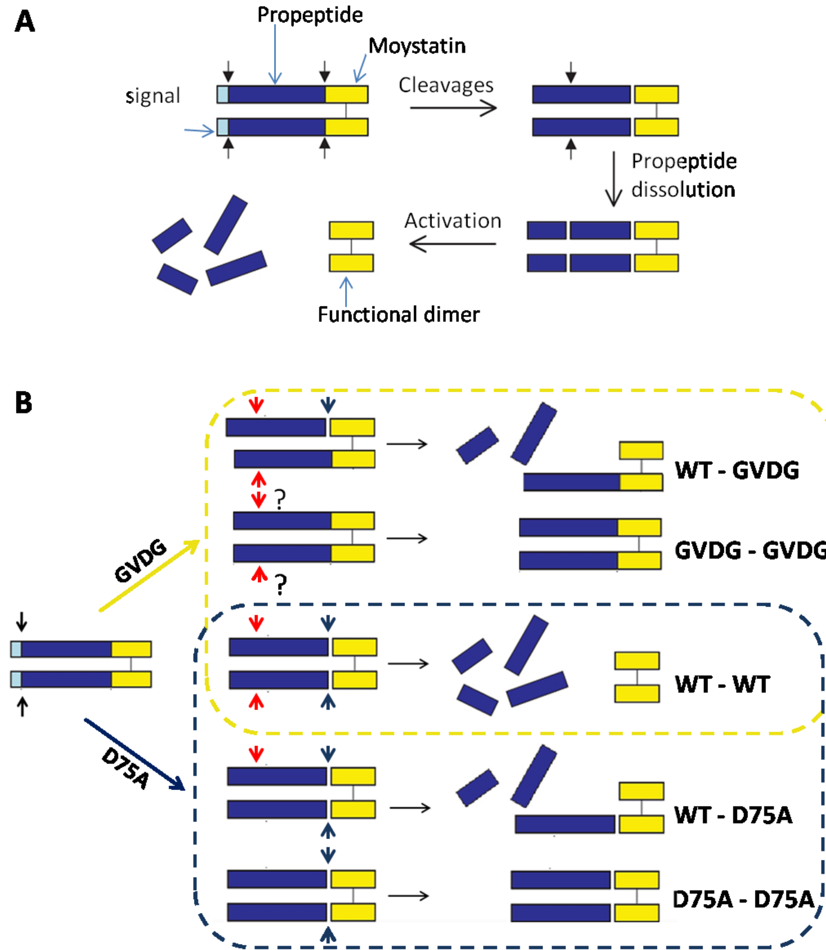

Figure 4. The processing of Myostatin precursor to generate mature dimers and mechanisms of dominant negative mutants in disrupting Myostatin function. A. After transcription, the precursor protein goes through three rounds of proteolytic processing (non-annotated short black arrows) while being secreted: removal of the signal peptide, separation of the propeptide and C-terminal Myostatin, and degradation of the covalently bound propeptide to release the functional Myostatin dimer from the LAP form to become active in receptor binding. Modified from Figure 3 of Se-Jin Lee, Annu. Rev. Cell Dev. Biol. 2004. 20:61-86. **B**. Although the dominant negative mutants to be tested in the pig have lost either the RSRR site (altered to GVDG) for proteolytic separation (blue arrow) of the propeptide and Myostatin or the 75th amino acid D critical for propeptide dissolution (D75A, A to C transversion, red arrow), they retain the ability to dimerize, with either themselves or the WT proteins. However, out of the three possible combinations of dimerization in each situation (encompassed by yellow or blue rounded rectangle), only WT-WT yields functional dimers, but the heterodimeric binding of mutants to WT monomers (WT-GVDG and WT-D75A) greatly diminish such combinations, thus exercising dominant negative effects. This figure was created in the style of Figure 3 from Se-Jin Lee, Annu. Rev. Cell Dev. Biol. 2004. 20:61-86 for better communication.

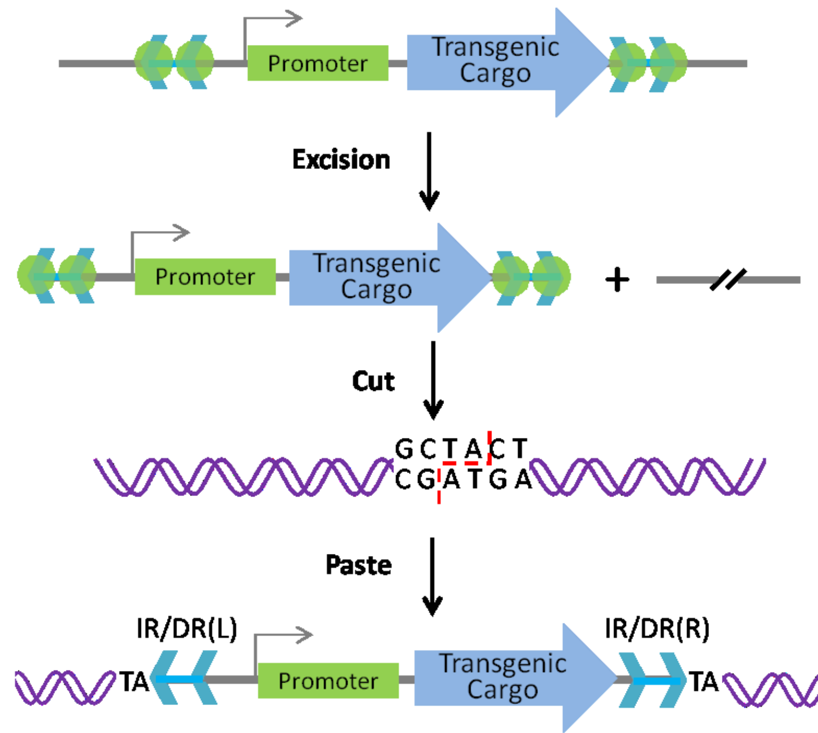

Figure 5. Mechanism of *SB* Mediated Transpositional Transgenesis. The *SB* transposase (light green circles) excise the engineered *SB* transposon from its original location after binding to the inverted terminal repeats with direct repeats IR/DR (L) and IR/DR(R) (colored in bright blue), which define the transposon. The transposases then recognize a TA dinucleotide in the target DNA (purple double helix) nearly randomly, generate a break (cut), and integrate the transposon into this target location (paste). During the transposition, the expression cassette consisting of a transgenic cargo (blue arrow) driven by a promoter (green rectangle) is transposed between two locations and the TA dinucleotide is duplicated in the target locus.

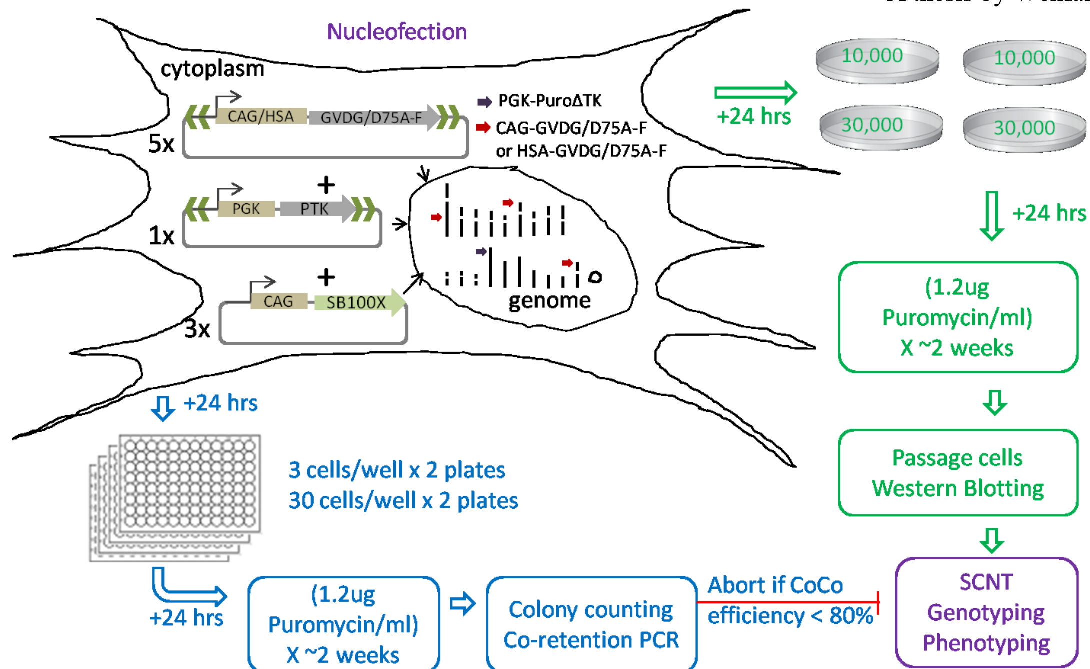

Figure 6. A flowchart depicting the procedure to generate transgenic pigs with ectopic expression of dominant negative Myostatin (GOI, gene of interest) using CoCo. Low passage pig fetal fibroblasts are transfected with plasmid mixes listed in Table 1, random integration of the selection marker (PTK) and GOI into the pig genome are represented by black and red arrows in the nucleus. 24 hours after nucleofection, cells are split and undergo two paths, CoCo efficiency quality control (blue), and preparation of clonable populations (green). For quality control, cells are plated on 96-well plates at densities shown and supplemented with untransfected cells to reach 1000 cells/ well density. In about two weeks of drug selection in Puromycin, resistant colonies reach confluency and are harvested for co-retention PCR to detect integration of PTK and GOI. If co-retention efficiency= (GOI^+ colonies) / (internal control+ colonies) < 80%, the experiment is aborted before SCNT. To prepare potential populations for SCNT, cells are plated on 10 cm dishes at densities shown and selected under Puromycin for about 2 weeks to reach confluency. These populations are then passaged and crude protein is extracted from each for Western Blotting to detect transgenic protein expression if applicable. If the cells pass the CoCo efficiency quality control and western blotting results are agreeable, they become nucleus donors for production of transgenic pigs with ectopic expression of the GOI through SCNT. When piglets are born, they are genotyped for the transgenes and phenotyped for their expression and effects on muscle growth.

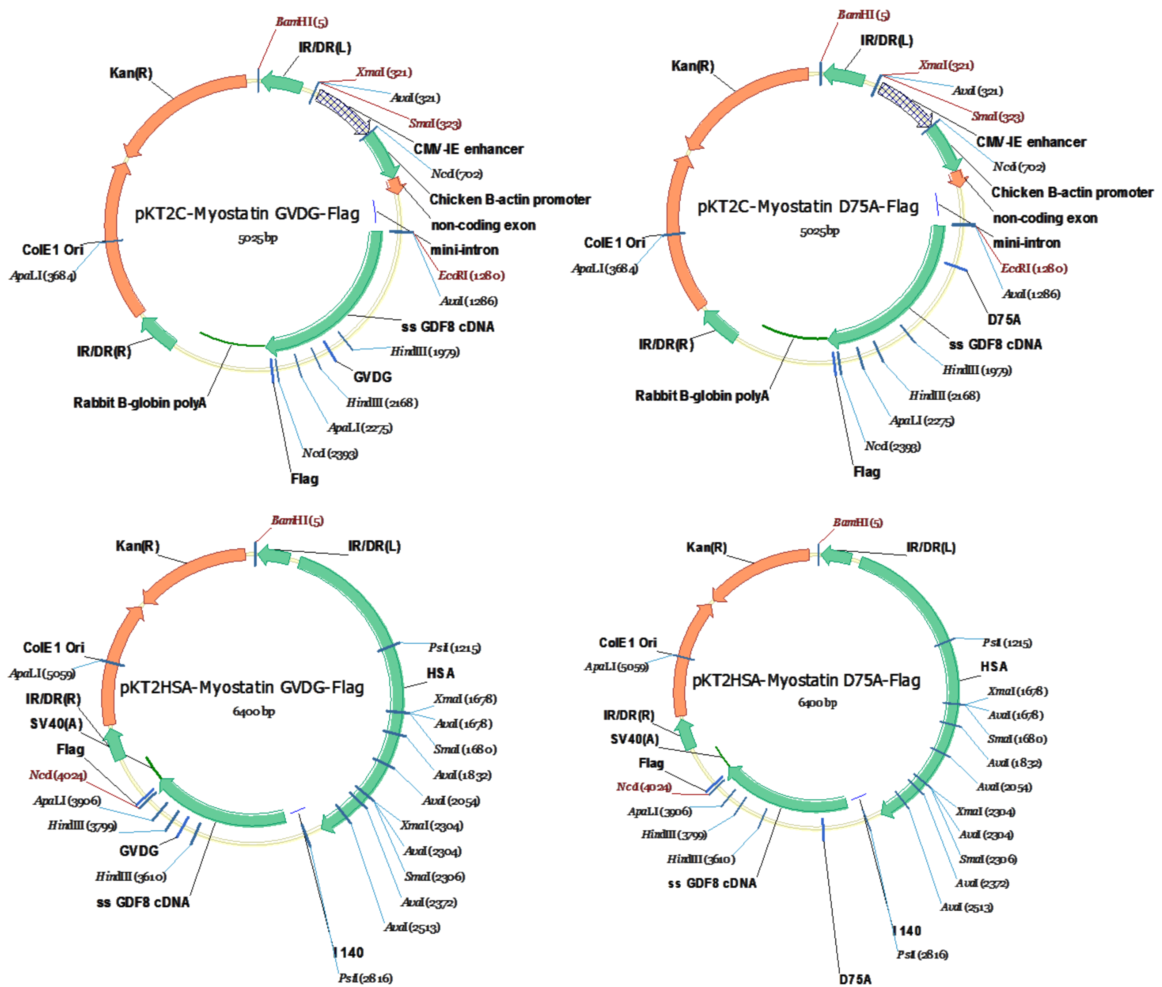

Figure 7. Physical maps of the transposon plasmids delivering cassettes that express the dominant negative alleles of Myostatin. Pig Myostatin cDNA (ssGDF8 cDNA) bearing one of the mutations lays downstream of either Chicken beta-actin promoter (CAG) or human skeletal actin promoter (HSA) and upstream of a polyadenylation signal (Rabbit B-globin polyA or SV40(A)). The expression cassettes are flanked by short green arrows representing IR/DR (L) and IR/DR(R) of *SB* transposon. All cDNA mutants are tagged with Flag epitope at the 3' or C-terminal of the peptide product to aid detection of transgenic expression.

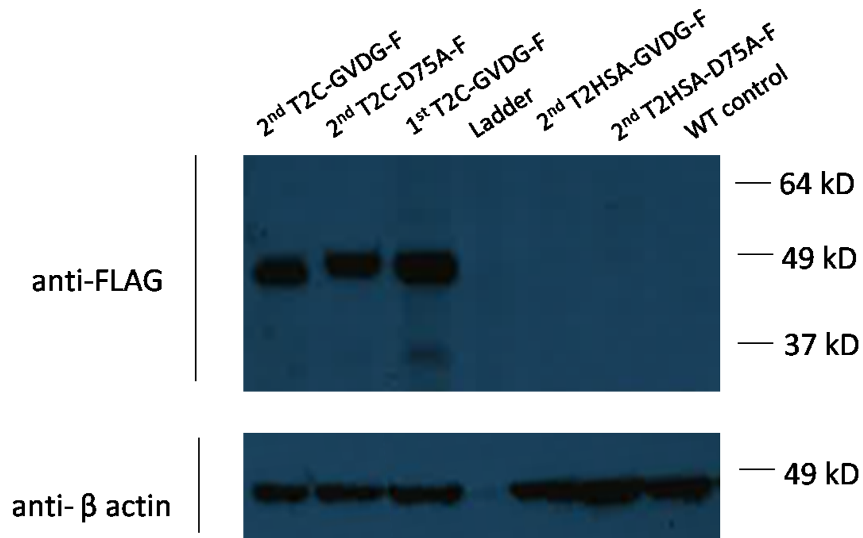

Figure 8. Western blotting results on crude protein extracts from first and second round transfections. All transfections tested here used male fibroblasts and transfected DNA mixes were listed in **Table 1.** The top image was obtained by hybridization with anti-FLAG antibodies to detect transgenic Myostatin expression. The bottom images were obtained by anti-β actin antibody treatment to the same protein extracts as internal control. Please refer to legend of **Table 1** for abbreviations. Protein extract from untransfected WT cells was used as negative control.

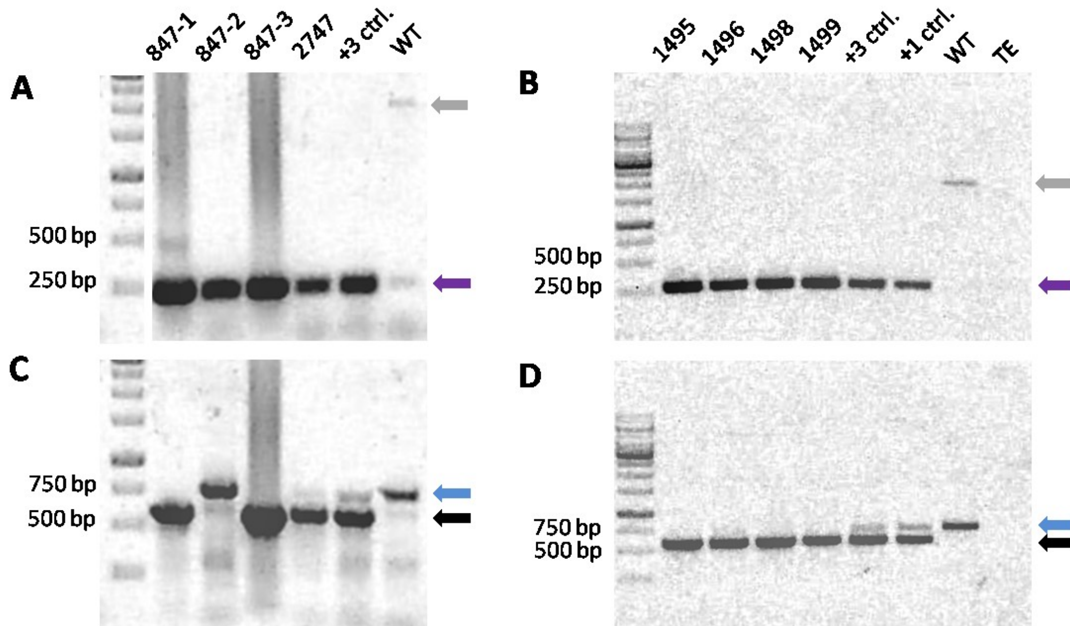

Figure 9. Genotyping results from PCR on piglets generated by SCNT using Round 2 male fibroblast populations as nucleus donors. A, C) results of PCR on genomic DNA samples from four dead piglets of two aborted late term pregnancies; **B, D**) results on four live-born pigs of one pregnancy. The top two gel images (**A, B**) show results from PCR enriched for Myostatin cDNA amplification using forward primer that binds to Exon 1 sequence and reverse primer to Exon 2 sequence. The purple arrows point to PCR products of cDNA amplification and the grey arrows indicate amplification of endogenous genomic DNA spanning Exon 1, Intron 1 and Exon 2. The bottom two images (**C, D**) display amplification results from PCR using primers targeting the HSA sequence. The black arrows specify PCR products from HSA amplification and the blue arrows point to unspecific amplification possibly from highly homologous endogenous genomic sequence. Pig IDs and control conditions are shown on top of gel images; +3 ctrl. and +1 ctrl. equal to PCR results using 3 copies or 1 copy of pKT2HSA-GVDG-F plasmid per WT cell as template.

Chapter 3 Targeting LDLR and Myostatin with Recombinant Adeno-Associated Virus in Swine

Targeting LDLR and Myostatin with Recombinant Adeno-Associated Virus in Swine

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Author contributions: WT designed and generated the targeting constructs, packaged the viruses, conducted the transductions, drug selections and screenings, and wrote the manuscript; DFC participlated in the screening for LDLR knockouts, made Figure 3 - 5, contributed to the writing of the "Results" section for LDLR, and conducted the Southern blotting; SCF supervised the study.

Recombinant Adeno-associated Virus has been harnessed as homologous templates for gene targeting with an efficiency ranging from 10^{-4} and 10^{-2} in various organisms. In this study, we aimed to use it to knockout the LDLR gene in pig to model familial hypercholesterolemia and atherosclerosis. We also attempted to introgress the D75A dominant negative allele to study porcine Myostatin biology and promote muscle growth. After several rounds of infection, selection and screening, we obtained targeted fibroblast colonies for both projects with efficiency between 7×10^{-6} to 2×10^{-4}.

After sequencing the Myostatin targeted colonies, we found that although the D75A SNP was placed only 198 bp away from the selection cassette, half of the targeted colonies did not harbor the SNP, indicative of short conversion tracks during recombination or sister chromatin exchange in these cases. Several factors affected the efficiency of gene targeting; the plating density on 96-well plates for colony formation had the biggest impact on our ability to detect targeted clones and isolate monoclonal colonies. According to *Poisson* distribution, plating cells to reach a 35% rate of confluency on 96-well plates is optimal for screening, since it minimizes the workload and maximizes the chance of obtaining monoclonal clones.

I. Introduction

One of the earliest high frequency gene targeting was achieved through recombinant Adeno-Associated Virus (rAAV) mediated homologous recombination (HR) in human fibroblasts (1). Compared to conventional gene targeting templates such as plasmids, the T shaped and hairpin-structured inverted terminal repeats (ITR) of the AAV vector promote its trafficking to the nucleus and stabilize the vector in the cellular environment. Besides, its single-stranded DNA genome enables direct pairing with the target sequence. All these features likely have contributed to the observed higher targeting efficiency ranging between 10^{-4} to 10^{-2} of total selected cells (1-3), compared to the 10^{-6} to 10^{-5} range obtained by plasmid templates (4-6).

rAAV mediated gene targeting has since been used to target various genes for medical research and applications. For example, it was utilized to knock out a mutated COL1A1 allele that is responsible for Osteogenesis Imperfect in patient specific mesenchymal stem cells and correct trisomy 21 Down Syndrome in patient derived iPS cells (2, 7). AAV gene targeting has also been harnessed to generate animal models for diseases, e.g. ferrets with knocked out *CFTR* gene to model cystic fibrosis (8) and bi-allelic disrupted *CFTR* pigs for the same disease (9). Phenotypic analysis of both animals revealed manifestation of a wide range of disease symptoms that are typical in the human condition (10-13). Among the several serotypes of AAV that have been developed for genetic manipulation, serotype 2 (AAV-2) is more often used and successful in gene targeting.

Relying on mouse models, we have gained substantial understanding in the pathophysiology of many diseases and developed effective treatment plans. However, for certain diseases or conditions, mouse models might not recapitulate the main spectrum of the conditions, e.g. cystic fibrosis (14, 15) and some inflammatory diseases (16). This calls for the development of large animal models. Owing to the high resemblance with humans in physiology, genetics and anatomy, the pig could provide information that is more inferable in general, not just for the previous conditions. Besides, pigs have biological advantages that make them more suitable for disease modeling than other large animals including cattle and sheep: pigs are multiparous, yielding 8-14 offspring per gestation instead of 1-2 calves per cow or ewe. In addition, pigs reach sexual maturity a lot earlier than the ruminants and the gestation period is shorter as well.

Low density lipoprotein (LDL) receptor (LDLR) is a membrane protein that mediates the endocytosis and clearance of cholesterol-rich LDL from the bloodstream. Abnormity in cholesterol metabolism and subsequent accumulation of cholesterol is directly linked to the development of atherosclerosis and cardiovascular conditions. This receptor is encoded by the

LDLR gene and over 900 mutations found in this gene are direct causes for familial hyper-cholesterolemia (17-21), which often leads to atherosclerosis and cardiovascular problems. Monogenic hypercholesterolemia caused by mono-allelic mutations in the *LDLR* gene is prevalent in 1 in 500 persons worldwide (20), but some of its pathophysiology is still unknown.

We hoped to develop genetically modified pigs to facilitate the study of and treatment for familial hypercholesterolemia and atherosclerosis byknocking out the *LDLR* gene through rAAV-2 mediated gene targeting. The targeting strategy is depicted in **Fig. 3A**. Theoretically, when the Neomycin selection marker is incorporated, the *LDLR* open reading frame (ORF) would be disrupted and suffer an early termination, rendering its ability to produce functional protein. With the aid of somatic cell nuclear transfer (SCNT), successful *LDLR* targeting *in vitro* could be transferred into pigs that are expected to manifest symptoms of the modeled diseases.

With a similar rAAV targeting approach, we attempted to introgress the D75A SNP into the Myostatin gene in the pig. Instead of ectopically express this dominant negative allele, we decided to replace the wild type gene with this SNP (**Fig. 6A**). This not only eliminates endogenous protein products with a normal inhibitory role in muscle development but also the expression of the dominant negative SNP is under control of the endogenous promoter achieving physiological levels of expression. Such a strategy might translate into a bigger boost in muscle growth and be more beneficial to the animal's health.

With the assistance from AAV, we were able to achieve *LDLR* knockout and D75A introgression at efficiency up to 10^{-4}. We initially had difficulty isolating monoclonal targeted *LDLR* clones because the densities we plated the transduced cells were too high on 96-well plates, such that targeted clones were confounded by "bystanders", colonies resistant to Puromycin due to random integrations instead of gene targeting. To our surprise, over one half of the Myostatin targeted clones were absent from D75A integration, suggesting a conversion track shorter than 198 bp or sister chromatin exchange post targeting.

II. Results

Targeting of LDLR

By inserting a PGK-Neo selection marker into Exon 4 of *LDLR*, we generated a HR cassette (**Fig. 1**) that was packaged into recombinant AAV (rAAV-LDLR E4-Neo) to replace endogenous *LDLR*, so that it would result in a truncated and non-functional protein product (**Fig. 3A**). The general timeline of our targeting procedures is summarized in **Figure 2**. To begin with, male primary fetal fibroblasts reaching complete confluency on a 6-well plate were incubated with 5,

25, 100, and 150 µl (per well) rAAV-LDLR E4-Neo viral supernatant, before being plated onto five 96-well plates at the density of 2000 cells/well from each condition. These cells were initially selected with 250 µg/ml G418 with a subsequent boost to 300 µg/ml six days later.

After a total of two weeks of selection, almost all the wells became 100% confluent from the 25, 100 and 150 µl transductions whereas approximately half of the wells from the 5 µl transduction were confluent, suggesting that some of these wells are monoclonal compared to 2-6 colonies per well in the previous conditions. Unfortunately, 3' junction PCR that would only yield a product from recombined alleles (**Fig. 3A**) did not detect any positive wells from the 5 µl plates, suggesting that homologous recombination had occurred in less than 1 in 250 G418 resistant colonies. We next screened cells from the 25 µl transduction plates and despite the likelihood of multiple colonies per well, we detected positive signal from 26 of the confluent wells (**Fig. 3B**). Signal intensity of the PCR varied significantly among positives and likely reflected the proportion of correctly targeted cells versus resistant "bystanders". In total, 7 of the 26 positives had strong signal and were sampled for whole genome amplification (WGA) and Southern blot analysis.

A second round infection was conducted to both male and female PFF around 40-50% confluency with 100 µl of the same viral supernatant. Transduced cells were plated at densities of 100 cells/well, 200 cells/well, and 500 cells/well with five replicates for each density and sex, supplemented with wild type cells to reach 1000 cells/well final density. After selection with 300 µg/ml G418 for two weeks, resistant colonies appeared in approximately 30 to 50 wells on each of the 100 cells/well and 200 cells/well plates (**Table 1**) while greater than 90 percent of wells in the 500 cells/well plates contained confluent cells, indicating that colonies from the lower densities were optimal for screening because more monoclonal colonies were expected to reside. Thus primary 3' junction PCR screening was performed on the 100 cells/well and 200 cells/well plates only and we identified 2 and 11 positive wells for male and female cells respectively (**Table 1**). The healthy (1 male and 7 female) colonies were cryopreserved and a portion were set aside for WGA/Southern blot analysis.

Subsequent PCR of both 5' and 3' junctions (**Fig. 4A**) from WGA DNA derived from the 7 positives of Round one and these 8 positives of Round two revealed positive signal in 3 of Round one positives and 1 and 5 of the Round two male and female colonies respectively (**Table 1** and **Fig. 4**). Identity of the junction PCR was confirmed by restriction digests with enzymes that would yield unique patterns for targeted and otherwise (**Fig. 4B, C**). Finally, the 15 positive clones from Round one and two were further analyzed by Southern blotting (**Fig. 5**). By using a

α-^{32}P labeled probe that hybridized to the region shown in **Figure 3A**, a WT internal control band was obvious for every colony tested. However, only six of them were also positive for the 4 Kb targeted band, namely colony No. 6 from Round one and No. 8, 10, 11, 13, and 15 from Round two. Furthermore, in pure heterozygous knockout colonies, we would expect a 50:50 ratio of intensity between targeted and wild type bands, but this only appeared to be the case for colonies 8, 10, 13, and 15, all from Round two.

Targeting of Myostatin

We adopted a similar strategy and procedures to introgress the D75A SNP into Exon 1 of the Myostatin gene in PFFs, by rAAV mediated HR with one major exception. Instead of inserting the PGK-Neo selection marker into the coding sequence, we placed it in the intron to avoid disruption of the Myostatin ORF (**Fig. 1 and 6A**). After packaging the HR cassette into AAV, we used the virus to infect both female and male PFFs. After two weeks of selection in 300 µl/ml of G418, we obtained 30 to 50 confluent wells from each 96-well plate that initially seeded with 100 cells/well or 250 cells/well (**Table 2**).

By conducting 3' junction PCR on samples from these wells, we identified 32 male and 24 female wells that yielded PCR products of the right size (**Table 2, Fig. 6B**). However, the intensities of the bands also varied and only 12 male samples and 11 of the female candidates produced strong bands as pointed out in the figure. We then cryopreserved these colonies, derived WGA samples from them and conducted both 5' and 3' junction PCR to further characterize the identities (**Fig. 7A**). 10 out of the 12 male candidates and 9 out of the 11 female candidates remained positive and their 3' PCR products were verified by *NCO*I digest; No. 4,5,17, 19 were eliminated due to lightness of the PCR bands. We did not screen the 625 cells/well plates because 60% of the wells were confluent, suggesting that the wells might harbor multiple colonies.

We then sequenced the 5' junction PCR products from 18 of the remaining candidates with strong products. To our surprise, even though they were all determined as targeted by three rounds of junction PCR, clearly only seven of them clearly incorporated the D75A SNP, although the SNP was placed merely 198 bp away from the left border of the selection marker (**Fig. 7D**). Eight of them did not contain the SNP at all thus deemed worthless and the remaining three appeared to be heterogeneous, the double peaks in the chromatogram indicated that some of the targeted alleles underwent DNA exhange with the wild type untargeted sister chromatin.

III. Discussion

Recombinant AAV mediated targeting enabled us to achieve gene knockout and introgression at frequencies up to 2 x 10^{-4}, at least a 10-fold enhancement over plasmid templates (4). The most apparent limitation of rAAV is its 4.9 Kb maximum cargo capacity to ensure optimal packaging and ability to infect (22); but compared to plasmid templates, rAAV could still be favorable in gene transfer or targeting because it might more efficiently avoid insertional mutagenesis from concatemers which are often concomitant with plasmids. However, random insertions are still prevalent because only 0.48% to 19.4% of G418 resistant colonies were identified as HR positive by primary junction PCR in our experiments (**Table 1 and 2**), and a good percentage of these candidates were eliminated due to non-targeted "bystanders" containing random integrations.

Multiple factors affect the AAV targeting efficiency, especially the design of the targeting vector, the cellular source and the drug selection strategy. We discovered that the reason why our previous attempts to target Exon 4 of *LDLR* were unsuccessful was due to illegitimate recombination at a repetitive element in Intron 4 of this gene. We then redesigned the AAV construct that avoided those repetitive elements around Exon 4 and achieved targeting. Thus, while designing the targeting vectors, it is helpful to pay closer attention to the target sequences and chromosomal location of choice to avoid negative positional effects that include repetitive elements and silenced regions etc (23, 24).

We chose fibroblasts to interrogate the gene targeting and introgression. These are readily available cells that are actively dividing; a feature that seems to be a prerequisite for high targeting efficiencies (1, 25). Maybe the phase of the cell cycle influences AAV targeting efficiency (26) and since the degree of confluency or cell contact affect the cycle stage of plated fibroblasts (27, 28), it might make sense to determine the optimal density for targeting or synchronize cells at a certain stage. AAV targeting efficiency could also be cell line dependent. We later conducted a third transduction to knockout *LDLR* using mixtures of five male PFF lines or four female PFF lines of similar passage. 5' and 3' junction PCR detected much higher rates of positive colonies, from 0.48% and 2.7% per selected female or male PFFs to 7.48% and 3.6% respectively. However, no subsequent analysis was performed to determine whether there was a bias towards certain cell lines in the candidates.

Studies including work with rAAV have proven that double strand breaks (DSB) in the target genome dramatically promote gene targeting (29-31).With the rapid development of site-specific endonucleases that can efficiently generate a DSB in almost any desired sequence, such as Zinc Finger Nucleases (ZFNs) (32, 33), Transcription Activator Like Effector Nucleases

(TALENs) (34, 35) and CRISPR/Cas9 (36-38), these enzymes could potentially be powerful to substantially enhance AAV mediated gene targeting by pre-treating the cells and generating a DSB.

To effectively obtain monoclonal targeted colonies, cells should be plated at an optimal density on the 96-well plates to maximize the chance of a G418 resistant well to be monoclonal. Since the distribution of resistant cells that give rise to colonies among wells follows the *Poisson* distribution, we can estimate the numbers of monoclonal versus polyclonal resistant wells on a certain plate based on the rate of confluency (R) after G418 selection. Let X be the estimated colony number in a certain well from a 96-well plate with a confluent wells (hence $R = a/96$), then the probability of that well to have k colonies can be calculated as:

$$\mathrm{P}(\mathrm{X} = \mathrm{k}) = \frac{\lambda^k e^{-\lambda}}{k!} \quad (\mathrm{k} = 0, 1, 2 \ldots \mathrm{n})$$

We already know that $\mathrm{P}(\mathrm{X} = 0) = \mathrm{e}^{-\lambda} = 1 - \mathrm{R}$, thus, the probability for a well to be monoclonal is $\mathrm{P}(\mathrm{X} = 1) = (R - 1)\ln(1 - R)$ or polyclonal $\mathrm{P}(\mathrm{X} \geq 2) = R - (R - 1)\ln(1 - R)$. So the estimated number of monoclonal or polyclonal wells from this plate would be:

$$Mono. = 96(R - 1)\ln(1 - R)$$

and

$$Poly. = 96(R - (R - 1)\ln(1 - R))$$

respectively.

As shown by the scatter pot generated with number points calculated from the formulas (**Fig. 8**), the number of polyclonal wells ($Poly.$, blue circles) goes up steadily when R increases or more wells are confluent. However, the number of monoclonal wells ($Mono.$, purple triangle) increases initially but starts to plateau when R is around 30-35%. The corresponding plating density might be optimal for HR screening because a lower rate would require more plates to screen while a higher rate would dramatically raise the risk of identifying candidates confounded by "bystanders". This might explain why the majority of *LDLR* knockout candidates from Round one failed to remain positive (**Fig. 5.**): those wells were seeded with a high density (2000 cells/well) and R was close to 1, most of the confluent wells were polyclonal according to the plot. Even though the junction PCR was sensitive enough to pick up low copy numbers of recombined alleles, Southern blotting was not when the majority of genomes were wild type (**Fig. 5**). By the way, Southern blotting would also be necessary to determine the wt: targeted ratio in those D75A candidates.

When we designed the vector to introgress D75A, we placed the SNP in maximum proximity to the selection cassette; however, the sequencing results claim this does not guarantee introgression (**Fig. 7D**). Around one half of the targeted colonies did not integrate the SNP, for them the conversion track between the genomic sequence and the HR template was probably smaller than 198 bp, the distance from the SNP to the selection marker. Another possibility is a second recombination between the recombined allele and the wt sister chromatin. However, we did take into account the possibility of spliceosome disruption by putting the selection marker 50 bp away from the exon-intron junction to avoid it; however, the possibility still exits. Ways to settle the possibility could be to make animals from these targeted cells and examine splicing or to eliminate the marker by Cre-loxP mediated recombination.

The net change to the targeted genome after Cre-recombination is the SNP and one copy of loxP, no selection marker is retained. Several ways might achieve the goal, one is to crossbreed animals cloned from these targeted cells with a pig line that express the Cre-recombinase, hoping that the progenies are marker-free and transmit this feature through their germline. However, since Cre-expressing animals might not be available and the crossbreeding cannot guarantee removal, one could try to achieve marker elimination *in vitro* prior to SCNT. Cre-recombinase fused to a TAT domain and a nuclease localization signal (TAT-NLS-Cre) is cell-permeable and can re-localize to the nucleus to induce loxP mediated recombination. This has been demonstrated in various cell types and embryos with efficiencies ranging from 70% to 100% (39, 40).

IV. Methods

Construction of the targeting vectors. To build the pAAV-MSTN E1-D75A-Neo vector, two touchdown PCR was conducted to amplify segments for the left HR arm with the D75A point mutation from pig genomic DNA, using primer sets ATGCGGCCGCTCCTCAAATGTTTGTCTAAATAATG (forward) + GGGCAAAAGTTGTCTTATAGCAGCTTTGCTAATGTTAGG (reverse) and CTGCTATAAGACAA-CTTTTGCCCAAAGCTCCTC (forward) + GCTCCAGCTTTTGTTCCCTTTAGGAACAACAGTCAGC (reverse). Then a fusion PCR was performed to obtain full length left HR arm with the point mutation using the underlined primers and PCR products from above. To obtain sequence for the right HR arm, another touchdown PCR was conducted using primer set CGCCCTATAGTGAGTCGTATTACCAG-TGTTTATGAGAAACAGATC (forward) + ATGCGGCCGCAGCAATGTGGATACTACAG (reverse). A second fusion PCR was performed using the primers highlighted in green and products from the first fusion PCR, the right HR arm and pNeDaKO Neo plasmid (Addgene ID 16413) as templates. The final segment from this fusion PCR was TOPO cloned, sequenced, digested with *Not*I (NEB) and cloned into the pAAV-MCS vector (Agilent) between the *Not*I sites replacing the original segment in the vector. This final targeting vector was confirmed by restriction digests.

To build the pAAV-LDLR E4-Neo vector, regular PCR was conducted to amplify the full length HR arm sequence from a segment identified in our swine BAC genome library, using primers TAAGCGGCCGCCTCTTGTATAGACATGTCTTTCA (forward) + TATGCGGCCGCTAGTCAGATT-CGTTAACCACTG (reverse). The gel isolated 1.2 Kb PCR product was digested with *NotI* restriction enzyme directly and cloned into the pAAV-MCS vector (Agilent) between the *NotI* sites, replacing the original insert. Afterwards, a 1.9 Kb fragment containing the PGK-Neo cassette flanked by loxP sites was prepared by digesting plasmid PL452 with *Xho*I and inserted into *LDLR* Exon 4 in the previous intermediate vector at the *Xho*I site. This final vector was then restriction digest confirmed and sequenced before packaging. All the PCR amplifications were carried out using Platinum taq HIFI polymerase (Invitrogen).

AAV packaging[3]. AAV-293 approaching 70% confluency on a 10 cm dish was transfected with 5 μg of each of the following plasmids, pAAV-RC, pAAV-helper, and pAAV-MSTN E1-D75A-Neo or pAAV-LDLR E4-Neo, using *Trans*IT-LT1 (Mirus). After two days of incubation, cells were scraped off the plate and transferred into a sterile 1.5 ml microcentrifuge tube in 1 ml of the culture media. After three freeze-thaw cycles, cell debris was pelleted in a microfuge at maximum speed for 3 minutes and the supernatant was applied directly to PFF cells or frozen at -80 °C for future transductions. Please refer to the "Methods" section of Chapter 5 and the Agilent online protocol (http://www.chem-agilent.com/pdf/strata/240071.pdf) for more information.

AAV transduction[4]. Low passage PFF cells were seeded on 6-well plates and infected when they reach around 40%, 70% or 100% confluency; media change was performed with 1 ml pre-warmed fresh media 2 hours ahead of transduction. Then 25 μl, 100 μl, or 150 μl of viral supernatant was added drop-wise to the PFF cells in culture and gentle rocking motion was applied to the plate to evenly distribute viral particles. 2 hours post infection, 3 ml of pre-warmed media was added to the culture and cells were incubated for 24 more hours before seeding on 96-well plates for colony formation.

Tissue culture and colony formation. AAV-293 cells and PFFs before drug selection were cultured in standard DMEM media supplemented with 10% FBS, 100 I.U./ml Penicillin and Streptomycin, and 2mM L-Glutamine (*GIBCO*). After selection has initiated, G418 was added to the media at a concentration of 300 μg/ml; media change was performed every 3-4 days. All cells were maintained at 37 °C in 5% CO_2.

To plate cells on 96-well plates, infected cells were trypsinized and seeded at densities of 2000 cells/well or 100, 250, 625, 1000 cells/well supplemented with uninfected cells to reach 1000 cells/well final density. G418 was added one day after plating and maintained until cryopreservation. When resistant

[3] The author would like to thank Mr. Brian Ruiz from Professor Eric Hendrickson's lab for sharing the plasmids for AAV packaging and teaching me how to package recombinant AAV viruses.

[4] Professor Scott McIvor shared his lab space with us to conduct our initial AAV targeting work before we obtained our own license to use AAV.

colonies emerged and reached 100% confluency, each was split: one-half for potential cryopreservation and the other half lysed for genotypic analysis.

After identified as HR candidate by junction PCR and restriction digest analysis, the colony was further expanded on a 24-well plate until confluency. One half of the cells were cryopreserved in liquid nitrogen for potential cloning in 1X freezing media containing DMEM supplemented with 20% FBS (*GIBCO*) and 10% DMSO (Sigma-Aldrich); the other half was used as template in whole genome amplification (WGA) for Southern blotting.

Sample preparation. To prepare DNA samples from Neomycin resistant colonies for PCR screening, one replica from each resistant colony was lysed in 25 µl of 1X PCR compatible lysis buffer containing 10 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.45% Tryton X-100(vol/vol), 0.45% Tween-20(vol/vol) freshly supplemented with 200 µg/ml Proteinase K. The lysates were processed in a thermal cycler running the following program: 55 °C for 60 minutes, 95 °C for 15 minutes, 4 °C hold. Cells harvested from HR candidate colonies were used in WGA using the REPLI-g Midi Kit (Qiagen). The manufacturer's instructions were closely followed.

5' and 3' HR junction PCR. 1 µl of each colony lysate was used as template in 5' or 3' junction PCR to identify HR candidates for further characterization. The primer pairs used in the screen are listed in **Table 3** and were added to 1X Mytaq Redmix (Bioline) with the templates to make complete reactions which were then resolved on 1% agarose gels. The intensities of HR bands from the samples were compared to those from 1500, 150, 15 or 0 copies of positive control plasmids with longer homologous sequences than the targeting vectors.

Restriction digests of junction PCR products. To verify the identities of junction PCR products, they were digested with selected restriction enzymes that were expected to result in unique patterns of cutting. Usually, 8 µl of PCR product and 3 to 6 I.U. of enzymes were included in a 20 µl digestion mix, which was incubated at 37 °C for 2 hours before being resolved on 1% agarose gel. *Nco*I, *EcoR*I, or *Xho*I was used to digest 3' junction PCR products amplified from D75A candidates, 5' or 3' junction PCR products from candidates with LDLR knockout respectively.

Southern blotting. To confirm the candidate LDLR knockout colonies and determine whether they were monoclonal, Southern blotting was conducted on WGA products from the samples. Briefly, 10 µg of WGA DNA was digested with 40 I.U. of *EcoR*I at 37 °C for 4 hours before being resolved by a 0.7% agarose gel running at 32 V overnight. The cross-linked DNA was then transferred via capillarity to positively charged nylon. The probe was labeled with α-^{32}P by amplifying the segment shown in **Figure 5A** with the Klenow fragment primed with a random Hexamer. Standard blocking, hybridization and washing procedures were followed and image was produced by phosphorimaging.

Sequencing and analysis. 5' junction PCR was conducted on 3' junction PCR identified colonies targeted with the MSTN E1-D75A-Neo cassette using primers GGAAACTGAGCACGATTTTCACG (forward) + CAGGACGTGACAAATGGAAGTAGCA (reverse). Sanger sequencing was performed on cleaned up

PCR products with the reverse primer. Multiple alignments were performed using Vector NTI software (Invitrogen) to analyze the sequencing results.

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Table 1: LDLR Targeting Frequency (second transduction)

Plating density (cells / well)	Sex	Total seeded wells	Neo^R colonies (% selected)	3' junction PCR positive (% colonies)	5'-3' PCR/RE positives (% colonies)	Targeting frequency (HR/selected)
100 or 200 †	♂	96*10=960	410 (0.28)	2 (0.48)	1 (0.24)	6.94×10^{-6}
100 or 200 †	♀	960	333 (0.23)	9 (2.7)	5 (3.78)	3.47×10^{-5}

A Neo^R colony is defined as one well of a 96-well plate containing G-418 resistant cells, regardless whether they are monoclonal or polyclonal;
PCR/RE stands for restriction digest of 5' or 3' junction PCR products;
Targeting frequency here is calculated by dividing PCR/RE positives with total plated cells.
† Combined results from 5 plates with 100 cells/well and 5 plates with 200 cells/well.

Table 2. Results of PCR screening for HR positive colonies in D75A targeting

Plating density (cells / well)	Sex	Total seeded wells	Neo^R colonies (% selected)	3' junction PCR positive (% colonies)	3' PCR/RE positives (% colonies)	Targeting frequency (HR/selected)
100	♂	96*3=288	70(0.24)	7(10.0)	10/10(100)	≥2.0 x 10^{-4}
250	♂	288	148(0.21)	25(16.9)		
625	♂	192	179(0.15)	ND	ND	ND
100	♀	288	75(0.26)	4(5.3)	9/9(100)	≥1.8 x 10^{-4}
250	♀	288	103(0.14)	20(19.4)		
625	♀	192	178(0.15)	ND	ND	ND

Since only colonies with dark HR bands shown in Figure 6 are characterized by 3' PCR/RE, the calculated targeting frequency is of minimal value.
Please refer to the legend of Table 1 for more information.

Table 3. Primers used in junction PCR for HR screening

3' D75A	Forward	GCAACTGCGTGCACTTCGTG
	Reverse	CAATCAGTCTGGAAGAAGGAACCC
5' LDLR KO	Forward	TCCCACAGTGTCTGTCACCTGCA
	Reverse	CTTTTGAAGCGTGCAGAATGCC
3' LDLR KO	Forward	GCTGACCGCTTCCTCGTGCTTT
	Reverse	TCCAATCCTAGCCTGTGACC

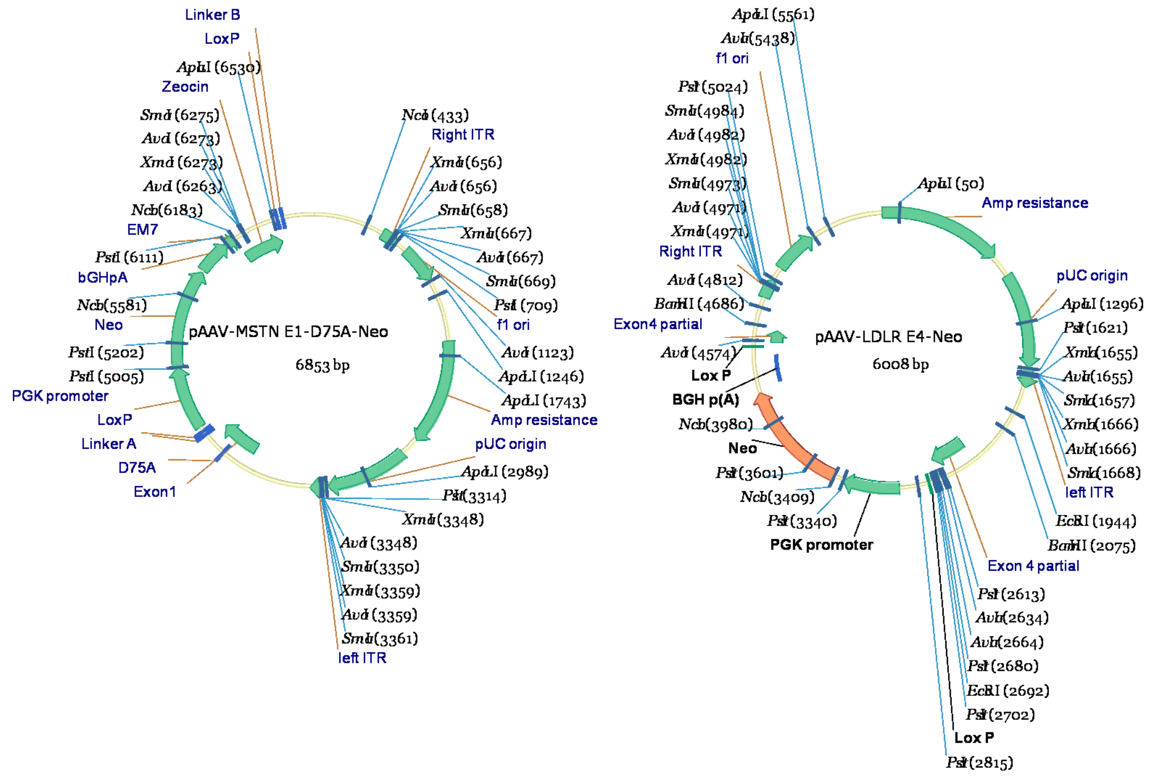


Figure 1. Physical maps of the two AAV targeting vectors. These are the targeting vectors used for packaging. Their key features are highlighted in blue or bold.

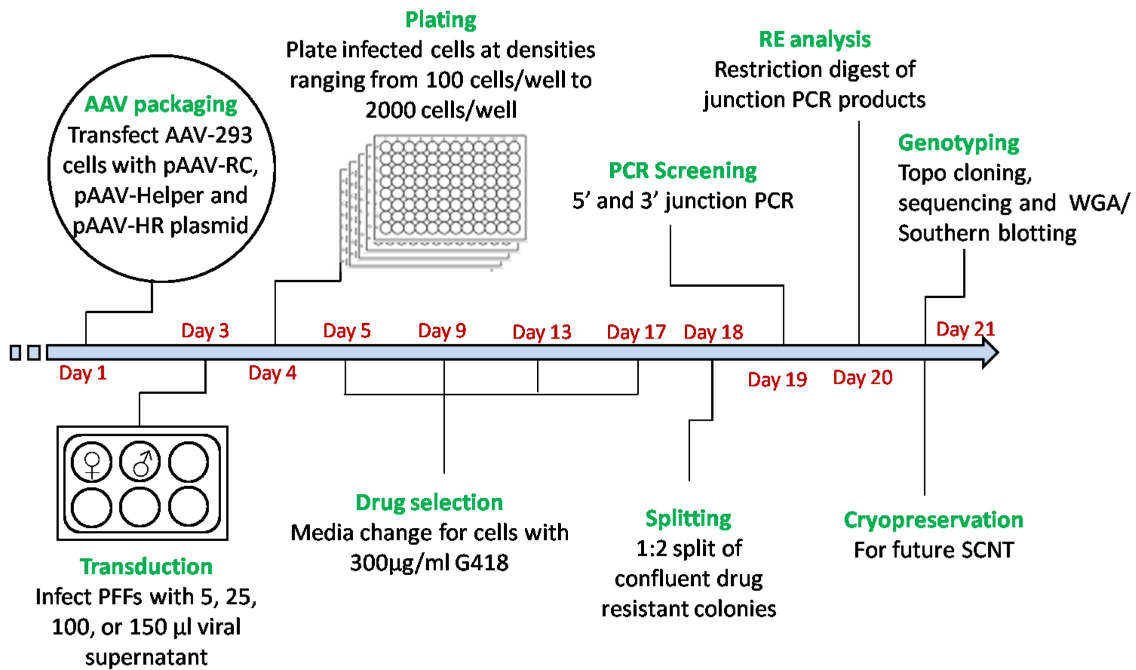
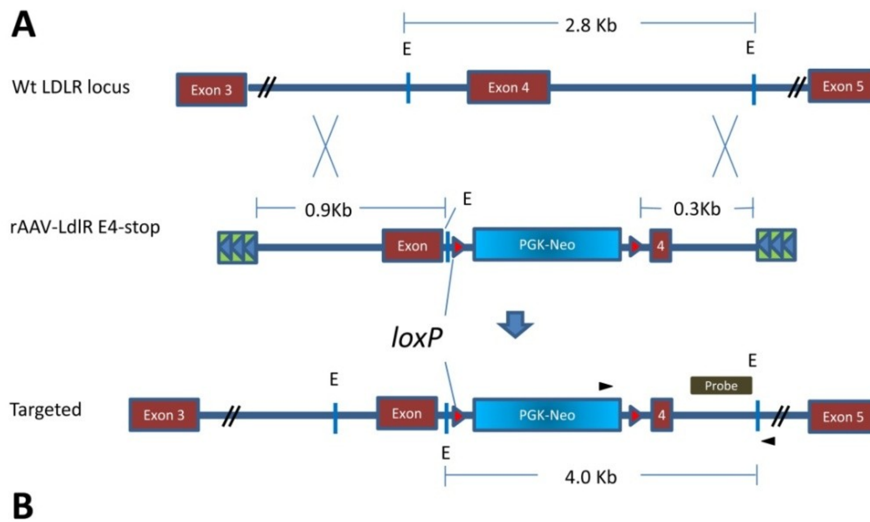


Figure 2. The timeline of rAAV mediated gene targeting. Along the axis of time lie the procedures for generating targeted cells. AAV-293 cells reaching 70% confluency on a 10 cm dish are transfected with plasmid components for rAAV packaging on Day 1. Two days later viruses are harvested and 25 - 150 µl fresh viral supernatant is added to PFFs reaching 70% confluency on a 6-well plate. One day after transduction (on Day 4), cells are plated out on 96-well plates supplemented with wild type cells to reach 1000 total cells/ well. One day after recovery, cells are selected with G418 and colonies emerge and reach confluency in about two weeks. Each colony is then split, one-half for cryopreservation and the other for DNA isolation for PCR/restriction digest analysis and other genotypic measurements to determine whether that colony is targeted and monoclonal.



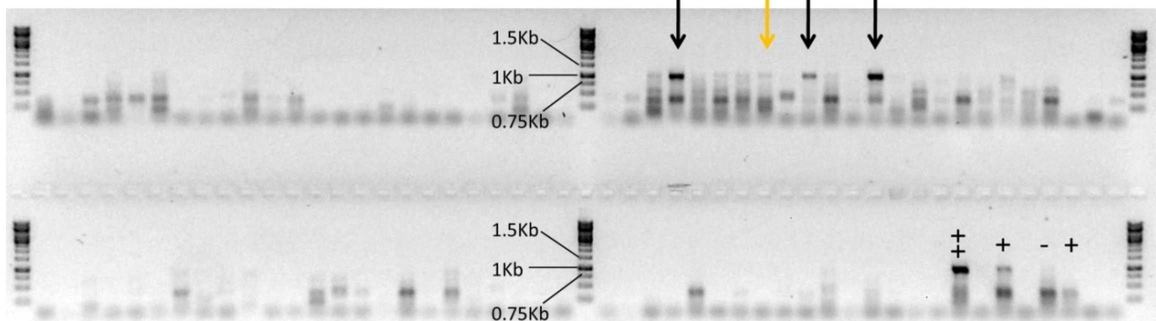


Figure 3. LDLR KO strategy and gel sample of PCR results from Round 1 transduction. A) This schematic shows both wild type (Wt) and a correctly targeted (Targeted) LDLR locus at exon 4 after homologous recombination with the rAAV-LDLR E4-Neo replacement cassette. The rAAV-LDLR E4-Neo cassette contains a floxed PGK-Neo selection cassette inserted within Exon 4 flanked by a 5' homology arm of 0.9 kb and a 3' homology arm of 0.3 kb. **B)** G-418 resistant colonies were screened for gene targeting by amplification of junctions between the LDLR locus and the PGK-Neo cassette (indicated as black triangles in panel **A**. Shown is the PCR result from one of the Round 1 25 µl transduction plates. Positive wells are expected to have a 1.1 Kb PCR product whereas negative colonies should have no product. Examples of strong (black arrows) and weak signal (yellow arrows) are shown. Positive (++ ~150 copies, + 15 copies) and negative (- wild type genomic DNA only) controls are shown in the bottom right corner of the gel.

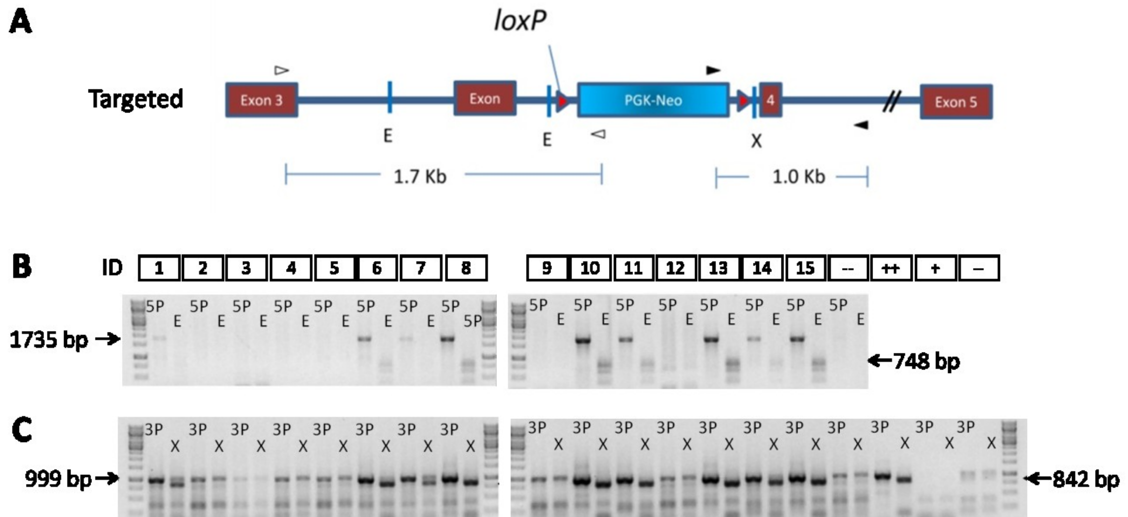


Figure 4. Confirmation of round 1 and 2 positives by PCR/Restriction analysis. A) This schematic shows a correctly targeted (Targeted) LDLR locus at Exon 4 after homologous recombination with the rAAV-LDLR E4-Neo cassette. PCR primers for screening 5' (open triangles) and 3' (filled triangles) junctions are shown. **B)** 5' junction PCR was performed on WGA DNA from 7 and 8 colonies identified in the primary PCR screen of round 1 and 2 respectively. PCR from correctly targeted clones will produce the expected 1735 bp (lanes "5P") and its identity is verified by restriction digest with *EcoR*I (labeled "E" in panel A) resulting in 3 fragments (748, 607 and 380 bp, lanes "E") of which the 748 bp band is indicated. WGA DNA from a negative colony was used as the negative control (--).**C)** 3' PCR from the same candidate colonies produces a band of 999 bp ("3P" lanes) and its identity is verified by restriction digest with *Xho*I resulting in 2 fragments of 842 (indicated) and 157 bp (lanes "X"). Two positive (++ ~150 copies, + 15 copies) and two negative controls (--, WGA DNA from a negative colony) are shown. While a band appears in both negative controls at approximately 999 bp, it is not cleaved by *Xho*I indicating amplification of a random DNA fragment of similar size rather than a positive result. This can also be observed in clones 2-5, 9 and 12. The PCR was not sensitive enough to detect the 15 copy positive (+) control. Sample ID for both panel B and C are shown on the top of panel B.

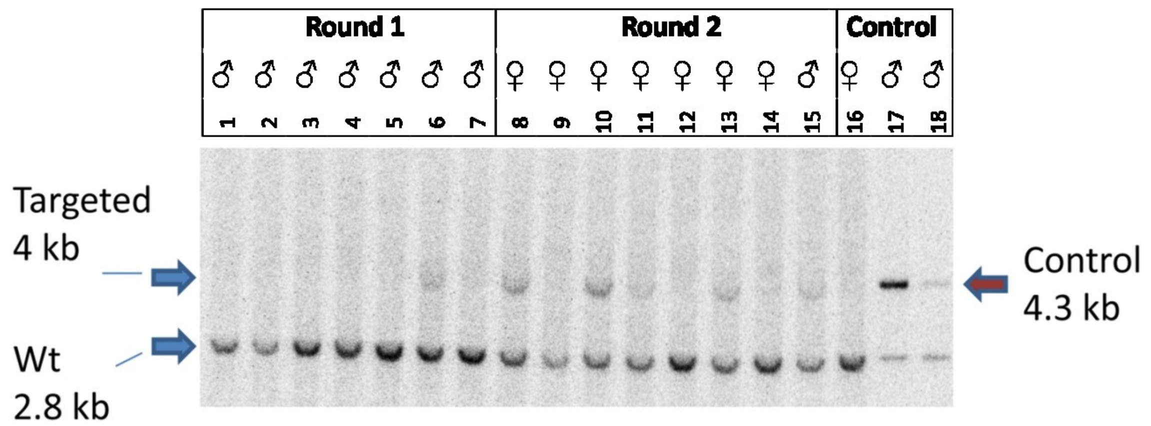


Figure 5. Confirmation of round 1 and 2 positives by Southern Blotting: Candidates identified by PCR (Figure 3 and 4**)** were subjected to WGA/Southern blotting. Restriction digest with *EcoRI* will release a fragment of 2.8 kb in wild type (Wt) cells and a 4.0 kb fragment for correctly targeted (Targeted) cells (see Figure 3 for schematic). Each colony identified confirmed positive by 5' and 3' junction PCR (Figure 4) displayed signal characteristic of a correctly targeted clone. Some variation in signal indicates not all colonies are pure; however, clones 8, 10, 13 and 15 appear to contain a majority of heterozygous LDLR knockout cells.

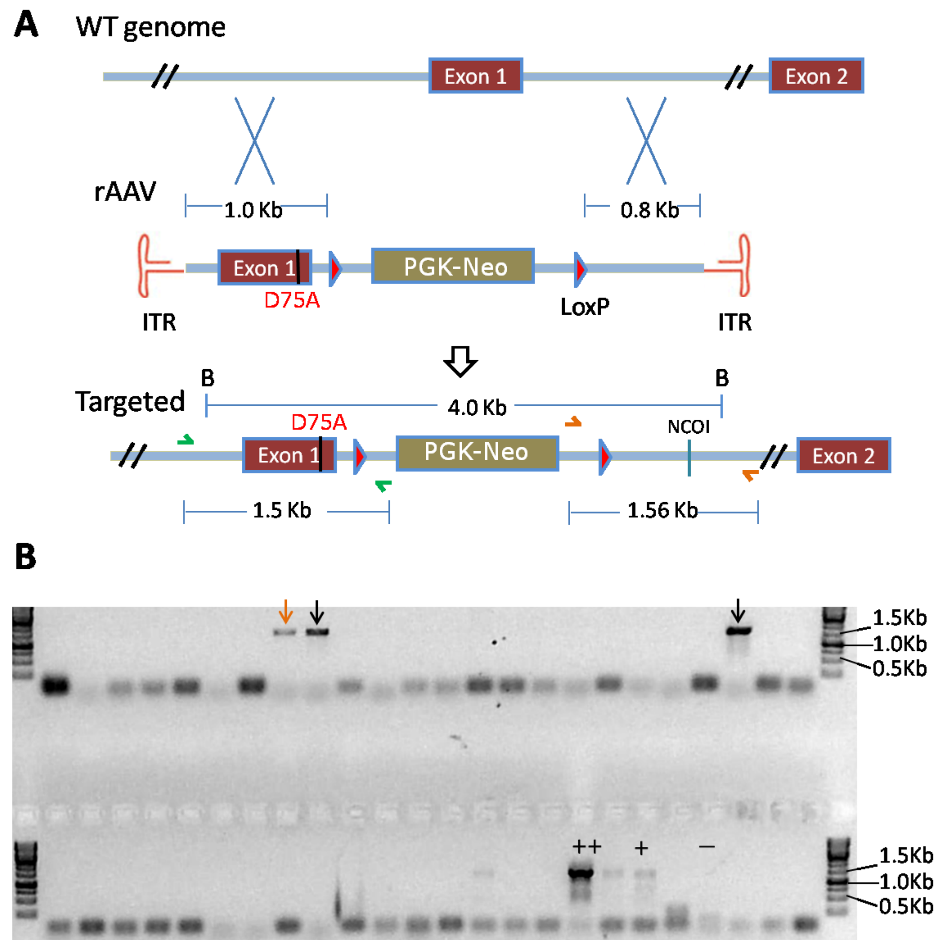


Figure 6. D75A introgression strategy and PCR/restriction digest results. A) This schematic shows both wild type (WT genome) and a correctly targeted (Targeted) Myostatin locus at Exon 1 after homologous recombination with the rAAV-MSTN E1-D75A-Neo replacement cassette. The rAAV-MSTN E1-D75A-Neo cassette contains a floxed PGK-Neo selection cassette inserted within Intron 1 flanked by a 5' homology arm of 1.0 kb and a 3' homology arm of 0.8 kb. The 5' homology arm is identical to the WT sequence except the desired SNP D75A in Exon 1(short black vertical line). **B)** Image sample of male G-418 resistant colonies screened for gene targeting by amplification of 3' junctions between the Myostatin locus and the PGK-Neo cassette (indicated as tan rectangles in panel **A** using primers shown as orange arrows at the bottom of the schematic. Positive wells are expected to have a 1.4 Kb PCR product whereas negative colonies should have no product. Examples of strong (black arrows) and weak signals (yellow arrows) are shown. Positive (++ ~1500 copies, + 150 copies) and negative (- wild type genomic DNA only) controls are shown in the bottom right corner of the gel sample.

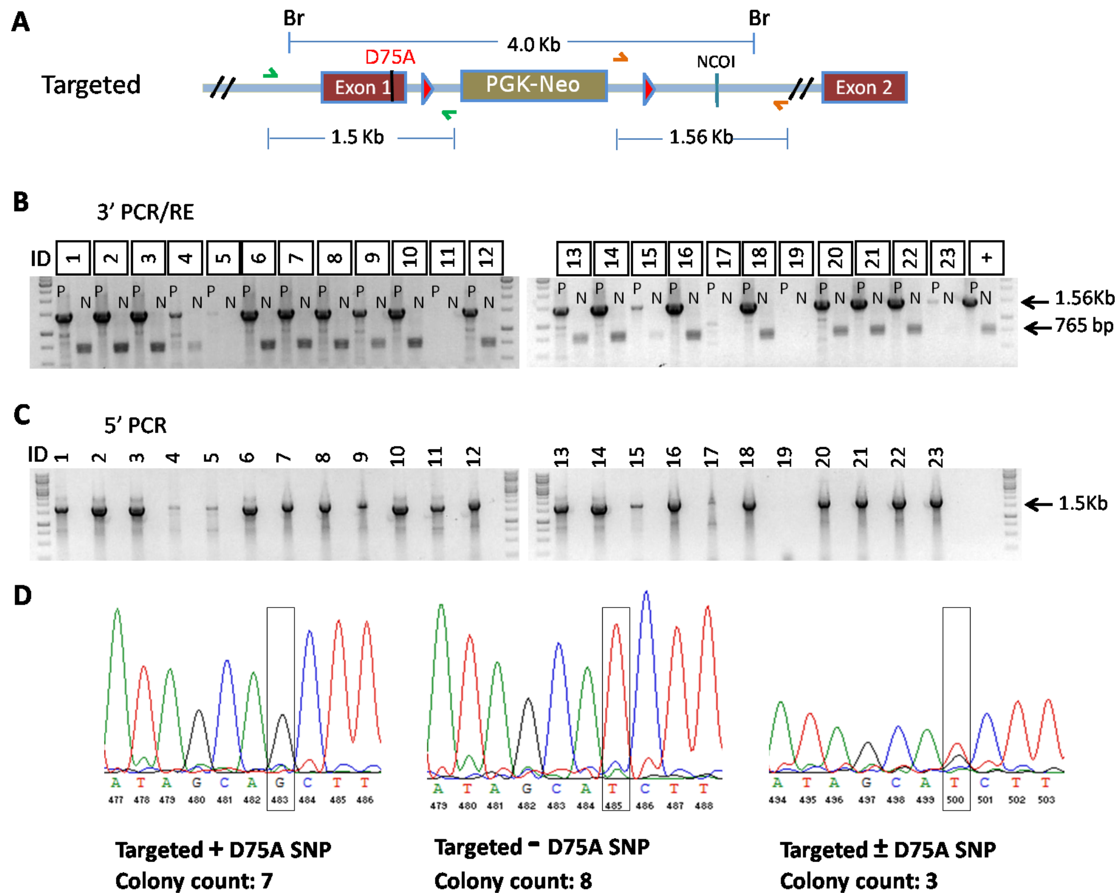


Figure 7. Confirmation of D75A HR positives by PCR/restriction digests analysis and sequencing. A) This schematic shows a correctly targeted (Targeted) Myostatin locus at Exon 1 after homologous recombination with the rAAV-MSTN E1-D75A-Neo cassette. PCR primers for screening 5' (green arrows) and 3' (orange arrows) junctions are shown. Borders (Br) mark the original 4.0 Kb HR cassette sequence. **B)** 3' junction PCR was performed on WGA DNA from 11 female and 12 male colonies identified in the primary PCR screen shown in Figure 6 with dark HR bands. PCR from correctly targeted clones will produce the expected 1.56 Kb band (shown in lanes labeled as "P") and its identity is verified by restriction digest with *Nco*I (marked by green vertical line in panel A), resulting in 2 fragments (765 and 804 bp; shown in lanes "N") of which the 765 bp band is indicated. ~150 copies of positive control plasmid were used as PCR template for "+" condition). **C)** 5' PCR from the same candidate colonies produces a band of 1.5 Kb. **D)** 18 fresh cleaned up PCR products shown in panel C (except No. 4, 5, 15, 17, 19) were sequenced with the reverse primer used in 5' junction PCR to determine whether the targeted genomes also harbored the D75A SNP. In the sense strand, D75A is caused by an A to C transversion, thus the corresponding anti-sense mutation is T to G. Three typical chromatographs are shown with identical sequences except: a G (D75A SNP), a T (WT SNP), and a G/T (D75A/WT SNP) at the same location, all highlighted with black rectangles. Sample IDs for both panel **B** and **C** are shown on the top of panel **B**. No. 1-11 were amplified from female-targeted cells and 12-23 from male cells.

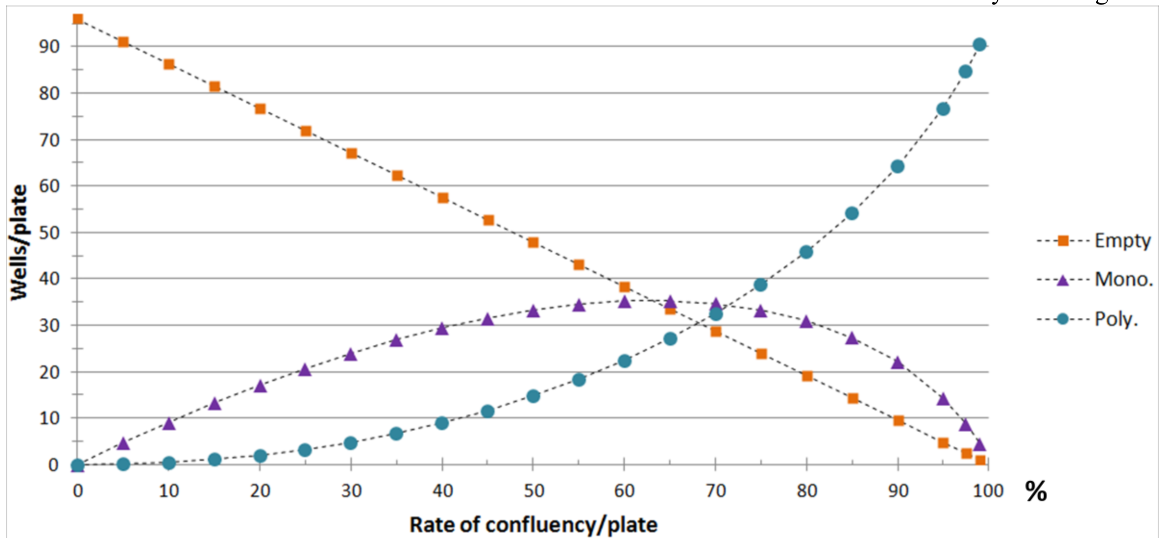


Figure 8. The change of numbers of wells that are empty, monoclonal, and polyclonal to the rates of confluency per plate. For each plate, when the Rate of confluency (=No. of confluent wells/96) goes up, the number of empty wells (orange rectangle) goes down and number of polyclonal wells (blue circles) goes up steadily. However, the number of monoclonal wells (purple triangle) starts to plateau when the rate of confluency is set around 35%, indicating that it is the optimal rate for screening to obtain monoclonal targeted colonies. The dotted lines are just for visualization only; they are not trend lines or graphed using the actual functions that generated the discrete numbers.

Chapter 4 Efficient TALEN-mediated Gene Knockout in Livestock

Efficient TALEN-mediated Gene Knockout in Livestock[5]

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Author contributions: DFC designed the research, performed the experiments, analyzed data and wrote the manuscript; WT designed and assembled all the TALENs, tested the activities of some, constructed pC-TAL+231 and RCIscript-TAL+231 plasmids, made Table S4, and analyzed part of the data; MC and DFV contributed new reagents and analytic tools; SGL performed research and analyzed data; DS, CP and CRL performed research; CBAW designed research and analyzed data; ; SCF designed research, analyzed data, and wrote the manuscript.

TALENs are programmable nucleases that join FokI endonuclease with the modular DNA-binding domain of transcription activator-like effectors (TALEs). Although zinc-finger nucleases (ZFN) enable a variety of genome modifications, their application to genetically engineering livestock has been slowed by technical limitations of embryo-injection, culture of primary cells and difficulty in producing reliable reagents with a limited budget. In contrast, we found that TALENs could easily be manufactured and that over half (23/36; 64%) demonstrate high activity in primary cells. Cytoplasmic injection of TALEN mRNA into livestock zygotes was capable of inducing gene knockout (KO) in up to 75% of embryos analyzed, a portion of which harbored bi-allelic modification.

We also developed a simple transposon co-selection strategy for TALEN-mediated gene modification in primary fibroblasts that enabled both enrichment for modified cells and efficient isolation of modified colonies. Co-selection after treatment with a single TALEN pair enabled isolation of colonies with mono- and bi-allelic modification in up to 54% and 17% of colonies, respectively. Co-selection after treatment with two TALEN pairs directed against the same chromosome enabled the isolation of colonies harboring large chromosomal deletions and inversions (10% and 4% of colonies, respectively).

TALEN-modified Ossabaw swine fetal fibroblasts were effective nuclear donors for cloning, resulting in the creation of the miniature swine containing mono- and bi-allelic mutations of the Low Density Lipoprotein Receptor gene as models of familial hypercholesterolemia. TALENs thus appear to represent a highly facile platform for the modification of livestock genomes for both biomedical and agricultural applications.

I. Introduction

The ability to knockout (KO) or precisely alter genes is fundamental for determining gene function and genetic engineering. For livestock, gene-KO strategies enable refinement of traits for xenotransplantation and biomedical products (1) and have been used to produce valuable models of human disease (2). There are further objectives in animal agriculture that would benefit from gene-KO including: functional characterization of high-impact genes identified in association studies, engineering disease resistance, reducing the threat of zoonotic disease transmission, alteration of production traits and enhancement of animal welfare. Until recently, homologous recombination (HR) in primary fibroblasts followed by somatic cell nuclear transfer (SCNT) was the exclusive method for the production of KO pigs and cattle. However, generation of KO cell lines by HR is inefficient and the length of time for gestation and reproductive maturation for livestock represent significant barriers to homozygous gene inactivation or the engineering of multiple loci. In addition, HR often requires the use of a linked selection-marker, which can confound functional studies (3) and/or complicate regulatory approval of engineered food products.

Zinc finger nucleases (ZFNs) offer an alternative platform for germline KO. ZFNs are fusion proteins consisting of a modular DNA-binding domain tethered to a FokI endonuclease monomer. When two ZFNs bind their target in an appropriate orientation, FokI monomers can dimerize and introduce a DNA double-strand break (4). Lesions are often repaired by non-homologous end-joining (NHEJ) that typically results in small insertions or deletions (indels) (5), two-thirds of which cause a frame-shift that disables encoded proteins. Thus, in contrast to HR where genes are disabled by targeted introduction of selection cassettes, ZFNs disable genes without introduction of exogenous DNA. Generation of germline KO has been successful in many model animal systems (6) as well as in pigs (7, 8). Particularly exciting in livestock was the report of ZFN-mediated, bi-allelic KO of the porcine *GGTA1* gene using commercial ZFN reagents (9) wherein bi-allelic null cells could be enriched by fluorescence-activated cell sorting (FACS) for the absence of a *GGTA1*-dependent surface epitope. Double-strand DNA breaks dramatically enhance homologous recombination (10) leading to homology-dependent repair of ZFN lesions for precise gene alteration in human cells (11), mice and rats (12, 13). While the potential of ZFN genome modification of livestock is great, design and assembly is labor-intensive and limited by available target sites (6).

Transcription activator-like effector nucleases (TALENs), like ZFNs, consist of assembled DNA-binding motifs coupled to the FokI nuclease (6, 14). Active, custom-designed TALENs have indel frequencies between 2-55% of targeted chromosomes (15, 16). As with ZFNs, TALEN-mediated double-strand breaks also stimulate HR in human cells at levels similar to those achieved with ZFNs (11, 15). Most importantly, TALENs appear to be superior to ZFNs in terms of simple and straightforward design and assembly strategies (17) such that manufacture of effective TALENs is significantly cheaper and faster than effective ZFNs. Here, we demonstrate efficiency and versatility of TALENs for a variety of genome modifications to livestock genomes. We have achieved mono- and bi-allelic KO of genes and large chromosomal rearrangements. Finally, towards our goal of producing a large animal model of atherosclerosis, we've used TALENs to develop Ossabaw miniature swine (18) containing inactivating alleles of the low density lipoprotein receptor (*LDLR*) gene. To our knowledge, this is the first report of genetic engineering and cloning of these valuable biomedical animals, and is also the first example of TALEN mediated gene modification of livestock.

II. Results

Evaluation of TALENs in livestock embryos. We chose to characterize TALEN efficiency for precise alteration of genomes in livestock embryos by using *in vitro*-prepared (IVP) bovine and porcine embryos. Three pairs of TALENs were generated using the +231 TALEN scaffold (17, 19) (Fig. 1). *In vitro* transcribed mRNA encoding each TALEN pair was injected into the cytoplasm of bovine embryos (≥50 embryos per condition) at ~19 hours post fertilization and were cultured *in vitro* until blastocyst stage (Table S1). Whole genome amplification (WGA) and genotypic analysis revealed indels in one blastocyst injected with *ACAN*11 and five injected with *ACAN*12 TALENs (Fig. 1b and Table S1); however, development was significantly impaired in conditions with the greatest indel frequency (max indel frequency ~10%, Table S1). Two groups have reported enhancement in TALEN activity using different N- and C- terminal truncations of the native TALE protein (15, 20). Thus, we created a truncated TALEN scaffold (GoldyTALEN; **GT**) compatible with the Golden Gate cloning procedure reported in Cermak *et. al.* 2011 (17) (Fig. 1a) for use in subsequent experiments. An additional TALEN pair, *GDF8*3.1, was developed using the GT scaffold. Zygotes were injected with TALEN mRNA plus 2ng/ul of EGFP mRNA as an indicator of successful injection. Indel analysis was performed on individual blastocysts by direct sequencing of PCR amplicons spanning the TALEN recognition site (example, Fig. S1). Mutation frequency of GT-*GDF8*3.1 TALENs significantly exceeded

previous injections. Six of 14 blastocysts (43%) injected with a low mRNA dosage (2ng/ul) displayed indels without a significant reduction in development rate (Table S1). Three of four blastocysts in the high dosage group (10ng/ul) displayed indels, with bi-allelic modification occurring in 2 of 3 mutant blastocysts (Fig. 1).

A single set of injections was also conducted in porcine zygotes using TALENs targeted to the porcine *RELA* gene (*p65*) for which a tolerance allele for African Swine Fever has been proposed (21). Zygotes were injected with a mixture including 20ng/ul of TALEN mRNA along with 5ng/ul of EGFP mRNA as an indicator of successful injection. In contrast to bovine injections where all EGFP injected embryos fluoresced, only 35% (71 of 214) were EGFP positive. WGA and PCR amplification was successful from 56 of the EGFP-positive embryos, and 16 of these (29%) revealed indels by Surveyor assay or sequence analysis. One-third of the mutants (6 of 16) were either homozygous or heterozygous bi-allelic mutants (Fig 1b).

TALEN function in livestock fibroblasts. Although our in vitro data demonstrates that direct embryo modification is likely to be a viable approach to livestock genome modification, the need to precisely engineer alleles led us to explore TALEN function in cultured livestock cells to meet two objectives, 1) to serve as a quality control measure for assembled TALEN pairs prior to injection and 2) to develop methods for TALEN-mediated genetic modification of livestock by cloning. To determine the best TALEN architecture for livestock fibroblasts, binding domains of six TALEN pairs were placed in the context of the +231 and GT scaffolds (Fig. 1a). Each TALEN pair was transfected into primary livestock fibroblasts, and the efficiency of genome modification was measured at day 3 by the Surveyor assay. The most active TALEN pairs, *DMD*E7.1 and *ACAN*12, displayed cleavage efficiencies of 38% and 25% (Fig. 2a). The TALEN scaffold had a significant effect on nuclease activity in fibroblasts. In total, 4 of 6 loci targeted with the +GT scaffold cleaved at 3.5% or greater while only the *DMD*E7.1 TALEN pair cleaved above 1% with the +231 scaffold (Fig. 2). As noted in previous studies (15, 22), a 72hr incubation at 30°C after transfection had a positive effect on target cleavage in livestock fibroblasts. We applied these findings to the design and testing of additional TALEN pairs. In total, 23 of 36 (64%) TALEN pairs were detectably active (> 1.0% NHEJ) at 15 genes scattered across the pig and cow genomes (autosomes and sex chromosomes, Table S2). Three quarters of the active pairs cleaved with high efficiency (19-40% NHEJ), with an average of 25%.

Extended culture and indel enrichment by transposon co-transfection. An ideal resource for cloning would be cell populations in which the majority of cells harbor indels that remain stable over extended periods in culture. While an average modification level of 25% (i.e., ~44% cells

with at least one modified allele; calculations described in Table 1) would be reasonable for direct somatic cell nuclear transfer of cell populations, a method to enrich for modified cells or isolation of specific modified clones would be useful. To this end, we evaluated the stability cells containing indels after an extended period in culture and tested an indel enrichment strategy based on transposon co-selection (23). To apply this approach to TALEN gene modification, a *Sleeping Beauty* transposon carrying a selection marker plus SB100X transposase was added to transfections with TALEN encoding plasmids at a ratio of 1:5. Transient TALEN expression and transposon integration can only occur in cells that have been successfully transfected, thus providing a mechanism for enrichment for transfected cells. In addition, selection is an extremely reliable method for clonal isolation in primary fibroblasts, a cell type averse to low culture density and dilutional cloning (24).

We first evaluated co-transfection with Mirus LT1, a commonly used cationic lipid transfection reagent. Whereas gene modification was below detection 14 days post-transfection without selection, genome modification levels in transposon-selected populations were 31%, 13% and 20% for *DMD*7.1, *DMD*6 and *LDLR*2.1 TALEN-pairs, respectively (Fig. 3). Thus, despite low transfection efficiency with cationic lipids (<5% for this experiment), efficient modification can be achieved using transposon co-selection. We next applied transposon co-selection to cells transfected by nucleofection where >90% transfection efficiency is routine. Without selection, the proportion of cells modified after 14 d in culture was lower (50-90%) than levels measured at day 3, suggesting an attrition of modified cells. In contrast, transposon co-selection was in all cases effective for maintenance of modified cells transfected by nucleofection but, with the exception of *ACAN*12, did not significantly increase the frequency of modified cells (Fig. 3c). Transposon co-selection thus appears to be an effective enrichment method when transfection efficiency is low and an effective maintenance method when transfection efficiency is high.

Isolation of mono- and bi-allelic KO clones. We have previously shown that transgenic primary fibroblast colonies can reliably be isolated and expanded when plated with wild-type fibroblasts and subjected to drug selection using the transposon co-selection (23). To evaluate this approach, we isolated puromycin-resistant colonies from cells treated with six TALEN pairs and evaluated their genotypes by the Surveyor assay or direct sequencing of PCR products spanning the target site (Table 1). The proportion of indel-positive clones was similar to our predictions calculated from %NHEJ measured in the parent populations 3d after transfection (Table 1). Bi-allelic modified clones were identified for five of six TALEN pairs, occurring in up to 35% of indel-positive cells (Table 1). Notably, among modified clones, the frequency of bi-alleic modification

exceeded predictions based on day 3 modification levels and the assumption that each chromosome cleavage/repair would be an independent event (observed 17-35% vs. predicted 10-16%). Among clones with bi-allelic modifications, a significant portion (15 of 23; 65%), were homozygous for the same indel, suggesting that sister chromatid exchange may be common (Fig. S2).

Chromosomal deletions and inversions with TALENs. We next investigated whether the simultaneous delivery of two TALEN pairs targeting the same chromosome could induce either large chromosomal deletions or inversions. We chose TALEN pairs *DMD*E6 and *DMD*E7.1 since a high percentage of Duchenne's Muscular Dystrophy (*DMD*) is caused by gross deletions (25), providing the opportunity to mimic the human condition in a porcine model. Day-3 gene modification levels were high for each TALEN pair (24% for *DMD*E6 and 23% *DMD*E7.1), albeit slightly lower than when either TALEN pair was transfected individually (Fig. 4b). To determine if the sequence between the two TALEN pairs had been deleted, we designed PCR primers to span this region. If the 6.5kb sequence had been removed, we expected to see a PCR product of ~500 bp. A fragment approximating 500 bp was observed in replicates in which both TALEN pairs were introduced, but was absent when either TALEN pair was introduced alone (Fig. 4c). We next assayed the cell population for inversion events by PCR amplification across presumptive new 5'- and 3'-junctions. Products were observed at the expected sizes for both the 5'- and 3'-junctions of the predicted inversion, but only when both TALEN pairs were introduced (Fig. 4d). Both deletion and inversion events were recovered at relatively high frequencies (10.3% and 4.1%, respectively; n > 1000; Table S4) in colonies generated using the transposon co-selection strategy. Deletion and inversion events occurred with remarkable fidelity, with 41 out of 43 (95%) of putative inversion-positive colonies confirmed by PCR amplification of both the 5'- and 3'-junctions. Sequencing of PCR products confirmed both deletion and inversion events with addition or deletion of very few nucleotides at their junctions (Fig. S3).

Production of TALEN-modified swine by cloning. Having demonstrated the ability to efficiently generate knockouts, gross deletions and inversion *in vitro,* we next sought to examine the utility of TALEN-modified swine cells for animal production. Transposon co-selected Ossabaw swine colonies with mono- and bi-allelic modification of the Class A domain 1 of the *LDLR* gene were pooled disproportionately (pools *A - 4 genotypes, B - 3 genotypes and C - 5 genotypes*) and cloned by chromatin transfer (26). Pregnancy was established in 7/9 transfers (1/2 for pool *A*, 2/3 for pool *B,* and 4/4 for pool *C*). Six pregnancies were maintained to term resulting in 18 liveborn piglets, including; one stillborn from pool *A*, 9 healthy and two stillborn piglets

from pool *B*, and 8 healthy, one stillborn, and one euthanized (due to a cloning defect) piglets from pool *C* (Fig. 5a). At 60+ days old all 17 piglets remained in good health. In total, 4 of 8 colony genotypes from pools *B* and *C* were represented in cloned offspring. Ten of the eleven piglets from pool *B* carried bi-allelic modifications of *LDLR* (B1, Fig. 5) derived from a colony harboring both an insertion-induced frameshift (289_290ins34) in one allele and a three base-pair deletion (285_287delATG) in the other allele, the former prematurely truncating the *LDLR* ORF, the latter removing a universally conserved aspartic acid residue (D47del) implicated in familial hypercholesterolemia (27, 28). A second colony genotype from pool *B* (B2; 211_292del128) was observed in a stillborn piglet. Two of 5 colony genotypes were represented in piglets from pool *C*, including 289_290del10 and 289_290insA, each resulting in frameshift mutations expected to prematurely truncate the *LDLR* ORF.

III. Discussion

Our examination of TALEN activity in livestock embryos produced some intriguing results. First, only one-third of pig zygotes cytoplasmically injected with the TALEN/EGFP mRNA fluoresced at a detectable level. While this low rate was unexpected, others have reported similar findings by either EGFP mRNA injections of porcine oocytes (29, 30) or cytoplasmic injection of plasmids into bovine zygotes (31). In contrast, all EGFP injected bovine embryos fluoresced. Pig and cattle injections took place at different locales; thus, we are unable to conclude whether this difference is due to technical or species specific differences. Regardless, we speculate that selecting for EGFP positive embryos will be a valuable first-level method for enrichment of TALEN modified embryos. Second, though we did not directly compare TALEN efficiency based on scaffold alone, the GT-TALENs had greater activity than the +231 scaffold in livestock zygotes. In contrast to +231 injected zygotes, bi-allelic modification was common (25% of modified embryos) in zygotes injected with GT-TALEN pairs in both pigs and cattle. Finally, there was a pronounced dosage response using the GT-bt*GDF8*3.1 TALENs in terms of activity (43% vs 75% mutant blastocysts) and development (24% vs 8% blast formation rate) in low and high dosage groups, respectively. However, the 5-fold difference between low and high dosage indicates TALENs function over a broad dosage range where a balance between activity and development could be sought. Thus, we predict that cytoplasmic injection of GT-TALEN mRNA will be an efficient platform for genetic modification of livestock.

While direct modification of zygotic genomes may have some advantages, somatic cell modification followed by cloning also provides a significant advantage by permitting the isolation

of cells containing precise modifications before the expense of animal production. We therefore evaluated the use of TALENs for gene modification in primary fibroblasts, the standard cell-type used for cloning. In agreement with others, we found the truncated GT-TALEN scaffold was superior for gene modification in primary fibroblasts (15, 20). The efficiency of TALEN modification could also be improved by culture at 30°C for 72 hours following transfection (22). Combined use of these enhancement strategies resulted in highly successful TALEN production; 64% of synthesized pairs were active in fibroblasts with a NHEJ frequency from 1.8-40%. Assuming a TALEN-pair introduces NHEJ at an average efficiency of 20%, about 5 heterozygous KOs could be recovered after screening only 24 colonies.

Our transposon coselection strategy (23) in cell populations was extremely successful for enrichment of indel-positive cells when transfection was inefficient, and for maintenance of indel-positive cells when transfection was efficient. When cationic lipid transfection was used, enrichment for modified cells was likely due to a result of the elimination of non-transfected cells, >95% of total cells. However, coselection was also capable of preventing an observed 50-90% loss of TALEN-modified cells after 14 d postnucleofection. The reason for modified-cell attrition is not clear, although it may be that without selection, cells with a low level of transgene expression have a growth advantage over cells with higher levels of expression. Given that coselection was able to enrich and maintain modified cells, it seems unlikely that nuclease off-target activity or toxicity (20, 32) is the cause for attrition. Instead, selection for antibiotic resistance is likely biased towards high levels of ectopic gene expression, thereby enriching for cells in which TALEN expression was also high, and cleavage more likely to have occurred. Potentially, transposition mobilizes the same factors that are involved with repair after double-stranded DNA cleavage by nucleases. Because transposition occurs in only a low percentage of transfected cells, as well cells in animal tissues (32-34), coselection might enrich for a sub-population of cells that were amenable to NHEJ.

By whatever mechanism, transposon coselection was very effective for the clonal isolation of both mono- and bi-allelic TALEN-modified cells. In fact, an analysis of coselected clones revealed the frequency of biallelic modifications to exceed predictions, assuming each TALEN-induced cleavage/repair was an independent event. This finding has also been observed for cells treated with ZFNs (11, 35, 36). Furthermore, approximately two thirds of the bi-allelic modified clones were homozygous for the same indel, suggesting that gene conversion of TALEN-mediated genetic changes from the sister chromatid is common, a bias previously observed by others (37). The efficiency of producing clones with biallelic KO observed here

represents a significant improvement over traditional approaches, for which line breeding or sequential targeting and recloning are required to generate homozygous KO animals (38). An additional advantage of nuclease-mediated biallelic KO is that linked selection markers are not theoretically required. For example, when the expression of the targeted gene results in a distinguishable epitope on the cell surface (9), cells harboring a biallelic KO can be isolated by FACS sorting. Alternatively, cotransfection of cells with a nonintegrating reporter system enabled the isolation of cells displaying evidence of nuclease activity at 3 d post transfection (39), although inefficient isolation of modified primary cell clones (two of six; both monoallelic indels) suggests low viability among sorted cells. In contrast, transposon coselection allowed efficient isolation of stable cells containing mono- and biallelic indels without FACS or a physically linked selection-marker. These targeted modifications can be easily segregated away from the selection transposons in a single generation of breeding during line propagation (23).

Structural variation in the form of chromosomal deletions, inversions and copy number variation accounts for a significant portion of human genetic variation (40). In this study, we found that large deletions and inversions could be generated in fibroblasts by a single co-transfection of two TALEN pairs that targeted the same chromosome. The efficiency of deletions and inversion in pig fibroblasts was similar to that reported by others using ZFN to generate chromosomal deletions in immortalized human cells (41, 42). However, whereas others found co-introduction of two ZFN pairs often resulted in unintended chromosomal rearrangements in addition to the desired rearrangement (42), we did not observe such events, perhaps because we were targeting the hemizygous *DMD* locus in male cells. The majority of useful rearrangements will likely occur on autosomes; therefore, founder lines will have to be carefully screened to avoid confounding rearrangements. Chromosomal rearrangements in livestock may also have applications beyond modeling of human disease. Deletions could be useful for functional query of gene clusters or used for deletion mapping of elusive genetic differences identified in association studies. Targeted inversions could also theoretically allow fixation of neighboring trait-determining alleles in a manner analogous to balancer chromosomes commonly used for genetic studies in lower eukaryotes. This could serve as a means to fix a desired trait/s in livestock for agricultural purposes or to eliminate the risks of environmental escape of genetically modified genomes based on non-disjunction.

The combination of TALENs plus the transposon components had no apparent impact on the utility of cells for cloning. We achieved a pregnancy rate of 78% with mono- and bi-allelic

modified Ossabaw fibroblasts, a rate similar to our previous results with transposon transgenic Landrace cells (23). This result is especially encouraging considering that the cloning of Ossabaw swine, which are superior models of metabolic syndrome, had not previously been reported. Of the 18 liveborn clones, 8 contained mono-allelic mutations and 10 contained bi-allelic modifications of the *LDLR* gene. These results demonstrate that TALENs can be used to generate animals with either mono-allelic or bi-allelic gene modification by cloning.

TALEN mediated genome engineering clearly has the capacity to revolutionize genetics and genome engineering in livestock species by introducing a variety of genomic changes including KO, bi-allelic KO, large chromosomal deletions/inversions, and potentially, precise allelic introgression. TALENs can be easily designed and assembled using molecular biology techniques available in most laboratories. We anticipate that their ease of use and versatility will rapidly expand the field of livestock genome engineering for a variety of purposes.

IV. Methods

See the supplementary text for full methods and Tables S4 and S5 for a complete list of TALENs and PCR primers used in this study.

All TALENs were designed using the TALE-NT software and assembled using methods described in Cermak et. al. 2011 (17) with some modifications. Four new backbone plasmids were generated to replace pTAL for the final step in TALEN assembly. The first pair of vectors pC-TAL+231 and pC-GoldyTALEN, direct expression of the +231 (19) and GoldyTALEN scaffolds from the mini Caggs promoter (43). The second pair of vectors, RCIscript-TAL+231 and RCIscript-GoldyTALEN, are useful for *in vitro* transcription of TALEN mRNA based on the pT3Ts vector previously described (44). The GoldyTALEN vectors have been made available through Addgene and are fully compatible with the Voytas Lab Golden Gate TALEN Kit also available through Addgene (#1000000016). Intermediary arrays were created and joined into these vectors as described (17).

TALEN mRNA was synthesized from SacI linearized RCIscript vectors using the mMessage Machine T3 Kit (Ambion) as previously described (44), and injected into the cytoplasm at specified concentrations. Fibroblasts were cultured and transfected using the Basic Fibroblast Nuclofection Kit (Amaxa Biosystems/Lonza) or Mirus LT1 reagent (Mirus) as previously described (44). TALEN activity was analyzed by Surveyor Assay (Transgenomic) and measurements were performed as described in Guschin *et. al.* 2010 (45). Indels were detected in embryos or individual colonies by Surveyor assay or direct sequencing. PCR assays described in the supplementary text were developed to detect large deletions and inversions.

TALEN design and assembly[6]. Briefly, intermediary arrays were produced for each TALEN pair that were compatible for Golden Gate cloning into one of four vectors; pC-+231-TAL; RCIscript-+231-TAL; pC-GoldyTALEN or RCIscript-GoldyTALEN. Arrays were joined in the above vectors in the as follows; 150 ng each pFUS_A, pFUS_B, pLR-X and the desired backbone were mixed in a 20 ul digestion/ligation reaction including 50 units T4 DNA ligase (New England Biolabs) and 10 units Esp3I (Fermentas) in 1X T4 ligase buffer (New England Biolabs). The reaction is incubated in a thermocycler for 10 cycles of 5 min at 37°C and 10 min at 16°C, then heated to 50°C for 5 min and then 80°C for 5 min. Two microliters of each reaction was transformed into *E.coli* and plated on LB-carbenicillin plates. Characteristics of each TALEN pair (ie. RVD sequence, spacer length) is provided in Table S4.

In vitro production of bovine and pig embryos[7]. Bovine ova were obtained from slaughterhouse derived ovaries were acquired through TransOva Genetics (MN, USA). Cumulus oocyte complexes were shipped overnight in maturation medium (TCM-199) supplemented with 10% fetal bovine serum (FBS), 0.01 units/ml of bovine follicle-stimulating hormone and bovine luteinizing hormone (Sioux Biochem, USA), 0.25 mM sodium pyruvate and 50 µg/mL gentamicin. Upon arrival, COCs were washed and fertilized as previously described (46). Approximately 19 hours following fertilization, presumptive zygotes were vortexed in in TL Hepes containing 0.4% bovine hyaluronidase to remove cumulus cells. High quality presumptive zygotes were selected and cultured in Bovine Evolve (Zenith Biotech, USA), supplemented with 4 mg/mL bovine serum albumin (Probumin, Millipore, USA) and 50 µg/mL gentamicin (Gibco, Invitrogen, USA) under mineral oil in a humidified atmosphere composed of 5% CO2, 5% O2 and 90% N2 at 38.5°C for up to 8 days post-fertilization and collected for indel analysis. Pig Ovaries were collected, washed with pre-warmed PBS and follicles aspirated. Washed oocytes were cultured in maturation medium for 44 hours (39°C, 5% CO_2), followed by gentle pipetting to remove cumulus cells and IVF for 6 hours (38.5°C in 5% CO_2). Zygotes were cultured in batches for 66 hours (5% CO_2, 38.5°C), following which they were placed individually into micro-droplets of medium under oil and assessed for GFP fluorescence. GFP positive zygotes were harvested for indel analysis.

Cell culture and Transfection. Fibroblasts were cultured in DMEM (high glucose) supplemented to 10% FBS, 20 mm GlutaMAX and 1X Penn/Strep solution (all from Invitrogen) and transfected by using the Basic Fibroblast Nuclofection Kit (Amaxa Biosystems/Lonza) or Mirus LT1 reagent (Mirus) as previously described (23). Briefly, each transfection included 500,000-1,000,000 fibroblasts, 2 ug each TALEN plasmid and 750 ng of transposon components (500 ng pKT2P-PTK; 200 ng pKC-SB100X; 50 ng pMAX-EGFP(Lonza)). For DMD deletion/inversion analysis, transfections included 1 ug each TALEN plasmid and 750 ng of transposon components. Nucleofection programs U-12 and U-23 were used for pig and cattle

[6] Dr. Colby Starker from Professor Dan Voytas' lab at the University of Minnesota assisted in initial TALEN assemblies.

[7] The authors would like to thank Dr. Mark Westhusin, Gayle Williamson, Jane Pryor and Ali Wilkerson from Texas A&M University for bovine zygote preparation and injection.

fibroblasts respectively. For Mirus LT1 transfections, 600 ng each TALEN and 300 ng transposon components were introduced into a well of a 6-well plate. Transfected cells were cultured 3 days at 37° or 30° C, as indicated, prior to splitting for indel analysis and plating extended culture, selection or colony isolation.

Individual colonies were collected by either selection in 96-well plates or 10 cm dishes. For 96-well plate selection, 5, 10 or 30 transfected cells were plated in one well supplemented with non-transfected cells for a total of 200-500 cells per well. To bias towards single colonies, confluent wells from plates where 30-50% of the wells contained a colony were selected. In 10 cm dishes, 500-1,000 transfected cells were co-plated with 10,000 non-transfected cells and individual colonies were aspirated under gentle trypsinization. Selection medium (1.0 ug/ml and 0.7 ug/ml for pig and cattle fibroblasts respectively) was applied 48 hours after plating and was replaced every 3-4 days.

Detection of gene modification. Transfected cells harvested at day 3 and 14 were prepared for PCR by pelleting and re-suspension in PCR-safe lysis buffer (10 mm Tris-Cl, pH 8.0; 2 mM EDTA; 2.5% Tween-20; 2.5% Triton-X 100; 100 ug/ml Proteinase K) at ~1,000 cells per microliter, followed by incubation at 50°C for 60 m and 95°C for 15 m. Typically, 1 ul of prepared lysate was used in a 25 ul PCR reaction using Platinum Taq DNA polymerase Hi-Fidelity (Invitrogen) for Surveyor Nuclease assay or MyTaq Red Mix (Bioline), all other applications, according to the manufacturer's protocol.

For quantification of TALEN activity in cell populations, 10 ul of a PCR reaction (300-700 bp amplicon) was treated 1 ul each Surveyor Nuclease and Enhancer (Transgenomic) according to the manufacturer's protocol. Products were resolved on 8-10% polyacrylamide gels and imaged by ethidium bromide staining. Densitometry measurements were performed using ImageJ and percent NHEJ was calculated as described in Guschin *et. al.* 2010 (45). For detection of gene modification in embryos, whole genome amplification (WGA) was performed on individuals using the Repli-G Mini kit (Qiagen), according to the "Amplification from blood or cells" method specified by the manufacturer, followed by target specific PCR and Surveyor assay (above) or direct sequencing of PCR amplicons. Gene modification in selected colonies was detected by direct sequencing of PCR amplicons and characterized by TOPO cloning (Invitrogen) and sequencing.

To detect deletions at the *DMD* locus, PCR was conducted on day 3 and colony lysates using primers *DMD*E6 NJ 5-1 and *DMD*E7 NJ 3-1. For detection of inversions at the *DMD* locus, PCR using two primer sets for detection of the 5' junction (DMDE6 NJ 5-1; DMDE7 NJ 5-1) and the 3' junction (*DMD*E6 NJ 3-1; *DMD*E7 NJ 3-1) was conducted on day 3 and colony lysates. PCR amplicons from day 3 pools were TOPO cloned (Invitrogen) to sequence junctions.

Animal Husbandry/ cloning. Pigs were cloned by CT under contract with Minitube of America under Recombinetics' IACUC protocol 1103A97232 at the University of Minnesota.

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Table 1. Genotype distribution in fibroblast clones

TALEN-pair	Species	% NHEJ day 3 (Surveyor)	Predicted % mod clones	Predicted % biallelic mod	Observed mod clones (%)	Observed biallelic mod (%)
LDLRE2.1	Pig ♂	19	34.5	3.6	30/81(37)	5/26 (19)
LDLRE2.1	Pig ♀	21.5	38.3	4.6	23/76 (30)	8/23 (35)[*]
LDLRE2.1	Pig ♂	14.4	26.7	2.1	12/94 (13)	2/12 (≥17)[†]
LDLRE2.1-2x[‡]	Pig	19.7	35.5	3.9	8/24 (33)	2/8 (≥25)[†]
LDLRE4.2	Pig ♂	20	36	4.0	4/48 (8.3)	1/4(25)[†]
LDLRE4.2	Pig ♀	19	34.4	3.6	8/47 (17)	0/8[†]
DMDE6	Pig	25	43.8	6.3	17/35 (49)	-
DMDE7.1	Pig	27	47	7.3	12/29 (41)	3/10 (30)
DMDE7.1-2x[‡]	Pig	22	39.2	4.8	22/41 (54)	7/22 (≥32)[*†]
GHRHR2.3	Pig	29	50	8.4	26/43 (60)	15/26 (≥58)[*§]
ACAN12	Cow	29	50	8.4	27/35 (77)	2/6 (NA)[¶]
btGDF83.1	Cow	17	31	2.9	7/24 (29)	0/7

If chromosome modification is an independent event, the frequency of bi-allelic modification can be predicted by the following equation: bi-allelic = (day3 %NHEJ)^{2}. By the same assumption, mono-allelic modifications (mod) can be predicted by this equation: mono-allelic modification = (day 3% NHEJ × 2) × (1 −day 3% NHEJ). Expected % mod clones (both mono- and bi-allelic) was calculated by summing mono- and bi-allelic modification proportions derived from the equations above.

[*]A 95% Confidence interval exceeds expected biallelic-null hypothesis that cleavage and repair are of each chromosome is an independent event.

[†]Biallelic KO were identified by sequencing of PCR products. Only overlapping or homozygous deletions can be identified using this technique.

[‡]Fibroblasts were transfected and recovered twice within 2 wk with the same TALEN-pair.

[§]Five of 15 biallelic colonies were confirmed as double frame-shift alleles.

[¶]Only colonies with distinguishable gross deletions in the PCR amplicon were analyzed.

Table S1. TALEN activity in bovine zygotes

Target	Trial	Scaffold	mRNA total ng/ul	Number Inj.	Blast rate	SURVEYOR Candidates/ assayed	NHEJ Confirmed
Non inj.	1	-	-	60	41%	-	-
Buffer	1	-	-	68	36%	-	-
ACAN11	1	231	10	67	22%	2/24	1/2
ACAN11	1	231	2	87	28%	1/32	0/1
ACAN12	1	231	10	57	33%	0/22	-
ACAN12	1	231	2	54	37%	1/23	1/1
PRNP3.2	1	231	10	65	14%	0/19	-
PRNP3.2	1	231	2	50	30%	0/17	-
Subtotal-				**380**		**4 (3%)**	**2/4 (1.5% assayed)**
ACAN12	2	231	10	59	5%	1/10	0/1
ACAN12	2	231	25	58	16%	3/16	2/3
ACAN12	2	231	50	59	2%	2/9	1/2
ACAN12	2	231	100	51	0%	1/10	1/1*
Subtotal-				**227**		**7 (16%)**	**4/7 (9% assayed)**
Non inj.	3	-	-	51	43%	-	-
Buffer	3	-	-	35	23%	-	-
GDF83.1	3	GT	2†	62	24%	-	6/14
GDF83.1	3	GT	10†	53	8%	-	3/4‡
Subtotal				**328**			**9/18 (50% assayed)**

*- 3 indels in one embryo
†- eGFP mRNA was added to a final concentration of 2 ng/ul.
‡- two bi-allelic modification
ACAN- Aggrecan, candidate for model of congenital achondroplasia.
PRNP- Major prion protein, implicated in spongiform encephalopathy.
GDF8- Growth differentiation factor 8 (myostatin), regulator of muscle growth.

Table S2. Activity of Livestock TALEN pairs in fibroblasts and predicted genotype distribution

Organism	Gene	Exon	TALEN ID	Day 3 NHEJ (%)	Bi-allelic Mod % (expected)	Het Mod (%) (expected)	Total Mod (%)
Sus Scrofa	*SRY*	3' end	ssSRY ds3.2	40	na	na	40
Sus Scrofa	*p65*	11	p65_11-1	39	15.2	47.6	62.8
Sus Scrofa	*DMD*	7	DMD7.1L+7.1R	38	14.4	47.1	61.6
Sus Scrofa	*AMELY*	Intron 2	ssAMELY1.1	30.5	na	na	30.5
Bos Taurus	*GGTA*	9	btGGTA9.1	30	9	42	51
Bos Taurus	*ACAN*	12	btACAN12L+12R	29	8.4	41.2	49.6
Sus scrofa	*DMD*	6	DMD6L+6R	25	6.3	37.5	43.8
Sus scrofa	*LDLR*	4	LDLR4.2L+4.2NR	22	4.8	34.3	39.2
Sus scrofa	*RAG2*	1	ssRAG2.1L+2.1R	21.6	4.7	34	38.7
Sus scrofa	*IL2Rg*	2	ssIL2RG2.1L+2.1R	21.3	4.5	33.5	38
Sus scrofa	*LDLR*	4	LDLR4.2 L+ NR (NI-NG)	20	4.0	32.0	36.0
Sus scrofa	*GHRHR*	2	ssGHRHR2.3	20	4.0	32.0	36.0
Sus scrofa	*GGTA*	9	ssGGTA9.1	20	4.0	32.0	36.0
Sus scrofa	*GGTA*	9	ssGGTA9.2	20	4.0	32.0	36.0
Sus scrofa	*SRY*	3' end	ssSRY ds3.3	20	na	na	20
Sus scrofa	*GDF8*	3	ssGDF8E3.1L +NR	19.2	3.7	31.0	34.7
Sus scrofa	*LDLR*	2	LDLRE2.1L+2.1R	19	3.6	30.8	34.4
Bos Taurus	*GDF8*	3	btGDF83.1 L+NR	19	3.6	30.8	34.4
Sus scrofa	*GDF8*	3	ssGDF8E3.4 L+ NR	12	1.4	21.1	22.6
Bos Taurus	*PRNP*	3	btPRNP3.1L+3.1R	4.7	0.2	9.0	9.2
Sus scrofa	*GDF8*	3	ssGDF83.2L+83.2R	3.5	0.1	6.8	6.9
Sus Scrofa	*p65*	11	p65_11-2	2.5	0.1	4.9	4.9
Sus Scrofa	*p65*	11	p65_11-3	1.8	0.0	3.5	3.6
Sus scrofa	*RAG2*	1	ssRAG2.2L+2.2R	0			
Sus scrofa	*DMD*	7	DMD7L+7R	0			
Sus scrofa	*LDLR*	4	LDLR4.1L+4.1R	0			
Sus scrofa	*LDLR*	4	LDLR4.3L+4.3R	0			
Sus scrofa	*ApoE*	2	ApoE2.1L+2.1R	0			
Sus scrofa	*HR*	2	ssHR2.1L+2.1R	0			
Bos Taurus	*GDF8*	3	btGDF83.1L+83.1R	0			
Bos Taurus	*ACAN*	11	btACAN11L+11R	0			
Bos Taurus	*PRNP*	3	btPRNP3.2NL+3.2R	0			
Sus scrofa	*DAZL*	3	ssDAZLe3.1	0			
Sus Scrofa	*GHRHR*	2	ssGHRHR2.2	0			
Sus Scrofa	*AMELY*	Intron 2	ssAMELY1.2	0			
Bos Taurus	*OFD1Y*		btY LH1	0			

Expected frequency of modification was calculated as follows: Mono-allelic = (Day 3 mod X 2) X (1-Day 3 mod).

Frequency of bi-allelic modification was calculated as follows: Bi-allelic = (Day 3 Mod)^2.

Expected % Mod clones was calculated by summing mono- and bi-allelic modification proportions.

Table S3. DMD deletions and inversion frequency

96-well Density	Colonies/Plate	Deletion Positive	Inversion Positive (5' and 3' Junction)
10/well	4	0/4	0/4
30/well	12	3/12	0/12
100/well	47	4/47	1/47
1000/well	470[A]	93/940	40/940
total		**100/1003 (10.3 %)**	**41/1003 (4.1%)**

A- Number is an estimated based on lower dilution plates.

Table S4. TALEN characteristics

Species	ID	Exon	%NHEJ D3, +231 scaffold	%NHEJ D3, GT scaffold	Spacer size	TALEN size and RVD sequence, left TALEN shown on top	
Sus Scrofa	ssSRY ds3.2			40	16	19	NI NG NI HD NI NG NG NG NG NI HD NI HD NI HD NI NG NI NG
						17	NI NN NN NG NG HD NI NN NN HD HD NI NG NG NI NI NG
Sus Scrofa	p65_11-1	11		39	16	16	NN HD HD HD HD HD HD HD NI HD NI HD NI NN HD NG
						16	NI NG NI NN HD HD NG HD NI NN NN NN NG NI HD NG
Sus Scrofa	DMD7.1L+ 7.1R	7	17	38	15	18	NN NN NI NI HD NI NG NN HD NI NG NG HD NI NI HD NI NG
						20	HD HD NI NN NG NI NN NG NG NG HD NG HD NG NI NG NN HD HD NG
Sus Scrofa	ssAMELY1.1	Intron 2		30.5	16	19	HD HD NG NG HD HD NI NI NN NN HD NG NG HD NG NI HD NI NG
						23	NI HD HD NI NI NN NI NI NG NN NG HD NG NG NI NG HD NI NI NI NI NN NG
Bos Taurus	btGGTA9.1	9		30	17	17	HD NG NN HD NN HD NG HD HD NG NG HD NI NI NI NN NG
						17	NN NG HD HD NG NN HD HD NI HD HD NG HD NG NG HD NG
Bos Taurus	btACAN12L +12R	12	1	29	15	20	HD HD NG NG NG HD HD NG HD HD NI NN NN NN NI NG HD HD HD NG
						22	NN HD NG HD HD NG HD NG NN NG NG NN HD NG NG HD NG HD HD NI NN NG
Sus Scrofa	DMD6L+6R	6		25	15	19	HD NG NI NG NI HD HD NG NI NN NN NG HD NI NI NI NI NI NG
						21	NI NN NG NG NG NN NG NG NN HD NI NI NG HD HD NI NN HD HD NI NG
Sus Scrofa	LDLR4.2L+ 4.2NR	4		22	16	20	HD HD NI NN NG NN HD NI NI HD NI NN HD NG HD HD NI HD HD NG
						20	HD NI NN NN NI NG HD NI HD HD NI NG HD NI HD NI NN NN HD HD
Sus Scrofa	ssRAG2.1L+ 2.1R	1		21.6	16	18	NI HD HD NG NG HD HD NG HD HD NG HD NG HD HD NN HD NG
						17	HD NG NI NI NN HD NG NN HD NG NG NG NG NN NI NI NG
Sus Scrofa	ssIL2RG2.1L +2.1R	2		21.3	14	18	HD HD HD NI NI NI NN NN NG NG HD NI NN NG NN NG NG NG
						20	HD HD NI NI NN NG NN HD NI NI NG NG HD NI NG NN NG NI HD NG
Sus Scrofa	LDLR4.2L+4.2 NR (NI-NG)	4		20	16	20	HD HD NI NN NG NN HD NI NI HD NI NN HD NG HD HD NI HD HD NG
						20	HD NI NN NN NI NG HD NI HD HD NI NG HD NI HD NI NN NN HD HD
Sus Scrofa	ssGHRHR2.3	2		20	15	20	HD HD HD HD NG NN HD HD HD NN NN HD NG NG NG HD NG NG HD NG
						15	HD HD HD HD HD NG HD NI HD HD NG NN NN HD NG
Sus Scrofa	ssGGTA9.1	9		20	16	19	HD NG NN HD NI NI NI NG NI HD NI NG NI HD NG NG HD NI NG
						19	HD HD NI HD HD NI NG NN NI NG NN NG NI NI NI NI NN NI NG

Table S4. Continued

Species	ID	Exon	%NHEJ D3, +231 scaffold	%NHEJ D3, GT scaffold	Spacer size	TALEN size and RVD sequence, left TALEN shown on top
Sus Scrofa	ssGGTA9.2	9		20	15	17 NN HD NN NG NG HD HD NG NG NG NI NI NI NN NG NN NG 17 NN NG HD NG NG NN HD HD NI HD HD NG HD NG NG HD NG
Sus Scrofa	ssSRY ds3.3			20	15	18 NI NG NI NG NN NI NI NI HD NG NN NI HD NI NN NG NI NG 19 HD HD HD NI NI NG HD NG NN NI NN NG NG HD NG NN NN HD NG
Sus Scrofa	ssGDF8E3.1 +83.1NR	3		19.2	15	20 HD HD HD NG NG NG NG NG NI NN NI NI NN NG HD NI NI NN NN NG 21 HD HD NI NI NI NI NG HD NG HD NG HD HD NG NN NN NI NG HD NG NG
Sus Scrofa	LDLRE2.1L+ 2.1R	2		19	15	17 HD NG HD HD NG NI HD NI NI NN NG NN NN NI NG NG NG 18 HD NN NN NI HD HD HD NN NG HD HD NG NG NN HD NI HD NG
Bos Taurus	btGDF83.1L +83.1NR	3		19	16	23 NN NG NN NI NG NN NI NI HD NI HD NG HD HD NI HD NI NN NI NI NG HD NG 19 NG HD NI NI NI NI NG HD HD NI HD NI NN NG NG NI NN NI NN
Sus Scrofa	ssGDF8E3.4 +83.4NR	3		12	16	21 NN NN NI NG NG NI NG NG NN HD NI HD HD HD NI NI NI NI NN NI NG 21 NI HD NI NI NI NG NG HD NI HD NI HD NG HD NG HD HD NI NN NI NN
Bos Taurus	btPRNP3.1L +3.1R	3	0	4.7	15	20 NN HD NI NI NN NI NI NN HD NN NI HD HD NI NI NI NI HD HD NG 16 NI NG HD NN NN HD NG HD HD HD HD HD HD NI NN NG
Sus Scrofa	ssGDF83.2L +83.2R	3	0	3.5	15	21 HD NG NI NI HD NG NN NG NN NN NI NG NG NG NG NN NI NI NN HD NG 17 HD NG NG NG NG NN NN NN NG NN HD NI NI NG NI NI NG
Sus Scrofa	p65_11-2	11		2.5	18	21 HD HD HD NI HD NG HD HD HD HD NG NN NN NN NN NN HD HD NG HD NG 16 HD NG NG HD NN NG HD HD HD HD HD NN NI NN NI NN
Sus Scrofa	p65_11-3	11		1.8	16	21 HD NI HD HD NI NI HD NN NN NG HD NG HD HD NG HD NG HD NN NN NN 16 HD HD NI NG NN NG HD HD NN HD NI NI NG NN NN NI
Sus Scrofa	ssRAG2.2L+ 2.2R	1		0	15	20 NN NN NG NI NI NG NI NI HD NI NG NN NN HD HD NG NG NI NI NG 20 NN HD HD HD NI NG HD NI NI NI NI NG NG HD NI NG HD NI NI NG
Sus Scrofa	DMD7L+7R	7	0	0	21	21 HD NN HD HD NI NI NN NG NI NG HD NI NN NG NG NI NN NN HD NI NG 18 HD HD HD NI NI NI NI NG NN HD NI HD NG NI NI HD HD NG
Sus Scrofa	LDLR4.1L+ 4.1R	4	0	0	15	17 HD HD NI HD NG HD HD NI NN HD NG NN NN HD NN HD NG 21 HD NN NG HD NI NN NI HD NG NG NN NG HD HD NG NG NN HD NI NN NG

Table S4. Continued

Species	ID	Exon	%NHEJ D3, +231 scaffold	%NHEJ D3, GT scaffold	Spacer size	TALEN size and RVD sequence, left TALEN shown on top	
Sus Scrofa	LDLR4.3L+ 4.3R	4		0	16	23 21	NN HD NI NI HD NI NN HD NG HD HD NI HD HD NG NN HD NI NG HD HD HD HD HD NG HD NN HD NI NN NG HD NI NN NN NI NG HD NI HD HD NI NG
Sus Scrofa	ApoE2.1L+ 2.1R	2		0	21	30 29	NN NG NG NN NI NN NG NN NI HD NI NI NG NG NG HD NG HD HD HD NG HD HD NG NN HD NI NN NN NG NI HD HD NI HD HD NI NI NI NN HD NI NI HD HD HD NI HD NI NN NI NI HD HD HD NG HD NI NG
Sus Scrofa	ssHR2.1L+ 2.1R	2		0	18	25 26	NN NG NG HD HD HD HD NI NN NN HD HD NG NN NN NG NG HD NI NG NI HD NG HD NG NN NN NG NI HD HD HD NI NI NN NN HD NG HD HD HD NI HD HD NG HD HD NG NN NN NG
Sus Scrofa	ssIL2RG3.1L +3.1R	3		0	15	18 20	HD NG NI HD HD NI NI NI HD NI NG NG NG NN NG NG NN NG HD HD NG NN HD HD NG HD HD NG NN NN NN NG NG HD HD HD NN NG
Bos Taurus	btGDF83.1L +83.1R	3		0	21	23 23	NN NG NN NI NG NN NI NI HD NI HD NG HD HD NI HD NI NN NI NI NG HD NG HD HD NI NI NI NI NN HD NG NG HD NI NI NI NI NG HD HD NI HD NI NN NG
Bos Taurus	btACAN11L +11R	11		0	15	21 21	NN HD NI NI NG HD HD HD NI NN NN HD NG NG HD NI HD HD NN NG NG NN NG NI NN NN HD NI NI NN NG NG HD HD HD NI NG NG HD HD NN NG
Bos Taurus	btPRNP3.2N L+3.2R	3		0	16	20 23	HD NI NI NG NN NN NI NI HD NI NI NI HD HD HD NI NN NG NI NI NN HD NI NN HD NI NN HD NG HD HD NG NN HD HD NI HD NI NG NN HD NG
Sus Scrofa	ssGHRHR2.2	2		0	15	20 20	HD HD HD NI NG HD NG HD NN HD HD NN NG HD HD NI NN NN HD NG HD NN NN HD HD NI NN HD NI HD NI NI HD NI NN HD HD HD NI NG
Sus Scrofa	ssDAZLe3.1	3		0	17	16 17	NN NN NI NG NN NI NI NI HD HD NN NI NI NI NG NG HD NG NG NG NG NI HD NG NN NI NI HD HD NI NG NI NG
Bos Taurus	btY LH1			0	16	17 17	HD HD NI NI NN HD NI HD NG NN NG NG NG NI NI NN NG NI HD NI NG HD NI NG HD NI NI NI NG NG NN NN NN NG
Sus Scrofa	ssAMELY1.2	Intron 2		0	15	19 17	HD NG NI HD NI NG HD NI NI NG HD NI HD NG NG NN NN HD NG NI NG HD HD NI NG NI HD HD NI NI NN NI NI NG NN NG

Table S5. PCR primers used in this study

TARGET ID	Forward	Reverse
btACAN11L+11R	TGGGGTGGGTCACTGGTAAGGA	GTGGCTGAAGTTACCCATGGCAG
btACAN12L+12R	TCTCACAAAGCAGAGCCTGA	GCCTGTGAAGTCACCACTGA
btGDF83.1L+83.1NR btGDF83.1L+83.1R	CCTTGAGGTAGGAGAGTGTTTTGGG	CTCATGAACACCCACAGCGATCTAC
btGGTA9.1	AAGCCTGCAGAAATCCCAGAGGTT	TTCGCCGAAGGGAATGTATGCTG
btPRNP3.1L+3.1R	TGATGCCACTGCTATGCAGTCATT	ACTGGGTTTGTTCCATTGACCGT
btPRNP3.2NL+3.2R	GGTTCTCTTTGTGGCCATGT	CACTCGCTCCATCATCTTGA
btY LH1	CCTCTTCCCAACTGGTACTCC	AAACAATGGAGGAGGCAGAA
btY LH3	TTGTTTACCGTCTCCCAACC	GAAAGAACAACACGGGGAGA
DMD6L+6R	CAGGGAGGAATCCAGGAATGATCT	TGGAACCGTCCTGGAGAAAAAGA
DMD7.1L+7.1R DMD7L+7R	GGAATATGGGCATGTGTTGTCAGTC	TGCAGTATACTTCATCCACGAGGCA
LDLR4.1L+4.1R LDLR4.2L+4.2NR LDLR4.2L+4.2NR (NI- LDLR4.3L+4.3R	AGATGAGTTCCGCTGCCAGGAC	CAGAACCACCTAAAGGCACTGGC
LDLRE2.1L+2.1R	CACAGCCGTAATAATGCCAGCTCC	CCTTCTCCGCCCACATCCTAATTC
p65_11-1 p65_11-2 p65_11-3	GCAATAACACTGACCCGACCGTG	GCAGGTGTCAGCCCTTTAGGAGCT
ssAMELY1.1 ssAMELY1.2	CTATTCTCGGAGGGCTTGCATCAA	GCCCACCCATTATTTCCTTTCCTG
ssApoE2.1L+2.1R	TGAGGGAATTACACGGACAATGGA	AACGAGCTGTTCAGAAGCAAGGAGT
ssDAZLe3.1	ATTTGGGCCCTGTTGAAAAC	ACTCACCCTTTGGACACACC
ssGDF83.2L+83.2R ssGDF8E3.1+83.1NR ssGDF8E3.4+83.4NR	AGGCGAAGACCTCAGGGAAATTTA	TTGATTGGAGACATCTTTGTGGGAG
ssGGTA9.1 ssGGTA9.2	CGTCTCTCATCCAGGATGGGGAA	TGCTCCCCGATGGTCTTCATGC
ssGHRHR2.2 ssGHRHR2.3	ATGGCCAACTCCTCCTCGGGTA	AGGTCACAGAGAAAGACGGGGAGG
ssHR2.1L+2.1R	TTGGCGACGGTTGGGCTGTA	AATGACGAGTCCAGCCCCTCCT
ssIL2RG2.1L+2.1R	CTCCCCCACTTCATTTTCTCCCC	GATTCCACAGTCCAGCCTCAGCTC
ssIL2RG3.1L+3.1R	GAGGCTGGACTGTGGAATCTGTGG	GTGTGGTCATTCCCCTGGCCTAGA
ssRAG2.1L+2.1R ssRAG2.2L+2.2R	CCCAGCTGCCTGGATTTTTGC	CCGTCCTCCAAAGAGAACACCCA
ssSRY ds3.2 ssSRY ds3.3	GCTCCTGGCCATCTCTTTGGTCA	TGCCTGCCTGCTTGCATCTCTCA
Primer *b*	CCTTGAGGTAGGAGAGTGTTTTGGG	
Primer *b'*	CTCATGAACACCCACAGCGATCTAC	
Primer *c*	CAAAGTTGGTGACGTGACAGAGGTC	
Primer *c'*	GCATCGAGATTCTGTCACAATCAA	
Primer *d*	GTGTGCCATCCCTACTTTGTGGAA	
DMD Deletion	CAGGGAGGAATCCAGGAATGATCT	TGCAGTATACTTCATCCACGAGGCA
DMD Inversion 5'	CAGGGAGGAATCCAGGAATGATCT	GGAATATGGGCATGTGTTGTCAGTC
DMD Inversion 3'	TGGAACCGTCCTGGAGAAAAAGA	TGCAGTATACTTCATCCACGAGGCA

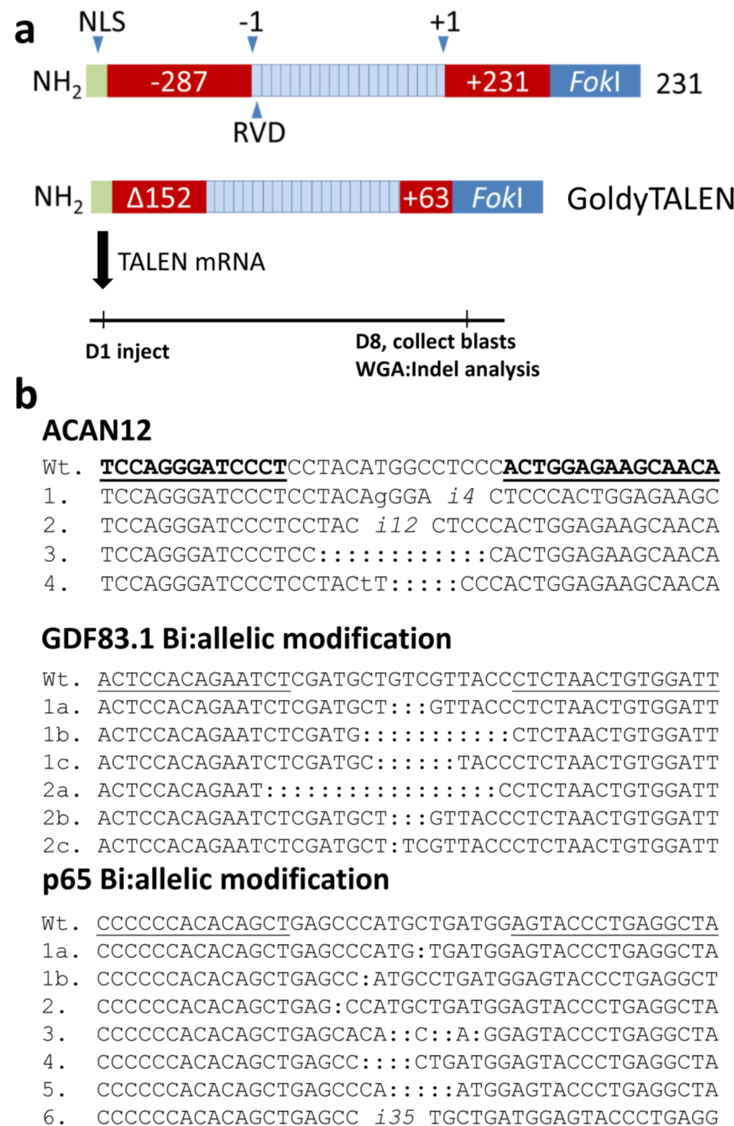
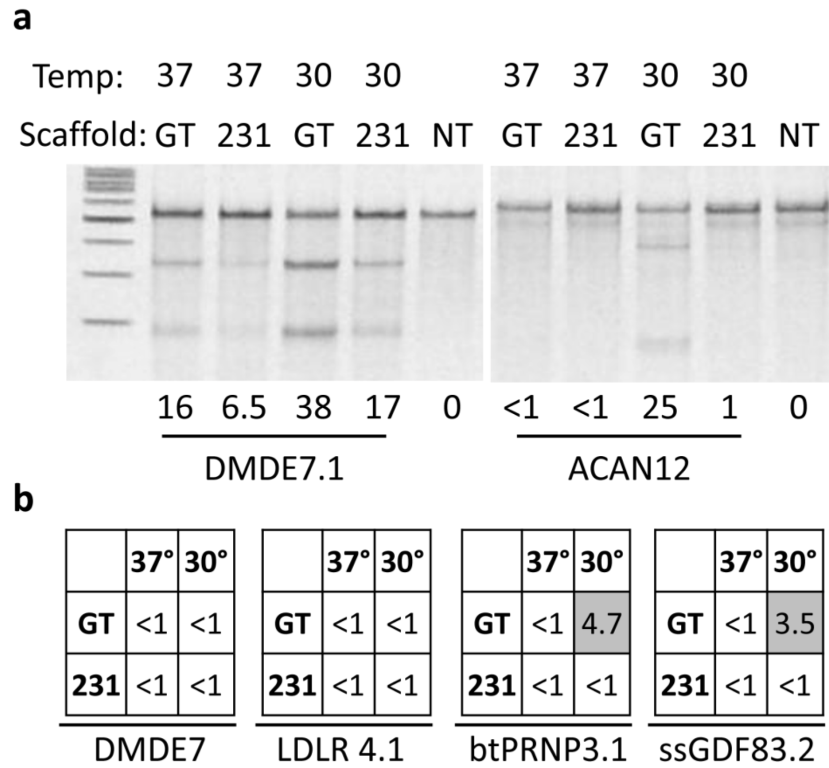


Fig. 1. TALEN activity in bovine embryos. a) TALENs for embryo injection generated in either the +231 or GoldyTALEN scaffold. Each scaffold shares a common SV40 nuclear localization signal (NLS) and C-terminal fusion of the FokI homodimer domain. Numbering is relative to the DNA-binding domain; the amino acid prior to the first repeat variable diresidue repeat (RVD) is labeled "-1" and the amino acid following the last RVD repeat is labeled "+1". Bovine or swine, *in vitro*-produced zygotes were injected with TALEN mRNA on day-1 (D1) and cultured *in vitro* to blastocyst formation. Individual blastocysts (blasts) were collected on day-8, subjected to whole genome amplification (WGA) and analyzed for indels. b) TALEN mediated indels in bovine (*ACAN*12 and *GDF8*3.1 injected) and porcine (*p65*-11.1 injected) embryos. Wild type (Wt) sequence is shown above with TALEN binding sites underlined. Both deletion and insertion (denoted with an *"i"* and number of base pairs) events were identified. Mismatch bases are indicated by lower-case text. Only bi-allelic modifications are shown for *GDF8*3.1 and *p65*-11.1 embryos. Embryos with a homozygous indel (same indel on each allele) are shown on a single line. Indel alleles of compound bi-allelic embryos (two or more unique indel alleles) are displayed on multiple lines (e.g. 1*a*, 1*b*). Some blastocysts were partially mosaic. Sequence analysis of embryos 1 and 2 injected with bovine *GDF8*3.1 TALENs revealed 3 unique indel alleles each; the number of reads for each allele is noted to the right of the sequence. In addition to indel alleles, 3 and 1 wild type sequence read was observed for *GDF8*3.1 embryos 1 and 2 respectively.



DMDE7

	37°	30°
GT	<1	<1
231	<1	<1

LDLR 4.1

	37°	30°
GT	<1	<1
231	<1	<1

btPRNP3.1

	37°	30°
GT	<1	4.7
231	<1	<1

ssGDF83.2

	37°	30°
GT	<1	3.5
231	<1	<1

Fig. 2. Comparison of TALEN scaffolds for gene-editing in livestock fibroblasts. a) The Surveyor assay was conducted on transfected fibroblasts transfected with either *DMD*E7.1 or *ACAN*12 TALEN pairs. Scaffold and temperature treatment is indicated above the gel and percent NHEJ is indicated below. Abbreviations, **NT** = not treated. b) Activity of four additional TALEN pairs, identified at the bottom of each matrix, with either the +231 or GT scaffold (left column in each matrix) at either 30° or 37° C.

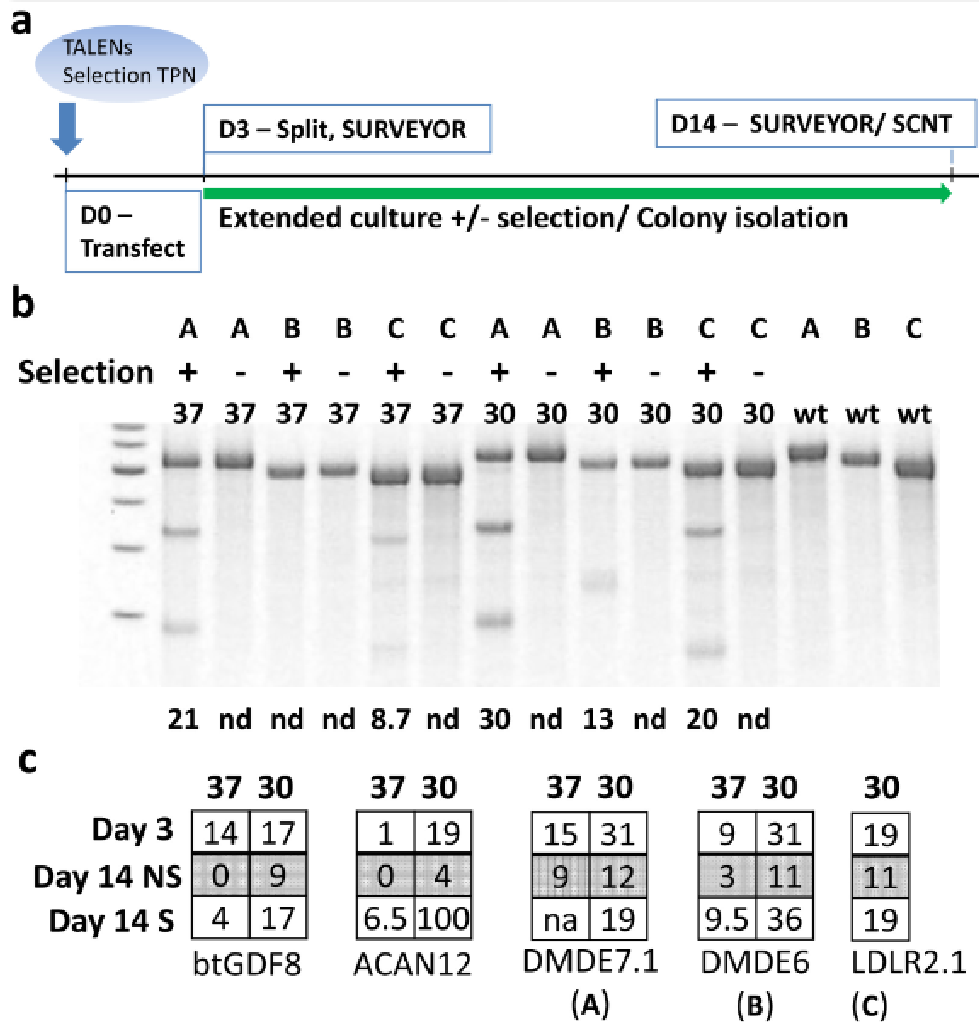


Fig. 3. Transposon co-selection for indel enrichment. a) The experimental timeline. Day zero (D0), cells were transfected with a mixture of plasmids including an expression cassette for each TALEN, a transposon encoding a selection marker, and transposase expression cassette. Transfected cells were cultured for 3 days at either 30° or 37° C prior to splitting, collection of a sample for Surveyor assay and re-plating for extended culture with and without selection for transposon integration. Cells cultured for 14+ days were collected for Surveyor assay. b) Fibroblasts were transfected using Mirus LT1 reagent and Surveyor assay was performed on day-14 populations. Temperature treatment, selection and TALEN identification (identified by single letters (A, B and C) as indicated in (c) are shown above the gel. c) Fibroblasts were transfected by nucleofection and the percent NHEJ was measured at day 3, and in day-14 non-selected (**NS**) and selected (**S**) populations. Temperature treatment is indicated above each matrix. Abbreviations: **nd** = not detected; **wt** = wild-type amplicon, Surveyor treated.

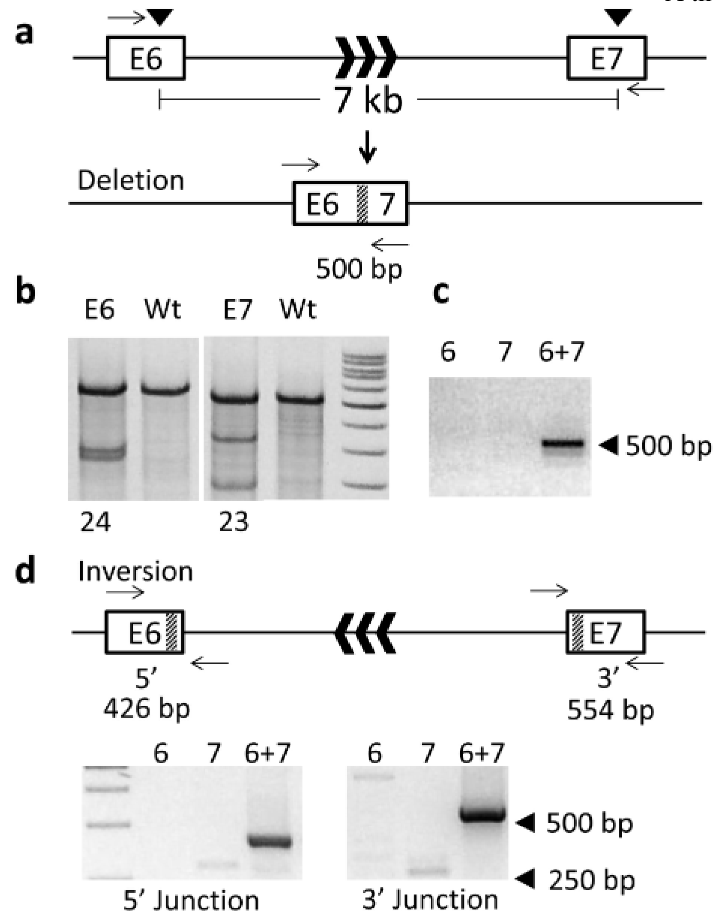


Fig. 4. TALEN induced deletions and inversions. a) *DMD* locus. Transcriptional orientation is denoted by black chevrons in intron-6. b) Surveyor assay of cells transfected simultaneously with TALENs targeted to exon 6 and 7 reveal NHEJ indels at both sites. c) PCR with primers (black arrows) flanking the presumptive deletion yield a ~500bp product when both exon-6 and exon-7 TALENs were introduced simultaneously, but not when transfected singly. d) The predicted outcome of an inversion event of the sequence between the TALEN target sites. Primers flanking the presumptive flanking sites at the 5'- and 3'- ends of the inversion locus are shown (black arrows) along with predicted product size. PCR products were observed at both 5'- and 3'- junctions only when both exon-6 and exon-7 TALENs were introduced simultaneously.

Wt. **CTCCTACAAGTGGATTT**GTGATGGGAACACCGA**GTGCAAGGACGGGTCCG**

B1: (289_290ins34; 285_287delATG) 10 born; 9 live

1. **CTCCTACAAGTGGATTT**GTGATGGGA_i34_ACACCGA**GTGCAAGGACGGGTCCG**
2. **CTCCTACAAGTGGATTT**GTG:::GGAACACCGA**GTGCAAGGACGGGTCCG**

B2: (211_292del128) One stillborn

1. AGGGAGTATGGTCAC::::::Δ128::ACCGA**GTGCAAGGACGGGTCCG**

C1: (289_290del10) 3 born (one stillborn, one euthanized due to clone defect)

1. **CTCCTACAAGTGGATTT**GTGATGGG:::::::::**GCAAGGACGGGTCCG**

C2: (289_290insA) 7 born; 7 live

1. **CTCCTACAAGTGGATTT**GTGATGGG**A**AACACCGA**GTGCAAGGACGGGTCCG**

Fig. 5. Genotypes of *LDLR*-knockout Ossabaw swine. The genotypes of 22 cloned piglets was evaluated by cloning and sequencing of amplicons spanning the *LDLR*2.1 target site. Four distinct genotypes were identified labeled and labeled **B1** and **B2** for pigs derived from the *B* pool and **C1** and **C2** for pigs derived from the *C* pool. The wild type sequence is shown above and the TALEN recognition sites are indicated in bold. Inserted bases are indicated by underlined text or denoted with an *"i"* and number of base pairs.

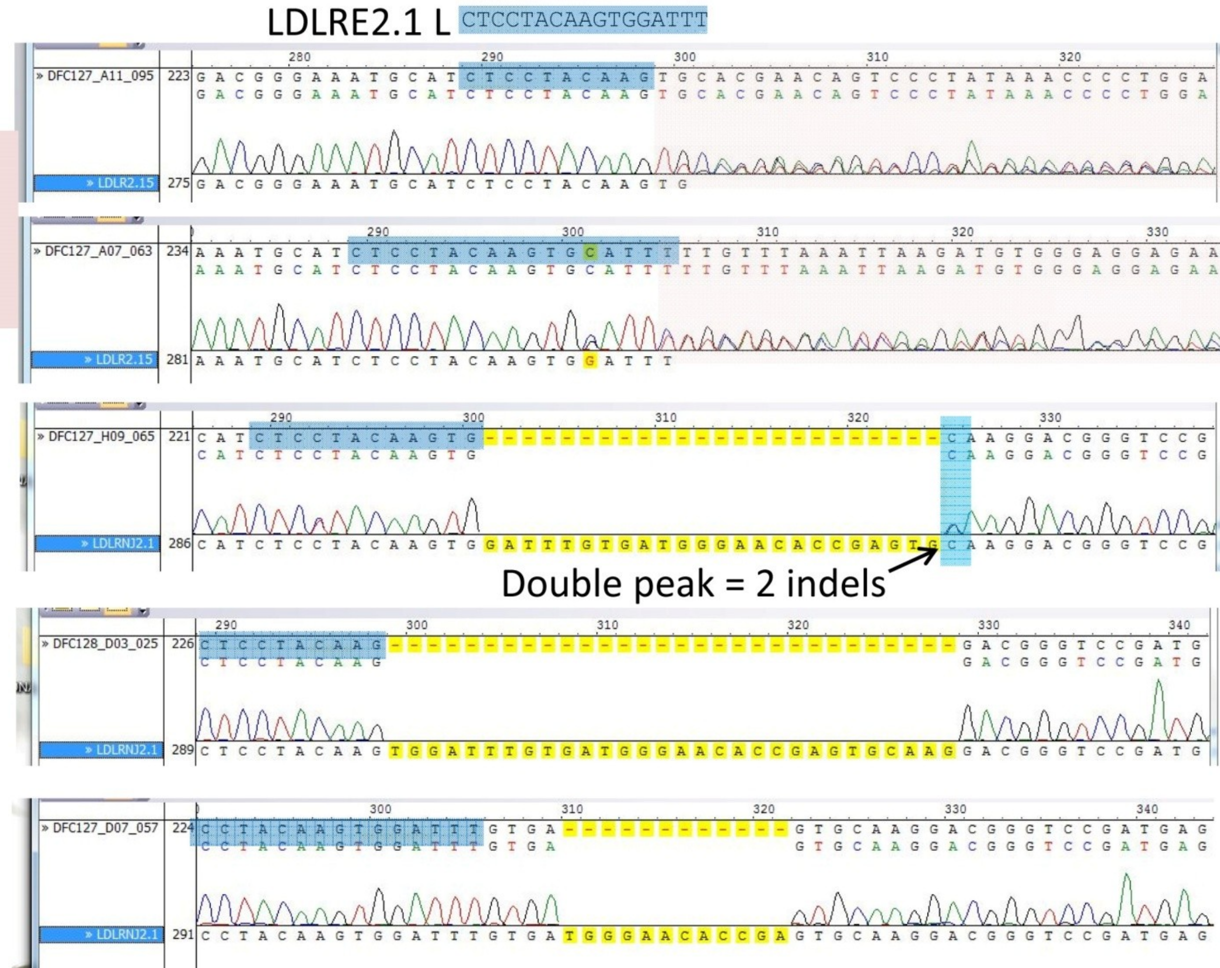


Fig. S1. Direct PCR sequencing for identification of indels. PCR amplicons from individual fibroblast colonies were purified, sequenced and compared to the wild type sequence. Mutation of one allele, or, non-overlapping mutations of both alleles will result in double sequence near the TALEN recognition sites (top). Overlapping bi-allelic mutations can be identified where differences between each allele can be identified by double peaks flanking the mutation site. Colonies with homozygous mutations do not display double peaks near the indel site.

DMDE7 Bi-allelic clones with homozygous indel

```
Wt.GCCACACAACGACTGGAACATGCATTCAACATCGCCAAGTATCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT
1. GCCACACAACGACTGGAACATGCATTCAACATCGCCA----TCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT     Δ4
2. GCCACACAACGACTGGAACATGCATTCAACATCGCCA-------GTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT     Δ7
3. GCCACACAACGACTGGAACATGCATTCAACATC----------AGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT     Δ10
4. GCCACACAACGACTGGAACATGCATTCAACATCGCCA357bpCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT     ins357
5. GCCACACAACGACTGGAACATGCATTCAACATCGCCAAGT|ATCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT    ins326
```

DMD Bi-allelic Two indels

```
Wt.GCCACACAACGACTGGAACATGCATTCAACATCGCCAAGTATCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT
6.
cCCACACAACGACTGGAACATGCATTCAACATCGCATaaTaTA|TCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT     Ins3 x5
GCCACACAACGACTGGAACATGCATTCAACATCGCCAA--ATCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT        Δ2 x5
7.
GCCACACAACGACTGGAACATGCATTCAACATC----------AGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT        Δ10 x7
GCCACACAACGACTGGAACATGCATTCAACATCGCCAAGTA|TCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT       Ins3 x1
8.
GCCACACAACGACTGGAACATGCATTCAACATCGCCAA---TCAGTTAGGCATAGAGAAACTACTGGATCCTGAAGGTT        Δ3 x8
GCCACACAACGACTGGAACATGCATTCAACATCGCCAAGTAT----TAGGCATAGAGAAACTACTGGATCCTGAAGGTT        Δ4 x3
```

4/8 Result in bi-allelic frameshift

LDLR E2 Bi-allelic Clones with homozygous indel

```
Wt.GGGAAATGCATCTCCTACAAGTGGATTTGTGATGGGAACACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG
1. GGGAAATGCATCTCCTACAAGTGGATTTGTGATGGGGAACACCGAGTGCAGGGACGGGTCATAAGAGTCCCTGGAGACGTG 2ins
2. GGGAAATGCATCTCCTACAAGTGGATTTGTG----GAACACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ4
3. GGGAAATGCATCTCCTACAAGTGGATTTGTG----GAACACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ4
4. GGGAAATGCATCTCCTACAAGTGGATTTGTG----GAACACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ4
5. GGGAAATGCATCTCCTACAAGTGGATTTG---------CACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ9
6. GGGAAATGCATCTCCTACAAGTGGAcTTG---------CACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ9
7. GGGAAATGCATCTCCTACAAGTGGATTTGTGA------------GTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ12
8. GGGAAATGCATCTCCTACAAGTGGATTTGTGA------------GTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ12
9. GGGAAATGCATCTCCTACAAGTG------------------------CAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ24
10.GGGAAATGCATCTCCTACAAG------------------------------GACGGGTCCGATGAGTCCCTGGAGAaG Δ30
```

LDLR E2 Bi-allelic Clones with two indels

```
11.GGGAAATGCATCTCCTACAAGTGGA----------------------------CGGGTCCGATGAGTCCCTGGAGACG Δ28 x2
   TGCTGATCCTGGCACTGAT-------------delta 116------caaGGACGGGTCCGATGAGTCCCTGGAGACG Δ116 x6

12.GGGAAATGCATCTCCTACAAGTGGA----------------------------CGGGTCCGATGAGTCCCTGGAGACG Δ28 x2
   TGCTGATCCTGGCACTGAT-------------delta 116------caaGGACGGGTCCGATGAGTCCCTGGAGACG Δ116 x4
   TAGACACAGGGAGTATGGTCACTTG------------delta 136------------3'CTGATTCCCACCGAGT 5'RA x1

13.GGGAAATGCATCTCCTACAAGTGGATTTG---------CACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ9 x5
   GGGAAATGCATCTCCTACAAGTGGATT---TGTGATG—GGAA---CACCGAGTGCAAGGACGGGTCCGATGAGTCCC
   GGGAAATGCATCTCCTAcAAGTGCAagGAGTGcaAgGACGGAATGCACCGAGTGCAAGGACGGGTCCGATGAGTCCC Ins7 x4

14.GGGAAATGCATCTCCTACAAGT----------Δ133--------TGCATTCcGCTGTGAATTAGGATGTGGGCGGAGA Δ133 x7
   GGGAAATGCATCTCCTACAAGTGGATTTGTGATG---ACACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ3  x2
Wt:1

15.GGCTCATTTCTCAGCTtGCA----------Δ161----------GTGCAAGGACGGGTCCGGTGAGTCCCTGGAGACG Δ161 x4
   GGGAAATGCATCTCCTACAAGTGGATTTGTGATac--ACACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACG Δ2 x5
Wt: 1
```

7/15 result in bi-allelic frameshift

Fig. S2. DMD and LDLR Bi-allelic modification alleles. Colonies with either homozygous modification alleles (ie. both alleles harbor the same mutation) or bi-allelic mutation with different mutations on each allele are displayed. For colonies with two indels, the number of times each allele was sequenced is displayed on the right. In some cases, a third mutation or single wild type allele was sequences indicating that not all colonies are 100% clonal. Frame shift alleles are indicated and mismatch nucleotides are denoted by lower-case text.

DMD Deletion Junctions

DMDE6, left TALEN underlined   DMDE7; right TALEN underlined

```
TCTATACCTAGGTCAAAAATGTAATGAAGAA-AGTATCAGTTAGGCATAGAGAAACTACTGGA
Replicate 1 O29_30
TCTATACCTAGGTCAAAAATGTAAT-----------TCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTAATGA---------TCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTAATGAGACAG-GTATCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGT-------------------TAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTA--------------------GGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTAAT------------CAGTTAGGCATAGAGAAACTACTGGA
Replicate 2 O28_30
TCTATACCTAGGTCAAAAATGTAATGAA-----GTATCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGCAATGAAGAAA--TATCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTAATGAATTAAA-TATCAGTTAGGCATAGAGAAACTACTGGA
TCAATACCTAGGTCAGAAATGTAATGAAGA---GTATCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTAAAG-------GTATCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTAATGAAAAGGGGTATCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAATT---------------ATCAGTTAGGCATAGAGAAACTACTGGA
TCTATACCTAGGTCAAAAATGTAA---------GAATCAGTTAGGCATAGAGAAACTACTGGA
```

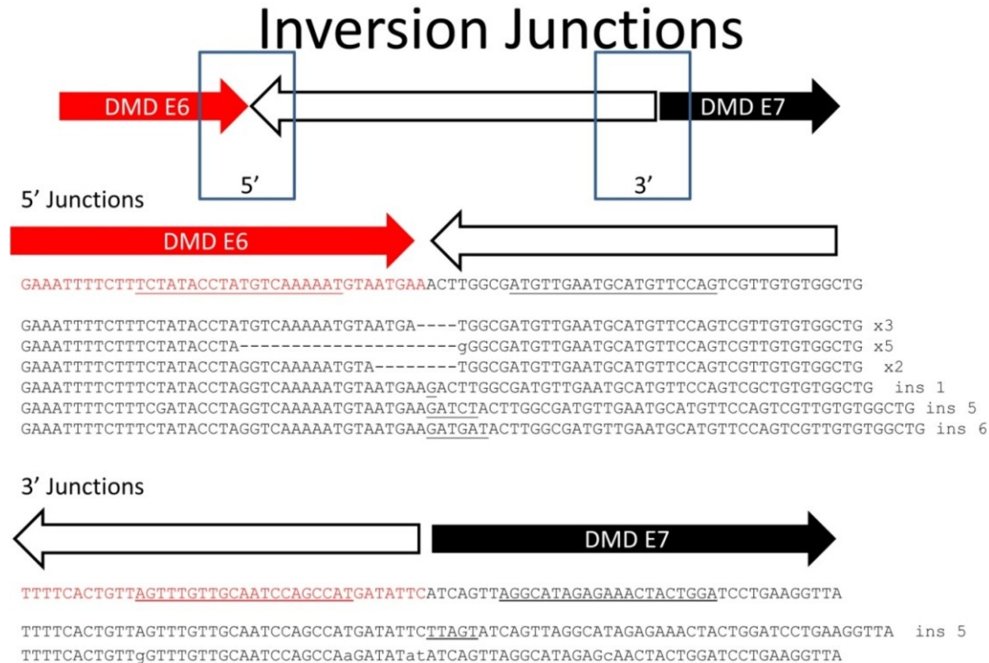


Fig. S3. ***DMD* deletion sequences.** *DMD* deletion junctions from replicate transfections are displayed. Above, exon 6 and 7 sequence is highlighted in green and yellow respectively and TALEN recognition sites are underlined. Inserted nucleotides are highlighted in red. ***DMD* inversion sequences.** A schematic of the DMD inversion allele is shown with the 5' and 3' junctions (boxed) that were analyzed by sequencing. Below, the predicted sequence for each fusion is shown corresponding fusion at the center of each spacer for the TALEN pairs. TALEN recognition sites are underlined. Sequenced inversion alleles from a transfected population are shown. The number of times each allele was sequenced is indicated at the right and inserted nucleotides are underlined. Mismatched nucleotides are denoted as lower-case text.

Chapter 5 Efficient Non-meiotic Allele Introgression in Livestock Using Custom Endonucleases

Efficient Non-meiotic Allele Introgression in Livestock Using Custom Endonucleases[8]

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Author contributions: WT designed, assembled and tested three fifths of the TALENs, designed and conducted experiments to isolate colonies with SNP-only introgression and integrate mloxP into btRosa26; WT also obtained most data presented in Figure 2 and 3, Supplementary figures 1, 4, and 10, and Supplementary table 2, made Table 1, supplementary Tables 2 to 5, Figures 2 and 3, and supplementary figures 1, 4 and 10; in addition, WT analyzed data and wrote part of the paper; DFC designed and conducted experiments for part of Table 1, generated data for and made Figures 4, 5, Supplementary figures 2, 3, 5 to 9, and Supplementary table 6, analyzed data and wrote part of the paper; CAL conducted the *POLLED* experiment and made Figure 1 and Supplementary table 1; JRG performed the Illumina sequencing analysis; DAW assembled TALENs and conducted some of the experiments; PH and SCF analyzed data and wrote part of the paper.

We have expanded the livestock gene-editing toolbox to include TALEN and CRISPR/Cas9 stimulated homology-directed repair (HDR) using plasmid, rAAV and oligonucleotide templates. Towards genetic dehorning of dairy cattle, we introgressed a bovine *POLLED* allele into horned bull fibroblasts. Single-nucleotide alterations or small indels were introduced into fourteen additional genes in pig, goat and cattle fibroblasts using TALEN mRNA and oligonucleotide transfection with efficiencies of 10-50% in populations. Several of the chosen edits mimic naturally occurring performance enhancing or disease resistance alleles including, for the first time, alteration of single basepairs. Up to 70% of fibroblast colonies propagated without selection harbored the intended edits of which over one-half were homozygous. Edited fibroblasts were used to generate pigs with knockout alleles in the *DAZL* and *APC genes* to model infertility and colon cancer. Our methods enable unprecedented meiosis-free intra- and inter-specific introgression of select alleles in livestock for agricultural and biomedical applications.

I. Introduction

Meeting global challenges presented by climate change and burgeoning populations requires development and application of biotechnologies that enhance productivity while reducing environmental footprints and improving animal welfare(1, 2). Since the first transgenic pig was created(3), more than 180 trials have been recorded to genetically engineer livestock in a variety of ways(2). Until recently, animal genetic engineering was accomplished by random insertions of expression cassettes which suffered from low efficiency and unpredictable expression, or homologous recombination (efficiency lower than 1 in 10^4) with linked selection markers.

Three recent technologies, zinc finger nucleases (ZFNs)(4),TAL effector nucleases (TALENs)(5) and clustered regularly interspaced short palindromic repeats/CRISPR associated endonuclease cas9 (CRISPR/Cas9)(6-8) have been utilized to disrupt gene-function by introducing small insertions and/or deletions (indels) into genomes of species mediated by non-homologous end-joining (NHEJ). Particularly, TALEN-induced gene disruption has been demonstrated in various species ranging from model organisms(9-12) to crops(13, 14), farm animals(15) and humans(16-18). However, indels introduced by NHEJ are variable in size and sequence which makes screening for functionally disrupted clones arduous(15) and does not enable precise alterations. TALEN or CRISPR/cas9 mediated homology-directed repair (HDR) supports the introduction of defined nucleotide changes; however, so far this has only been demonstrated in lower eukaryotic models including yeast(19), zebrafish(20) and very recently mice(21, 22).

Here we show precise, high frequency editing of fifteen genes in pig, goat, and cattle genomes. In many cases, the gene-edits are indistinguishable from alleles that exist within a species or clade and represent the first demonstration of marker-free, non-meiotic allele introgression. This work demonstrates that high-efficiency and precise gene-editing can be achieved in commercially important loci in the genomes of livestock utilized for agricultural or biomedical purposes.

II. Results

Introgression of the *POLLED* allele into the genome of horned dairy cattle breeds

We previously demonstrated an efficient system for deriving TALEN-mediated NHEJ knockouts in livestock fibroblasts and embryos(15) and theorized that similar methods would be suitable for derivation of cells with HDR-mediated genetic changes. Accordingly, we used TALENs to stimulate HDR with plasmid and recombinant adeno-associated Virus (rAAV) donor templates

designed to introduce a naturally occurring 11bp deletion or the Belgian Blue mutation(23-25) at position 821 of *GDF8* (821del11) (**Fig. S1**). Individual colonies with the intended mutation were recovered in 1 and 13 percent of colonies treated with the plasmid and rAAV templates, respectively (**Fig. S1d**). These encouraging results inspired us to attempt the introgression of more complex alleles into livestock fibroblasts.

To protect the welfare of dairy farm operators and cattle, horns are routinely manually removed from the majority of dairy cattle in the U.S. and Europe. De-horning is painful, elicits a temporary elevation in animal stress, adds expense to animal production(26) and, despite the intent of protecting animals from subsequent injury, the practice is viewed by some as inhumane. Some beef breeds are naturally horn-free (e.g., Angus), a dominant trait referred to as *POLLED* (27). Two allelic variants conferring *polledness* have recently been identified on chromosome 1(28). Meiotic introgression of the *POLLED* trait into horned dairy breeds can be accomplished by traditional crossbreeding, but the genetic merit of the resulting animals would rank lower due to the admixture of unselected (inferior) alleles for net merit (milk production) into the population. We undertook the non-meiotic introgression of the Celtic *POLLED* allele (duplication of 212 bp that replaces 10bp), referred to as Pc, into fibroblasts derived from horned dairy bulls. We constructed a plasmid HDR template containing a 1594bp fragment including the Pc allele from the Angus breed (**Fig. 1a**). TALENs were designed such that they could cleave the *HORNED* allele but leave the Pc allele unaffected. In addition, after finding that one pair of TALENs delivered as mRNA had similar activity compared to plasmid expression cassettes (**Fig. S2**), we chose to deliver TALENs as mRNA to eliminate the possible genomic integration of TALEN expression plasmids. Five of 226 colonies (2%) passed each PCR test shown in **Fig. 1b** to confirm introgression of Pc. Three of the five clones were homozygous for Pc introgression and confirmed by sequencing to be 100% identical to the intended allele (**Table S1**).

Reliable oligonucleotide templated allele introgression in pig, goat and cattle genomes

While plasmid templates were effective for introgression of Pc and *GDF8* alleles, many desirable alleles correspond to SNP or small indels that ideally would not rely on the use of double-stranded DNA templates that could randomly integrate into the genome. Additionally, while introgression of the *GDF8* 821del11 allele with AAV was efficient, viral methods are unlikely to be popular with consumers of animal protein. Recent studies have demonstrated that single stranded oligonucleotides from 40-100nt in length could serve as effective templates for HDR when stimulated by double-strand breaks (DSBs) in DNA(20, 21, 29-31). Accordingly, we

sought to determine whether this approach could stimulate HDR in primary fibroblasts from pigs and cattle, cellular substrates suitable for the creation of gene-edited animals by cloning. We designed oligonucleotide templates to overlap their cognate TALEN-binding sites and to introduce a 4bp indel into the spacer region for RFLP analysis. Primary fibroblasts were transfected with plasmid- or mRNA-encoded TALENs plus oligo templates and incubated 3 days at either 30 or 37°C.

We found that TALENs delivered as mRNA consistently out-performed plasmid in cells incubated at 30°C, as suggested by Day3 Surveyor (**Fig. 2**). Despite appreciable levels of TALEN activity measured by the Surveyor assay, HDR was consistently higher (> 2-fold) when TALENs were delivered as mRNA compared to plasmids. We speculated that this could have been a kinetic issue; i.e. TALENs from mRNA were more rapidly translated allowing utilization of the template prior to oligo degradation. However, a time-course experiment showed little difference in the onset of HDR between TALENs encoded by plasmid versus mRNA (**Fig. S3**). Among replicates using TALEN mRNA at 30°C, the levels of cumulative mutation and total HDR were similar, suggesting the majority of mutant alleles correspond to the intended introgression.

In our previous study, TALEN-induced indels declined 50-90% after extended culture without the aid of selection(15). In contrast, HDR levels at four loci were roughly equivalent when measured at days 3 and 10 without selective enrichment, indicating that these HDR indel alleles were stable in culture (**Fig. 2**). The consistently high rate (25-50%) and stability of gene edits at all four loci suggested that edited cells should be recoverable by dilution cloning without selective enrichment. Indeed, analysis of circa 1,000 colonies for defined indel alleles in eight separate loci revealed a recovery rate of 10-65% (average 42%) where up to 32% of the colonies are homozygous for the intended edit (**Table 1**).

Differential stability of gene-edits

We were extremely successful in isolating individual colonies with custom indel alleles, however, introgression of SNPs presented a greater challenge. As indicated in **Table 1,** both day-3 levels of HDR (7-18%) and the isolation of cellular clones with the intended SNP alleles (3-15%) within cattle and swine *GDF8* or pig *p65* was significantly lower than for indel alleles, where HDR ranged from 10 to about 50%. We hypothesized that indels were likely more stable than SNP because the introduction or elimination of 4bp in the TALEN spacer region would be expected to reduce re-cleavage of the locus, consistent with known constraints on TALEN spacer length(17).

Accordingly, the introgression of large insertions (*lox*P) was extremely efficient and stable, and comparison of HDR frequencies with oligo within the same locus suggested that even a 4bp insertion allele was more efficient than SNP alleles (**Fig. S4**). Sequence analysis also revealed that nearly half of the isolated SNP-positive colonies for *GDF8* or pig *p65* harbored concomitant indels expected to change TALEN spacing (**Table S2**). Regardless, we were able to recover colonies with homozygous conversion of G938A in *GDF8* (both pigs and cattle) and T1591C in pig *p65* at up to nearly a 5 percent level without any additional changes to the locus (**Table 1** and **Table S2**). We were also able to introgress SNP alleles for the sheep *FecB* and *Callipyge* loci into the goat genome. This ability to precisely alter a single nucleotide is significant and unprecedented.

As a comparison, we were also able to design CRISPR gRNAs that overlapped the T1591C site of *p65* and sought to compare introgression using the two platforms. Despite efficient production of DSB at the intended site, CRISPR/Cas9–mediated HDR was lower than 6 percent at day-3 and below detection at day-10 (**Fig. S5**). Recovery of modified clones with CRISPR–mediated HDR was also lower than with TALENs even though the TALENs cut 35bp away from the SNP site (**Table 1**). Analysis of CRISPR/Cas9 induced targeting at a second locus, ssAPC14.2, was much more efficient, but still did not reach the level of HDR induced by TALENs at this site, circa 30 versus 60% (**Fig. S6**).

Strategies for stabilizing introgressed SNP alleles

Given the conservation of the 5'-thymidine nucleotide immediately preceding TAL-binding sites(32, 33), we reasoned that altering these bases in the oligo (referred to as blocking mutations (BM)) would inhibit re-cleavage of edited alleles. Surprisingly, we found that BMs had no significant impact on the maintenance of SNP alleles at the pig *LDLR* or *GDF8* loci (**Fig. 3a**). This suggested that either the conversion tract for oligo-templated HDR is quite short and does not incorporate the BM, or that altering the 5'-thymidine does not completely abolish TALEN activity.

To examine this in greater detail we conducted Illumina next generation sequencing of 200-250bp amplicons flanking the target sites from populations of cells transfected with oligos and TALEN mRNA. The results from five loci in pigs and cattle show that insertion alleles were in general more prevalent and stable in the population (**Fig. 4**). Whereas BMs had little influence on the preservation of intended alleles in culture, there was a slight bias towards incorporation of BMs in SNP edited alleles versus insertional edits (**Fig. S7**). Consistent with our colony analysis,

reads sorted on the basis of incorporating the intended SNP (iSNP), G938A or T1591C conversion in *btGDF8* and *p65,* revealed that nearly half of the reads with the iSNP had an additional mutation (iSNP+Mut) (**Fig. 4b**), the majority of which were indels (**Fig. S7 and S8**). The majority of iSNP *btGDF8* reads with indels in the spacer also contained one or both BM (**Fig. S8**), demonstrating that modification of the conserved 5'-thymidine was not able to suppress re-cleavage and subsequent indel generation. Thus, this base must be less critical to TALEN-binding than suggested by conservation, and provides a molecular basis for the inability of BMs to preserve alleles as described above.

Another strategy to reduce re-cutting of the SNP edits is to design TALENs such that their binding sites overlap the target SNPs. We evaluated the influence of RVD/nucleotide mismatches within the TALEN-binding site for introgression of G938A SNP into cattle *GDF8.* Two pairs of TALENs were generated, one that bound the wildtype "*G*" allele (btGDF83.6-G) and another that bound the intended "*A*" allele (btGDF83.6-A) (**Fig. 3b**). HDR with each TALEN pair was similar at day-3 whereas levels measured at day-12 were significantly higher using the TALENs that bound the wildtype "*G*" allele, indicating that re-cleavage was more prevalent with btGDF83.6-A which targets the repaired allele perfectly. Different RVD/nucleotide mismatches will likely have greater influence on maintenance of HDR alleles since the NN-RVD used for the wildtype "*G*" TALENs is able to bind both G and A nucleotides. For modification of porcine *EIF4GI*, we found that three RVD/nucleotide mismatches were sufficient for protection of the HDR-edit as nearly 70% of isolated colonies contained an edited allele, greater than half of those being homozygotes (**Table 1** and **Fig. S9**). Thus, the intentional alteration of the target locus to resist re-cleavage is an effective strategy for preserving edits.

Ultimately, gene-editing is a dynamic process. TALEN cleavage and re-cleavage are in flux with repair by NHEJ, HDR with oligo template, and HDR with the sister chromatid as template. We hypothesized that the observed loss of SNP alleles might be reduced by extending the hypothermic treatment, slowing cell proliferation long enough to outlast the burst of TALEN activity from TALEN mRNA transfection. Indeed, this extension almost tripled the level of SNP HDR-edited alleles recovered after extended culture (**Fig. S10**).

Production of biomedical model pigs with gene-edited alleles

We chose two gene-edited loci in the porcine genome to carry through to live animals – *DAZL* and *APC*. Colonies with HDR- or NHEJ- edited alleles of *DAZL* or *APC* were pooled for cloning by chromatin transfer. Each pool yielded two pregnancies from three transfers, of which one

pregnancy each was carried to term. A total of eight piglets were born from *DAZL* modified cells, each of which reflected genotypes of the chosen colonies consistent with either the HDR allele (founders 1650, 1651 and 1657) or deletions resulting from NHEJ (**Fig. 5a**). Three of the *DAZL* piglets, founders 1655-1657, were stillborn. Of the six piglets from *APC* modified cells, one was stillborn, three died within one week, and another died after 3 weeks leaving only founder 1661. The lack of correlation between genotype and survival suggests that early death was related to cloning rather than gene-edits(34). All six *APC* piglets were heterozygous for the intended HDR-edited allele and all but one either had an in-frame insertion or deletion of 3bp on the second allele (**Fig. 5a, b**). Remaining animals are being raised for phenotypic analyses of spermatogenesis arrest (*DAZL*$^{-/-}$ founders) or development of colon cancer (*APC*$^{+/-}$ founders).

III. Discussion

The data in **Table 1** demonstrates that combining mRNAs encoding TALENs and oligonucleotides templates for directing HDR achieves several key benchmarks for a precision genome-editing strategy: 1) only target nucleotides were changed and mRNA transfection avoids unintended integration of plasmid DNA; 2) selection markers are unnecessary, an important aspect relative to acceptance of edited livestock for human consumption; 3) gene edits were efficient; from about 10% for SNPs to above 50% for some larger alterations, and 4) the method was highly reliable at every locus tested, targeted alteration of 16/16 sites (15 genes) was achieved. The efficiency and precision reported here is astonishing compared to what was feasible only a few years ago(2).

There are two concerns in gene editing, stabilizing the changes at the targeted site and avoiding modification of unintended sites. With regard to the first, we found evidence that HDR-edits directing single basepair changes, i.e., SNPs, could be lost (**Figs. 3** and **4b**). Based on the prediction that a thymidine preceding the targeted DNA sequence influences TAL binding(32, 33), we attempted to block re-cleavage of introgressed alleles by introducing BMs. However, we found that BMs did not prevent TALEN activity and re-cleavage of edited alleles (**Fig. 3** and **Figs. S7 and S8**). In contrast, introduction of multiple SNPs or additional sequence (**Figs. 2** and **Fig. S9**) resulted in more stable HDR-edits. Extension of hypothermic culture also resulted in the stabilization of introgressed SNP alleles. Since hypothermia slows cell proliferation primarily by prolonging G1-phase of the cell cycle(35), it may be that this treatment differentially favors oligo-HDR versus sister chromatid templated repair in a cell-cycle dependent manner. Regardless of the mechanism, this approach offers a straight-forward strategy for recovering cells with

precise introgression of SNP alleles. Regarding the second concern, the frequency of modification at unintended sites is exceedingly low and in the context of livestock breeding, will be lost by independent segregation in subsequent generations(2).

Gene-editing for single-step introgression of valuable alleles into livestock
A variety of objectives were achieved by precise gene editing (**Table 1**). Knockout of genes of biomedical relevance was accomplished by interrupting coding sequences with 4bp indels. This strategy was very reliable and generally resulted in HDR-edits in about 40% of the clones (range 26-60%), and of those, up to one-third were homozygotes. At similar frequencies, we integrated a modified *loxP(mloxP)* site into *ROSA26,* a presumptive *safe harbor* locus, and *SRY* loci in cattle and pigs that can be used as a *landing pad* for insertion of novel sequences in livestock via recombinase-mediated cassette exchange(36, 37). Previously, only NHEJ edits had been demonstrated for the Y chromosome of livestock(15), however, TALENs are clearly suitable for direct stimulation of knockout/knock-in, a capability we are exploiting in livestock, and recently demonstrated in mice(38).

Of more immediate value, we achieved efficient non-meiotic introgression of native alleles between species or breeds. Inter-species introgression is impossible by breeding and intra-species crossbreeding for allele introgression is either insufficient or deteriorating to other performance merits due to the genetic admixture. Our non-meiotic introgression includes the double-muscling mutations of *GDF8* (SNP G938A(23, 25) or 821del11(23-25) from Piedmontese and Belgian Blue cattle respectively) into the genome of Wagyu cattle and Landrace pigs. For improvement of animal welfare, we transferred the Pc allele for polledness from a beef producing breed into cells from horned dairy cattle. We also transferred a candidate SNP allele for African swine fever virus resilience T1591C of *p65*(39) from warthog to the genome of conventional swine cells and introgressed sheep SNPs responsible for elevated fecundity (FecB; *BMPR-IB*)(40) and parent-of-origin dependent muscle hypertrophy (Callipyge)(41) into the goat genome. Non-meiotic allele introgression has not previously been possible without selective enrichment, and our efficiencies are at least 10^5-fold higher than results previously obtained with selection(42, 43). Such high levels of unselected single-allele introgression suggests it will be feasible to alter multiple alleles in a single generation of farm animals, decreasing the impact of long generation intervals on genetic improvement.

Implications for animal breeding and human medicine

High throughput genome sequencing coupled with detailed phenotyping provides unprecedented opportunities for the identification of specific alleles that affect livestock performance, as well as both locus-centric and whole-genome based selection for improvement of animal genetics (44). Our results suggest that gene-editing can be incorporated into selection programs to accelerate genetic improvement when selective breeding is either inefficient or impossible. Non-meiotic introgression provides a genetic method to precisely crossbreed for large effect alleles without compromising the genetic merit of indigenous or purpose bred populations by whole genome admixture(2). By allowing single-generation introgression of select alleles, in the near future gene editing will accelerate genetic improvement based on local ecologies and needs that may derive from climate change and emerging diseases.

For the first time we used customized endonucleases to generate live animals with precise edits at two independent loci. Pigs edited to disrupt the *DAZL* gene can serve as a model for studying the restoration of human fertility by germ cell transplantation, or for the production of genetically modified offspring by transfer of genetically modified germline stem cells as demonstrated in pigs(45), goats(46) and rodents(47, 48). Gene edited alleles of *APC* could also provide a size-relevant model of colon cancer for pre-clinical evaluation of therapeutics, surgical intervention or detection modalities. These results, coupled with our previous findings(15), demonstrate relative ground breaking ease in introducing genetic modifications that mimic natural polymorphisms or human disease alleles into livestock.

Our work also has implications for personalized medicine, demonstrating the ability to develop precisely engineered large animals for tailored biomedical models for testing drug, device, and cellular therapeutics, and potentially as resources for xenogeneic and autologous therapeutic cells and organs. Finally, the precision and high efficiency we have achieved in altering either single nucleotides or small sequences in livestock fibroblasts is likely to be applicable to correcting disease-causing mutations in patient-derived human fibroblasts, a resource for conversion to induced pluripotent stem cells for use in cellular therapy(49), without employing exogenous marker genes for selection.

IV. Methods

Please refer to the Supplement and our previous paper(15)for detailed information.

Briefly, TALENs were assembled using the Golden Gate assembly protocol and library(50). TALEN-encoding mRNA was synthesized in vitro using the mMESSAGE mMACHINE® T3 Kit (Ambion). The CRISPR/Cas9 site-specific endonucleases employed in our HDR experiments were

generated based on the Church lab system and methods(8). All the transfections were conducted using the Neon transfection system (Invitrogen) unless otherwise stated. The frequency of total mutations in a TALEN or CRISPR/Cas9 transfected population was assessed by the Surveyor Mutation Detection Kit (Transgenomic) and rates of mutation (% Surveyor) was calculated by the Guschin method(51); %HDR was evaluated by RFLP or PCR instead. Colonies listed in **Table 1** were obtained by dilution cloning from corresponding populations without any drug selection. Population samples were sequenced on a Illumina MiSeq sequencer after PCR amplification to dissect the dynamics of HDR. All the RVD sequences of TALENs used in this paper, all oligo sequences for HDR, and all PCR primers are listed in **Tables S1,S3,S4,S5,S6**. Pigs were cloned by CT under contract with Minitube of America under its Animal Welfare Assurance protocol #A4520/01.

TALEN designing and production. Candidate TALEN target DNA sequences and RVD sequences were identified using the online tool "TAL Effector Nucleotide Targeter" (https://tale-nt.cac.cornell.edu/about). Plasmids for TALEN DNA transfection or in vitro TALEN mRNA transcription were then constructed by following the Golden Gate Assembly protocol(1) using pC-GoldyTALEN (Addgene ID 38143) and RCIscript-GoldyTALEN (Addgene ID 38143) as final destination vectors(2). The final pC-GoldyTALEN vectors were prepared by using PureLink® HiPure Plasmid Midiprep Kit (Life Technologies) and sequenced before usage. Assembled RCIscript vectors prepared using the QIAprep Spin Miniprep kit (Qiagen) were linearized by SacI to be used as templates for in vitro TALEN mRNA transcription using the mMESSAGE mMACHINE® T3 Kit (Ambion) as indicated previously(2). Refer to **Supplementary Table 3** for the RVD sequences of all the TALENs used in this paper. Modified mRNA was synthesized from RCIScript-GoldyTALEN vectors as previously described(2) substituting a ribonucleotide cocktail consisting of 3'-0-Me-m7G(5')ppp(5')G RNA cap analog (New England Biolabs), 5-methylcytidine triphosphate pseudouridine triphosphate (TriLink Biotechnologies, San Diego, CA) and adenosine triphosphate and guanosine triphosphate. Final nucleotide reaction concentrations are 6 mM for the cap analog, 1.5 mM for guanosine triphosphate, and 7.5 mM for the other nucleotides. Resulting mRNA was DNAse treated prior to purification using the MEGAclear Reaction Cleanup kit (Applied Biosciences).

CRISPR/Cas9 design and production. Gene specific gRNA sequences were cloned into the Church lab gRNA vector (Addgene ID: 41824) according their methods(3). The Cas9 nuclease was provided either by co-transfection of the hCas9 plasmid (Addgene ID: 41815) or mRNA synthesized from RCIScript-hCas9. This RCIScript-hCas9 was constructed by sub-cloning the XbaI-AgeI fragment from the hCas9 plasmid (encompassing the hCas9 cDNA) into the RCIScript plasmid. Synthesis of mRNA was conducted as above except that linearization was performed using KpnI.

Donor repair template preparation

BB-HDR (1,623bp) plasmid. A 1,695bp fragment encompassing the Belgian Blue allele was PCR amplified (btGDF8 BB 5-1: **5'-**CAAAGTTGGTGACGTGACAGAGGTC; btGDF8 BB 3-1: 5'-

GTGTGCCATCCCTACTTTGTGGAA) from Belgian Blue genomic DNA and TOPO cloned into the PCR 2.1 vector (Life Technologies). This plasmid was used as positive control template for analytical primer sets and for derivation of the 1,623bp BB-HDR template by PCR with following primers (BB del HR 1623 5-1: 5'-GATGTATTCCTCAGACTTTTCC; BB del HR 1623 3-1: 5'- GTGGAATCTCATCTTACCAA) and TOPO cloned as before. Each plasmid was sequence verified prior to use. Transfection grade plasmid was prepared using the Fast-Ion MIDI Plasmid Endo-Free kit (IBI Scientific).

rAAV packaging. BB-HDR was cloned into pAAV-MCS and packaged into using the Adeno-Associated Virus Helper-Free system (Agilent). Briefly, a 10cm dish AAV-293 cells was transfected with 5 µg each: pAAV-Helper, pAAV-RC and the AAV-BB-HDR plasmid. Two days post transfection, the cells were removed from the plate by scraping into 1 ml of growth media. Viral particles were released by 3 freeze-thaw cycles prior to centrifugation at maximum speed in a microcentrifuge for 5 minutes. The supernatant was aspirated and used directly for infection of target cells.

Pc *HDR template.* A 1,784bp fragment encompassing the Celtic *POLLED* allele was PCR amplified (F1: 5'-GGGCAAGTTGCTCAGCTGTTTTTG; R1- 5'-TCCGCATGGTTTAGCAGGATTCA) from angus genomic DNA and TOPO cloned into the PCR 2.1 vector (Life Technologies). This plasmid was used as positive the control template for analytical primer sets and for derivation of the 1,592bp HDR template by PCR with following primers (1594 F: 5'-ATCGAACCTGGGTCTTCTGCATTG; R1: 5'-TCCGCATGGTTTAGCAGGATTCA) and TOPO cloned as before. Each plasmid was sequence verified prior to use. Transfection grade plasmid was prepared using the Fast-Ion MIDI Plasmid Endo-Free kit (IBI Scientific) and 5 µg or 10 µg was transfected along with 2 µg HP1.3 TALEN mRNA.

Oligonucleotide templates. All oligonucleotide templates were synthesized by Integrated DNA Technologies, 100 nmole synthesis purified by standard desalting, and resuspended to 400 µM in TE. See **Supplementary Table 4** for the complete list of oligo templates.

Tissue culture and transfection. Pig, cattle or goat fibroblasts were maintained at 37 or 30 °C (as indicated) at 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 I.U./ml penicillin and streptomycin, and 2mM L-Glutamine. For transfection, all TALENs, CRISPR/Cas9 and HDR templates were delivered through transfection using the Neon Transfection system (Life Technologies) unless otherwise stated. Briefly, low passage Ossabaw[9], Landrace, Wagyu, Holstein[10] or goat[11] fibroblasts reaching 100% confluence were split 1:2 and harvested the next day at 70-80% confluence. The goat cells used in this study derived from a 35-40 day male Nubian x Boer fetus. Each transfection was comprised of 500,000-600,000 cells resuspended in buffer "R" mixed with plasmid DNA or mRNA and oligos and electroporated using the 100ul tips by the following parameters: input Voltage; 1800V; Pulse Width; 20ms; and Pulse Number; 1. Typically, 2-4 µg of TALEN expression plasmid or 1-2 µg of TALEN mRNA and 2-

[9] Dr. Michael Sturek provided Ossabaw swine cells.

[10] We thank Drs. Jared Decker and Jerry Taylor for Angus DNA and Dr. Brian Crooker for dairy cattle cells.

[11] We thank Dr. Irina Polejaeva for goat cells.

3 μM of oligos specific for the gene of interest were included in each transfection. Deviation from those amounts is indicated in the figure legends for both TALENs and CRISPR/Cas9 experiments. After transfection, cells were divided 60:40 into two separate wells of a 6-well dish for three days' culture at either 30 or 37°C respectively. After three days, cell populations were expanded and at 37°C until at least day 10 to assess stability of edits.

Plasmid and rAAV HDR in Wagyu Fibroblasts. Low passage Wagyu fibroblasts were cultured to 70-90% confluence and transfected by Nucleofection (Lonza) with 2 µg each TALEN expression plasmid (btGDF83.1L+NR, **Supplementary Table 3**) along with 750 ng of *Sleeping Beauty* transposon components as previously described(2). For conditions where plasmid HDR template was used, 2 µg of BB-HDR plasmid was also included in the transfection. Transfected cells were split between two wells of a 6-well plate for culture at 30 or 37°C. For conditions using rAAV HDR template, 150 µl of viral lysate was added to each well 2 hours post transfection. After incubation for three days, cells were harvested by trypsinization, a portion of which were lysed for analysis of HDR at day 3, and the remainder were plated for colony isolation as previously described(2).

Dilution cloning: Three days post transfection, 50 to 250 cells were seeded onto 10 cm dishes and cultured until individual colonies reached circa 5mm in diameter. At this point, 6 ml of TrypLE (Life Technologies) 1:5 (vol/vol) diluted in PBS was added and colonies were aspirated, transferred into wells of a 24-well dish well and cultured under the same conditions. Colonies reaching confluence were collected and divided for cryopreservation and genotyping.

Sample preparation: Transfected cells populations at day 3 and 10 were collected from a well of a 6-well dish and 10-30% were resuspended in 50 µl of 1X PCR compatible lysis buffer:10 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.45% Tryton X-100(vol/vol), 0.45% Tween-20(vol/vol) freshly supplemented with 200 µg/ml Proteinase K. The lysates were processed in a thermal cycler using the following program: 55°C for 60 minutes, 95°C for 15minutes. Colony samples from dilution cloning were treated as above using 20-30 µl of lysis buffer.

Surveyor mutation detection and RFLP analysis. PCR flanking the intended sites was conducted using Platinum Taq DNA polymerase HiFi (Life Technologies) with 1 µl of the cell lysate according to the manufacturer's recommendations. Primers for each site are listed in **Supplementary Table 5**. The frequency of mutation in a population was analysed with the Surveyor Mutation Detection Kit (Transgenomic) according to the manufacturer's recommendations using 10 ul of the PCR product as described above. RFLP analysis was performed on 10 µl of the above PCR reaction using the indicated restriction enzyme. Surveyor and RFLP reactions were resolved on a 10% TBE polyacrylamide gels and visualized by ethidium bromide staining. Densitometry measurements of the bands were performed using ImageJ; and mutation rate of Surveyor reactions was calculated as described in Guschin et al. 2010(4). Percent HDR was calculated via dividing the sum intensity of RFLP fragments by the sum intensity of the parental band + RFLP fragments. For analysis of *mloxP* insertion, small PCR products spanning the

insertion site were resolved on 10% polyacrylamide gels and the insert versus wild type alleles could be distinguished by size and quantified. RFLP analysis of colonies was treated similarly except that the PCR products were amplified by 1X MyTaq Red Mix (Bioline) and resolved on 2.5% agarose gels.

For analysis of clones for introgression of the *GDF8* G938A-only (oligos lacked a novel RFLP), colonies were initially screened by a three primer assay that could distinguish between heterozygous and homozygous introgression. Briefly, lysates from pig or cattle colonies were analysed by PCR using 1X MyTaq Red Mix (Bioline) using the following primers and programs. Cattle *GDF8* (Outside F1: 5'-CCTTGAGGTAGGAGAGTGTTTTGGG, Outside R1: 5'-TTCACCAGAAGACAAGGAGAATTGC, Inside F1: 5'-TAAGGCCAATTACTGCTCTGGAGACTA; and 35 cycles of (95°C, 20 s; 62°C, 20 s; 72°C, 60 s). Pig *GDF8*: Outside F1: 5'-CCTTTTTAGAAGTCAAGGTAACAGACAC, Outside R1: 5'-TTGATTGGAGACATCTTTGTGGGAG, Inside F1: 5'-TAAGGCCAATTACTGCTCTGGAGATTA; and 35 cycles of (95°C, 20 s; 58°C, 20 s; 72°C, 60 s). Amplicons from candidates were sequenced directly and/or TOPO cloned (Life Technologies) and sequenced by Sanger sequencing.

To detect TALEN-mediated HDR at with the BB-HDR template, either 1 μl or 1 μl of a 1:10 dilution of PCR-lysate (1,000 cells/ul) was added to a PCR reaction with PCR primers bt GDF8 BB 5-1 (primer "c") and primer "c' " (BB-Detect 3-1- 5'-GCATCGAGATTCTGTCACAATCAA) and subjected to PCR with using 1X MyTaq Red mix (Bioline) for 40 cycles (95°C, 20 s; 66°C, 20 s; 72°C, 60 s). To confirm HDR in colonies identified by the above PCR, amplification of the entire locus was performed with primers bt GDF8 BB 5-1 and bt GDF8 BB 3-1 followed by TOPO cloning (Life Technologies) and sequencing.

Detection of Pc introgression was performed by PCR using the F1 primer (see above) and the "P" primer (5'-ACGTACTCTTCATTTCACAGCCTAC) using 1X MyTaq Red mix (Bioline) for 38 cycles (95°C, 25 s; 62°C, 25 s; 72°C, 60 s). A second PCR assay was performed using (F2: 5'-GTCTGGGGTGAGATAGTTTTCTTGG; R2- 5'-GGCAGAGATGTTGGTCTTGGGTGT). Candidates passing both tests were analysed by PCR using the flanking F1 and R1 primers followed by TOPO cloning and sequencing.

Detection of *FecB* introgression was performed as previously described for sheep(5). *Callipyge* introgression was detected by an AvaII RFLP assay using primers indicated in **Supplementary Table 5**.

Amplicon sequencing and analysis. DNA was isolated from transfected populations and 100-250 ng was added to a 50 μl Platinum *Taq* DNA Polymerase High Fidelity (Life Technologies) assembled per the manufacturer's recommendations. Each sample was assigned a primer set with a unique barcode to enable multiplex sequencing (**Supplementary Table 6**). A portion of the PCR product was resolved on a 2.5% agarose gel to confirm size prior to PCR cleanup using the MinElute PCR Purification Kit (Qiagen). Samples were submitted to the University of Minnesota Genomics Center where they were quantified and pooled into a single sample for sequencing. The single combined sample was spiked with 25% PhiX (for sequence diversity) and sequenced on an Illumina MiSeq sequencer generating 150 base-

pair paired-end reads. Read quality was assessed using FastQC (www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Read-pairs with overlapping ends were joined using fastq-join from the ea-utils package(6). A custom perl script was used to demultiplex the joined reads and count insert types. Exact matches to the forward and reverse primers were required in the demultiplexing step.

Cloned animals were genotyped by RFLP assay and sequencing.

V. Conflict of interest statement

DFC, CAL, DAW, PH and SCF either have equity and/or work for Recombinetics, Inc., a new biotech company that receives funding from the NIH to develop gene-editing in commercial livestock.

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Table 1. Frequencies for recovery of colonies with HDR alleles

Reagent	ID	Species	Mutation type	nt change	aa change	Day 3 % HDR	HDR+ (%)	Bi-allelic HDR+ (%)
TALEN	ssLDLR2.1*	Pig ♀	Ins/FS	141(ins4)	47ΔPTC	38	55/184 (30)	4/184 (2)
TALEN	ssDAZL3.1†	Pig ♂	Ins/FS	173(ins4)	57ΔPTC	25	34/92 (37)	8/92 (9)
TALEN	ssDAZL3.1 Rep	Pig ♂	Ins/FS	173(ins4)	57ΔPTC	30	42/124 (34)	7/124 (6)
TALEN	ssAPC14.2†	Pig ♂	Ins/FS	2703(ins4)	902ΔPTC	48	22/40 (55)	4/40 (10)
TALEN	ssAPC14.2 Rep	Pig ♂	Ins/FS	2703(ins4)	902ΔPTC	50	57/96 (60)	19/96 (20)
TALEN	ssAPC14.2 Ld	Pig ♂	Ins/FS	2703(ins4)	902ΔPTC	34	21/81 (26)	1/81 (1)
TALEN	ssTp53	Pig ♂	Ins/FS	463(ins4)	154ΔPTC	22	42/71 (59)	12/71 (17)
TALEN	ssRAG2.1	Pig ♂	Ins/FS	228(ins4)	76ΔPTC	47	32/77 (42)	13/77 (17)
TALEN	btRosa1.2‡	Cow ♂	*Ins/mloxP*	ins34	NA	45	14/22(64)	7/22(32)
TALEN	ssSRY3.2	Pig ♂	*Ins/mloxP*	ins34	NA	30	ND	ND
TALEN	ssKissR3.2	Pig ♂	Ins/FS	322(ins6) 323(del2)	107ΔPTC	53	57/96(59)	17/96(18)
TALEN	btGDF83.1	Cow ♂	del/FS	821 (del11)	FS	~10	7/72 (10)	2/72 (3)
TALEN	ssEIF4GI14.1	Pig ♂	SNPs	G2014A T2017C C2019T	N672D L673F	52	68/102(67)	40/102(39)
TALEN	btGDF83.6N	Cow ♂	SNPs	G938A T945C	C313Y	18	8/94 (9)	3/94 (3)
TALEN	btGDF83.6N§	Cow ♂	SNP	G938A	C313Y	NA	7/105 (7)	2/105 (2)
TALEN	ssP65.8	Pig ♂	SNP	T1591C	S531P	18	6/40 (15)	3/40 (8)
TALEN	ssP65.8 Rep	Pig ♂	SNP	T1591C	S531P	7	9/63 (14)	5/63 (8)
TALEN	ssGDF83.6§	Pig ♂	SNP	G938A	C313Y	NA	3/90 (3)	1/90(1)
TALEN	caFecB6.1	Goat ♂	SNP	A747G	Q249R	17	17/72 (24)	3/72 (4)
TALEN	caCLPG1.1	Goat ♂	SNP	A→G	Extra-genic	4	ND	ND
CRISPR	ssP65 G1s	Pig ♂	SNP	T1591C	S531P	6	6/96 (6)	2/96 (2)
CRISPR	ssP65 G2a	Pig ♂	SNP	T1591C	S531P	5	2/45 (4)	0/45
CRISPR	APC14.2 G1a	Pig ♂	Ins/FS	2703(ins4)	902ΔPTC	32	ND	ND

Abbreviations: FS = frame shift; SNP = single nucleotide polymorphism; PTC = premature termination codon; Rep = independent replicate; ND = not determined.
Ld: Low dosage, 100 ng versus the typical 1-2 μg of TALEN mRNA was used for this replicate to reduce the number of homozygous *APC* mutants.
*: Homozygous null *LDLR*$^{-/-}$ could not be propagated in culture.
†. HDR positive colonies from these replicates were used to generate founder animals by cloning (Fig. 5)
‡. A modified *loxP* site (*mloxP*) was inserted into *btROSA26* and *ssSRY*; positive colonies were identified by PCR.
§: Only the target SNP was introduced into these genes, hence, RFLP analysis was not applicable; A tri-primer PCR combined with Sanger sequencing was designed to identify positive colonies (see Online Methods).

Supplementary Table 1: Sequence analysis of clones introgressed with the POLLED allele

Clone	Haplotype predicted by PCR	Allele	Homology Template	% Identity*	Number of INDELs
HP8 P6 A2	Heterozygous	*HORNED*	5 µg plasmid HP1594bp	86.8%	2
		POLLED		100%	0
HP8 P3 B5	Heterozygous	*HORNED*	5 µg plasmid HP1594bp	99.4%	1
		POLLED		100%	0
HP7 P4 A1	Homozygous	*POLLED*	10 µg plasmid HP1594bp	100%	0
HP14-30 P2 B4	Homozygous	*POLLED*	5 µg plasmid HP1594bp	100%	0
HP14-30 P3 B6	Homozygous	*POLLED*	5 µg plasmid HP1594bp	100%	0

* From primer pair HP1748F to HP1748R

Supplementary Table 2: Sequence analysis of SNP-introgressed cellular clones

Colony ID	Allele 1	Allele 2
btGDF8 G938A only		
C8	iSNP	iSNP
E3	iSNP	iSNP + Indel
A12	iSNP	WT
E7	iSNP	Indel
H5	iSNP	Indel
G8	iSNP + indel	WT
H3	iSNP +indel	Indel
D3	WT	WT
H10	WT	WT
ssP65		
B4	iSNP	iSNP
D4	iSNP	iSNP
D8	Homozygous iSNP + indel	
E7	Homozygous iSNP + indel	
B6	Homozygous iSNP + indel	
ssP65 Rep		
A8	iSNP	iSNP
E9	iSNP	WT
D2	iSNP	WT
C7	Homozygous iSNP + indel	
A4	Homozygous iSNP + indel	
D1	Homozygous iSNP + indel	
D7	iSNP + indel	indel
A3	iSNP + indel	WT
B7	iSNP + indel	indel
A10	Indel	WT
ssGDF8 G938A only		
4C6	iSNP	iSNP

Supplementary Table 2 Continued

3A1	iSNP + indel	WT
2D2	iSNP + indel	WT
3D2	ND	ND

iSNP = Intended SNP

Supplementary Table 3. TALEN sequences

TALEN pair	TALEN RVD sequence	DNA Target sequence (Sense strand)
ssLDLR2.1	HD NG HD HD NG NI HD NI NI NN NG NN NN NI NG NG NG HD NN NN NI HD HD HD NN NG HD HD NG NG NN HD NI HD NG	CTCCTACAAGTGGATTTGTGATGGGAACACCGAGT GCAAGGACGGGTCCG
btGDF83.1L+NR	NN NG NN NI NG NN NI NI HD NI HD NG HD HD NI HD NI NN NI NI NG HD NG NG HD NI NI NI NI NG HD HD NI HD NI NN NG NG NI NN NI NN	GTGATGAACACTCCACAGAATCTCGATGCTGTCGT TACCCTCTAACTGTGGATTTTGA
ssDAZL3.1	NN NN NI NG NN NI NI NI HD HD NN NI NI NI NG NG HD NG NG NG NG NI HD NG NN NI NI HD HD NI NG NI NG	GGATGAAACCGAAATTAGAAGTTTCTTTGCTAGAT ATGGTTCAGTAAAAG
ssAPC14.2	NN NN NI NI NN NI NI NN NG NI NG HD NI NN HD HD NI NG NN NI HD HD HD NI NN NI NI NG NG NG HD NG NN NG	GGAAGAAGTATCAGCCATTCATCCCTCCCAGGAAG ACAGAAATTCTGGGTC
ssTp53	NN NN HD NI HD HD HD NN NG NN NG HD HD NN HD NN HD HD NI NG NN NG NI HD NG HD NG NN NI HD NG NG	GGCACCCGTGTCCGCGCCATGGCCATCTACAAGAA GTCAGAGTACATG
ssKissR3.2	NN HD NG HD NG NI HD NG HD NG NI HD HD HD HD NN HD NI HD NI NG NN NI NI NN NG HD NN HD HD HD NI	GCTCTACTCTACCCCCTACCAGCCTGGGTGCTGGGC GACTTCATGTGC
ssEIF4GI14.1	HD HD NN NG HD HD NG NG NG NN HD HD NI NI HD HD NG NG NG NN NN NN NN NN HD HD HD NI HD NN NN NG NG NN HD NG	CCGTCCTTTGCCAACCTTGGCCGACCAGCCCTTAGC AACCGTGGGCCCCCA
btGGTA9.1	HD NG NN HD NN HD NG HD HD NG NG HD NI NI NI NN NG NN NG HD HD NG NN HD HD NI HD HD NG HD NG NG HD NG	CTGCGCTCCTTCAAAGTGTTTAAGATCAAGCCTGA GAAGAGGTGGCAGGAC
ssRAG2.1	NI HD HD NG NG HD HD NG HD HD NG HD NG HD HD NN HD NG HD NG NI NI NN HD NG NN HD NG NG NG NG NN NI NI NG	ACCTTCCTCCTCTCCGCTACCCAGCCACTTGCACAT TCAAAAGCAGCTTAG
ssIL2Rg2.1	HD HD HD NI NI NI NN NN NG NG HD NI NN NG NN NG NG NG HD HD NI NI NN NG NN HD NI NI NG NG HD NI NG NN NG NI HD NG	CCCAAAGGTTCAGTGTTTGTGTTCAATGTTGAGTA CATGAATTGCACTTGG
btGDF83.6-A	NN HD NG HD NG NN NN NI NN NI NI NG NI NG NI NG NN NI NN NN NI NG NI HD NG NG NG NG	GCTCTGGAGAATATGAATTTGTATTTTTGCAAAAGT ATCCTCAT
btGDF83.6-G	NN HD NG HD NG NN NN NI NN NI NI NG NNNG NI NG NN NI NN NN NI NG NI HD NG NG NG NG	GCTCTGGAGAATGTGAATTTGTATTTTTGCAAAAGT ATCCTCAT
ssGDF83.6	NI HD NG NN HD NG HD NG NN NN NI NN NI NN NG NN NG NN NI NN NN NN NG NI NG NG NG NG NG NN NG	ACTGCTCTGGAGAGTGTGAATTTGTATTTTTACAAA AATACCCTCAC

Supplementary Table 3 continued		
btRosa1.2	HD NG HD NN HD NI NG NG NN HD HD HD NI HD NG HD NG HD NG HD NG HD HD NI HD HD HD NG NI HD HD NG	CTCGCATTGCCCACTGGGTGGGTGCTTAGGTAGGT AGGGTGGAGAGAG
ssSRY3.2	NI NG NI HD NI NG NG NG NG NI HD NI HD NI HD NI NG NI NG NI NN NN NG NG HD NI NN NN HD HD NI NG NG NI NI NG	ATACATTTTACACACATATATATGAAACTGACAGT ATTAATGGCCTGAACCT
caFecB6.1	NI HD NI NN NI NN NN NI NN NN HD HD NI NN HD NG NN NN NG NG HD NI NG HD NI NI HD NI HD HD NN NG HD NG NN NI NG NI NG	ACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAA TATATCAGACGGTGTTGATG
caCLPG1.1	NN NI NN NI NN HD NN HD NI NN NN NI NI NG HD HD NI NN NN HD NG NN NI HD NI NN NN NG NN NN NG HD HD HD NI NN HD	GAGAGCGCAGGAATCCAGGCGCAGGGGCCCGAGG GCTGGGACCACCTGTCAG
btHP1.3	NG NG NG HD NG NG NN NN NG NI NN NN HD NG NN NN NI NI NI NI NN NI NN NI NN NG NG NG NG NN NI NG	TTTCTTGGTAGGCTGGTATTCTTGCTCTTTAGATCA AAACTCTCTTTC
ssP65_11.1	NN HD HD HD HD HD HD HD NI HD NI HD NI NN HD NG NI NG NI NN HD HD NG HD NI NN NN NN NG NI HD NG	GCCCCCCCACACAGCTGAGCCCATGCTGATGGAGT ACCCTGAGGCTAT
ssP65.8	HD NG HD HD NG HD HD NI NG NG NN HD NN NN NI NN NI NG HD NG NN NI HD NG HD NI NN NI NI NN	CTCCTCCATTGCGGACATGGACTTCTCAGCCCTTCT GAGTCAGATC

Abbreviations: ss= *Sus scrofa*; bt= *Bos taurus*; ca= *Capra aegagrus.*
Note: RVD sequences for left and right TALEN monomers are shown top and bottom respectively oriented from the N to C terminus. Red text indicates TALEN binding sites

Supplementary Table 4. Oligonucleotide HDR templates

TALEN pair	ssODN design	Sequence
ssLDLR2.1	46_SNPs BamHI	CCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTC
ssLDLR2.1	90_SNPs BamHI	TGCCGAGACGGGAAATGCATCTCCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTCCG ATGAGTCCCTGGAGACGTGC
ssLDLR2.1	90_ins4_BM BamHI	CCGAGACGGGAAATGCACCTCCTACAAGTGGATTTGTGATGGATCCGAACACCGAGTGCAAGGACGGGTC CGCTGAGTCCCTGGAGACGT
ssLDLR2.1	90_SNPs_BM BamH1	TGCCGAGACGGGAAATGCACCTCCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTCCG CTGAGTCCCTGGAGACGTGC
ssLDLR2.1	60_SNPs_BM BamH1	TGCACCTCCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTCCGCTGAG
ssLDLR2.1	86_del4_BM BamH1	TGCCGAGACGGGAAATGCACCTCCTACAAGTGGATTTGGGATCCACCGAGTGCAAGGACGGGTCCGCTGA GTCCCTGGAGACGTGC
ssDAZL3.1	90_ins4_BM BamHI	AATTCTTCTCCATAGACGGATGAAACCGAAATTAGAAGTTGGATCCTTTGCTAGATATGGTTCAGTAAAAG GAGTGAAGATATTCACAGA
ssAPC14.2	90_ins4_BM HindIII	CCAGATCGCCAAAGTCACGGAAGAAGTATCAGCCATTCATCCCTCCCAGTGAAGCTTACAGAAATTCTGGG TCGACCACGGAGTTGCACT
ssTp53	90_ins5_BM HindIII	AGCTCGCCACCCCCGCCGGGCACCCGTGTCCGCGCCATGGCCATCTAAGCTTAAAGAAGTCAGAGTACATG CCCGAGGTGGTGAGGCGCT
ssKissR3.2	90_ins4 HindIII	GTGCTGCGTGCCCTTTACTGCTCTACTCTACCCCCTACCAGCCTAAGCTTGTGCTGGGCGACTTCATGTGCA AGTTCCTCAACTACATCC
ssEIF4GI14.1	90_SNP-NL-DF EagI	CCCAGACTTCACTCCGTCCTTTGCCGACTTCGGCCGACCAGCCCTTAGCAACCGTGGGCCCCCAAGGGGTG GGCCAGGTGGGGAGCTGCC
btGGTA9.1	90_del4_BM HindIII	GCCTTTGATAGAGTTGGGTCCCCTGCGCTCCTTCAAAGTGTTTAAGCTTCTGAGAAGAGGTGGCAGGACCTC AGCATGATGCGCATGAAG
ssRAG2.1	90_ins4_BM HindIII	CTCTAAGGATTCCTGCCACCTTCCTCCTCTCCGCTACCCAGACTAAGCTTTGCACATTCAAAAGCAGCTTAG GGTCTGAAAAACATCAGT
ssIL2Rg2.1	90_ins4_BM HindIII	TTCCACTCTACCCCCCCCAAAGGTTCAGTGTTTTGTGTAAGCTTCAATGTTGAGTACATGAATTGCACTTGG GACAGCAGCTCTGAGCTC
btRosa1.2	41_mLoxP 75bp total	CTCGCATTGCCCACTGGGTGATAACTTCGTATAGCATACATTATAGCAATTTATGGTGCTTAGGTAGGTAGG GTG
btRosa1.2	60_LoxP 94bp total	GGGACTCTCGCATTGCCCACTGGGTGGGTATAACTTCGTATAATGTATGCTATACGAAGTTATGCTTAGGTA GGTAGGGTGGAGAGAGACTTGG
ssSRY3.2	DS 3.2 mLoxP 86bp total	ATACATTTTACACACATATATATGAAAATAACTTCGTATAGCATACATTATAGCAATTTATCTGACAGTATT AATGGCCTGAACCT

Supplementary Table 4. Continued		
caFecB6.1	FecB-A-G	AAAGTGTTCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCGGACGGTGTTGATGAG GCATGAAAACATCTTGGGC
caCLPG1.1	CLPG A- G AvaII (loss of)	TGCTGAGAGCGCAGGAATCCAGGCGCAGGGGCCCGAGGGCTGGGGCCACCTGTCAGATCCTTTCCCCAGCT GAAGGCAGGGTGTGGGTGA
btGDF83.1	71_del11	GGAGAGATTTTGGGCTTGATTGTGACAGAATCTCGATGCTGTCGTTACCCTCTAACTGTGGATTTTGAAGC
btGDF83.6-G	90_SNPs_BM EcoRI	CTAAAAGATATAAGGCCAATTACCGCTCTGGAGAATATGAATTCGTATTTTTGCAAAAGTATCCTCATCCCC ATCTTGTGCACCAAGCAA
btGDF83.6-G	90_SNPs EcoRI	CTAAAAGATATAAGGCCAATTACTGCTCTGGAGAATATGAATTCGTATTTTTGCAAAAGTATCCTCATACCC ATCTTGTGCACCAAGCAA
btGDF83.6-G	90_SNP	CTAAAAGATATAAGGCCAATTACTGCTCTGGAGAATATGAATTTGTATTTTTGCAAAAGTATCCTCATACCC ATCTTGTGCACCAAGCAA
ssP65.8	90_SNP XmaI	GGGCCTCTGGGCTCACCAACGGTCTCCTCCCGGGGGACGAAGACTTCTCCTCCATTGCGGACATGGACTTCT CAGCCCTTCTGAGTCAGA
ssP65-S-P (CRISPR)	S-P-HDR XmaI	GCTCCCACTCCCCTGGGGGCCTCTGGGCTCACCAACGGTCTCCTCCCGGGGGACGAAGACTTCTCCTCCATTGCGGACATGGACTTCT CA
ssGDF83.6	90_SNPs_BM EcoRI	CCCAAAAGATATAAGGCCAGTCACTGCTCTGGAGAGTATGAATTCGTATTTTTACAAAAATACCCTCACCC TCATCTTGTGCACCAAGCA
ssGDF83.6	90_SNPs EcoRI	CCCAAAAGATATAAGGCCAGTTACTGCTCTGGAGAGTATGAATTCGTATTTTTACAAAAATACCCTCACAC TCATCTTGTGCACCAAGCA
ssGDF83.6	90_SNP	CCCAAAAGATATAAGGCCAGTTACTGCTCTGGAGAGTATGAATTTGTATTTTTACAAAAATACCCTCACACT CATCTTGTGCACCAAGCA

Oversized red text represents intended SNPs; regular size red text stands indicates BMs or nucleotide changes to generate restriction sites for RFLP screening; orange texts indicates insertions; blue text indicates TALEN or CRISPR binding sites; novel restriction sites are underlined.

Supplementary Table 5. Primers used for Surveyor or RFLP analysis

TALEN pair	Primer Forward 5’ to 3’	Primer Reverse 5’ to 3’
ssLDLR2.1	CACAGCCGTAATAATGCCAGCTCC	CCTTCTCCGCCCACATCCTAATTC
btGDF83.1	CCTTGAGGTAGGAGAGTGTTTTGGG	CTCATGAACACCCACAGCGATCTAC
ssDAZL3.1	ATTTGGGCCCTGTTGAAAAC	ACTCACCCTTTGGACACACC
ssAPC14.2	CAGTGTTGCCCAGCTCCTCTTCA	GCGTGTGAGTGGGCAGTAGAGCTT
ssTp53	TATAGCGATGGTGAGTGGGCGG	AAGGCCACGGACAAACCCCTCT
ssKissR3.2	AAGGATGTCAGCACCTCTCTGGGG	ACCCACCCGGACTCTACTCCTACCA
ssEIF4GI14.1	GGAGCCAGAGGTCCTGAAAGAGTTG	TGAGTCAGCCAACCTGTGACACCA
ssIL2Rg2.1	CTCCCCCACTTCATTTTCTCCCC	GATTCCACAGTCCAGCCTCAGCTC
ssRAG2.1	CCCAGCTGCCTGGATTTTTGC	CCGTCCTCCAAAGAGAACACCCA
btGGTA9.1	AAGCCTGCAGAAATCCCAGAGGTT	TTCGCCGAAGGGAATGTATGCTG
btRosa1.2	CGCCTGTCAGTTACAGCCTCG	CAGCCCTACCTCCCCGTGG
ssSRY3.2	GCTCCTGGCCATCTCTTTGGTCA	TGCCTGCCTGCTTGCATCTCTCA
caCLPG1.1	CTGCTCAGAGAGGCCAGATGCT	TGCTGGCAGGAGAGACGGTTA
btGDF83.6-G **btGDF83.6-A**	CCTTGAGGTAGGAGAGTGTTTTGGG	CTCATGAACACCCACAGCGATCTAC
ssP65_11.1 **ssP65.8**	GCAATAACACTGACCCGACCGTG	GCAGGTGTCAGCCCTTTAGGAGCT
ssGDF83.6	AGGCGAAGACCTCAGGGAAATTTA	TTGATTGGAGACATCTTTGTGGGAG

Supplementary Table 6. Amplification primers for Illumina sequencing

btGDF8	btGDF8-DS F1	TTTGGGCTTGATTGTGATGA
	btGDF8-DS F1_A	ATCACGTTTGGGCTTGATTGTGATGA
	btGDF8-DS F1_B	CGATGTTTTGGGCTTGATTGTGATGA
	btGDF8-DS F1_C	TTAGGCTTTGGGCTTGATTGTGATGA
	btGDF8-DS R1	AACCTCTGGGGTTTGCTTG
ssLDLR	ssLDLR2-DS F1	GGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_A	ATCACGGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_B	CGATGTGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_C	TTAGGCGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_D	TGACCAGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_E	ACAGTGGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS R1	TTCCCACCGAGTCTATCACC
ssAPC	ssAPC14 DS F1	TAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_A	ATCACGTAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_B	CGATGTTAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_C	TTAGGCTAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_D	TGACCATAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_E	ACAGTGTAGGCAACTACCATCCAGCAACAG
	ssAPC NJ 14 R1	GCGTGTGAGTGGGCAGTAGAGCTT
ssP53	ss tp53 E6 NJ F1	CTCCCCTGCCCTCAATAAGCTGTT
	ss tp53 E6 NJ F1_A	ATCACGCTCCCCTGCCCTCAATAAGCTGTT
	ss tp53 E6 NJ R1	TGGGAATGAGGGGTTTGGCAG
P65	ssP65-DS-F1	TGAGGCTATAACTCGCTTGG
	ssP65-DS-F1_A	ATCACGTGAGGCTATAACTCGCTTGG
	ssP65-DS-R1	ATCCGTAAGTGCTGGCTCTG
Barcode for multiplexing is underlined.		

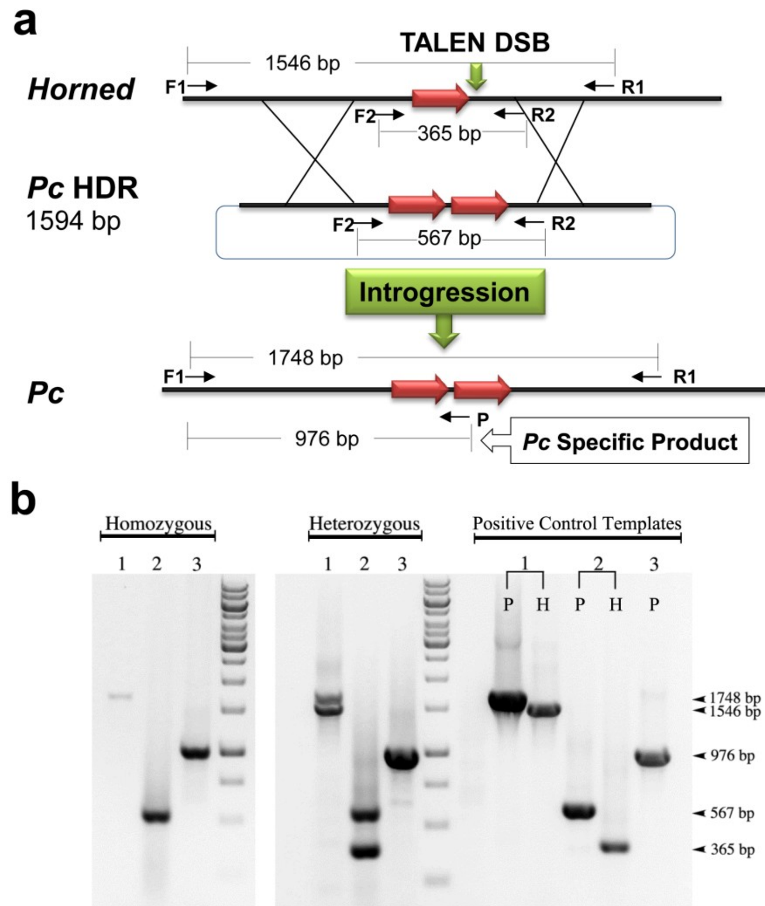


Figure 1. TALEN-mediated introgression of *Polled*. a) A schematic of the strategy to introgress the Pc allele into Holstein (*HORNED*) cells. The Pc allele, bottom, is a tandem repeat of 212bp (red arrow) with a 10bp deletion (not shown). TALENs were developed to specifically target the *HORNED* allele (green vertical arrow) which could be repaired by homologous recombination using the Pc HDR plasmid. Primer sets used in "panel b" depicted on schematic. **b)** Representative images of colonies with homozygous or heterozygous introgression of Pc. Three primer sets, indicated by number on figure, were used for positive classification of candidate colonies: Set 1 – F1+R1, Set 2 – F2+R2, and Set 3 – F1+P (Pc specific). Amplicons generated using positive control templates [(P) plasmid template containing sequence verified Pc 1748 bp insert between primers F1+R1; (H) Holstein bull genomic DNA] are included on the right. Identity of the PCR products was confirmed by sequencing F1+R1 amplicons.

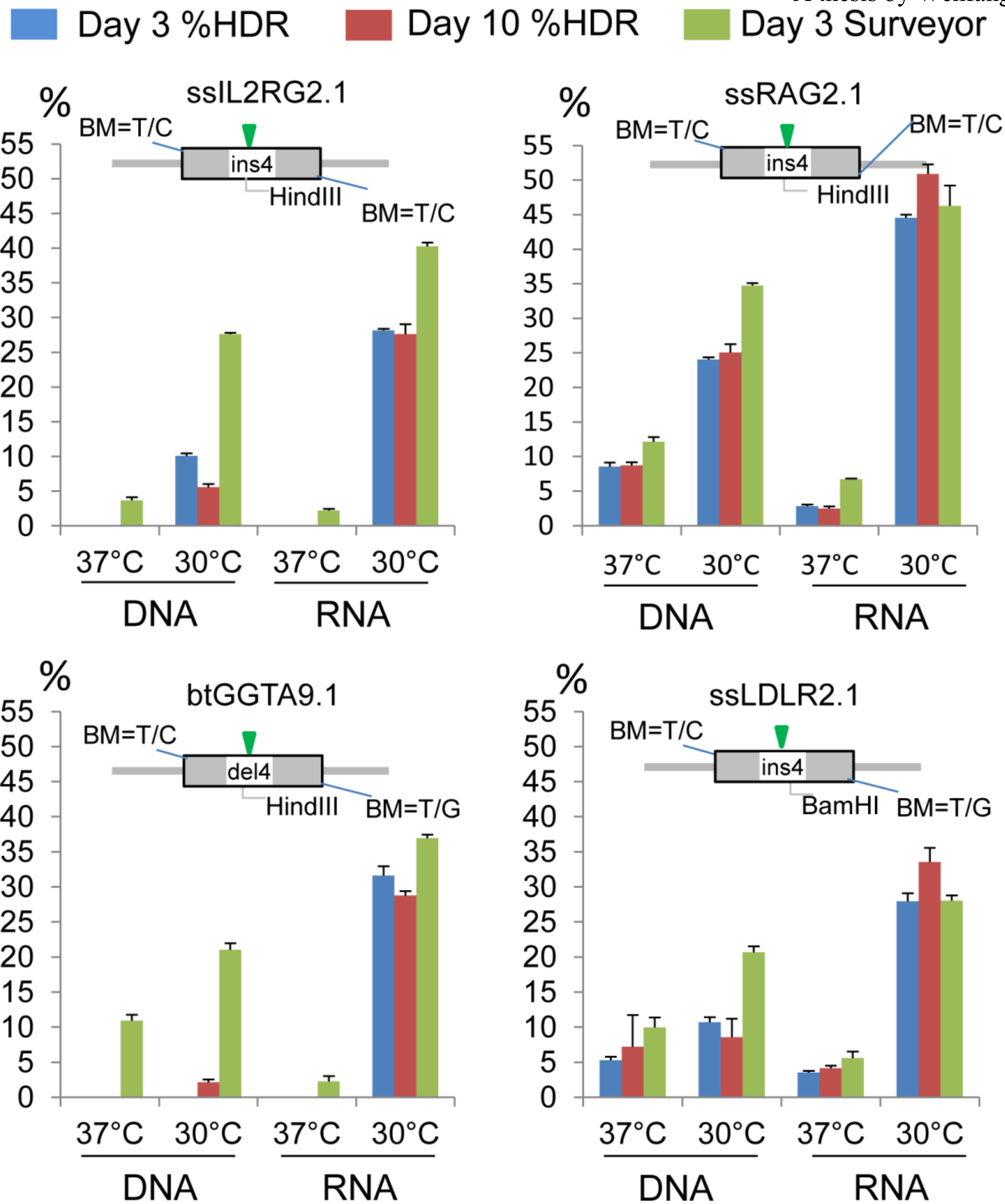


Figure 2. An mRNA source of TALENs stimulates efficient and consistent HDR using an oligo donor. Each chart displays results of targeting a specific locus in fibroblasts (e.g. *ssIL2RG*; "ss" for *Sus Scrofa* and "bt" for *Bos Taurus*) using oligo donor templates and TALENs delivered as plasmid DNA or mRNA. A diagram of the oligo template is shown (inset) where the shaded box represents the TALEN binding site and the spacer is shown in white. Each oligo contains either a 4bp insertion (ins4) or deletion (del4) that introduces a novel restriction site for RFLP analysis. Presumptive blocking mutations (BM) replace the conserved -1 thymidine (relative to the TALEN binding site) with the indicated nucleotide. Fibroblasts were transfected with either TALEN encoding plasmids (3 µg) or mRNA (1 µg) along with 3 µM of their cognate oligos homologous template (**Table S4**). Cells were then incubated at 37°C or 30°C for three days prior to expansion at 37°C until day 10. TALEN activity was measured by Surveyor assay at day 3 (Day 3 Surveyor) and HDR was measured at days 3 and 10 by RFLP analysis (Day3 %HDR and Day10 %HDR). Each bar displays the average and standard error of the mean (SEM) from three replicates.

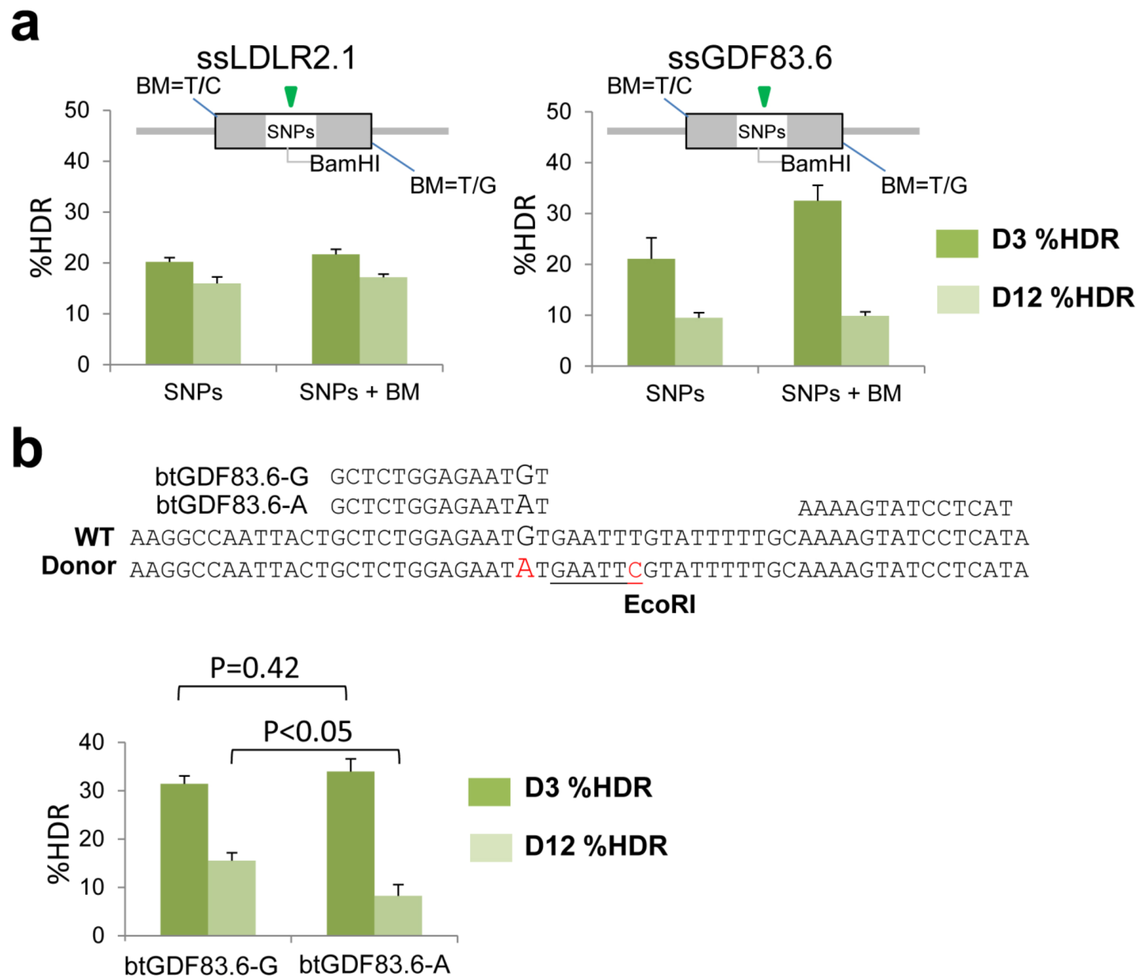


Figure 3. SNP introgression using oligo donors. a) The influence of blocking mutations (BM) on maintenance of HDR alleles was evaluated in pig *LDLR* and *GDF8*. Each oligo was designed to introduce the same SNPs/restriction site plus or minus blocking mutations. HR was quantified in transfected populations initially cultured at 30°C for three days and further maintained at 37°C until day 12 by RFLP assay. The average and SEM (n=3) is shown. **b)** Introgression of myostatin C313Y into Wagyu fibroblasts. The C313Y missense mutation is caused by a G-A transition (indicated by oversized text) at nucleotide 938 of bovine myostatin(23-25). The HDR template also includes a T to C transition (red) to introduce a novel EcoRI site for RFLP screening. Two left TALENs were designed against the locus, btGDF83.6-G, targeting the wild type alelle (Wt), and btGDF83.6-A targeting the mutant allele (C313Y); both share a common right TALEN. Transfection, culture and measurement were conducted as above. The average and SEM for btGDF83.6-G (n=30) and btGDF83.6-A (n=5) represent twelve and three biological replicates, respectively. A two-sided student's t-test was used to compare averages between groups; the p values are indicated.

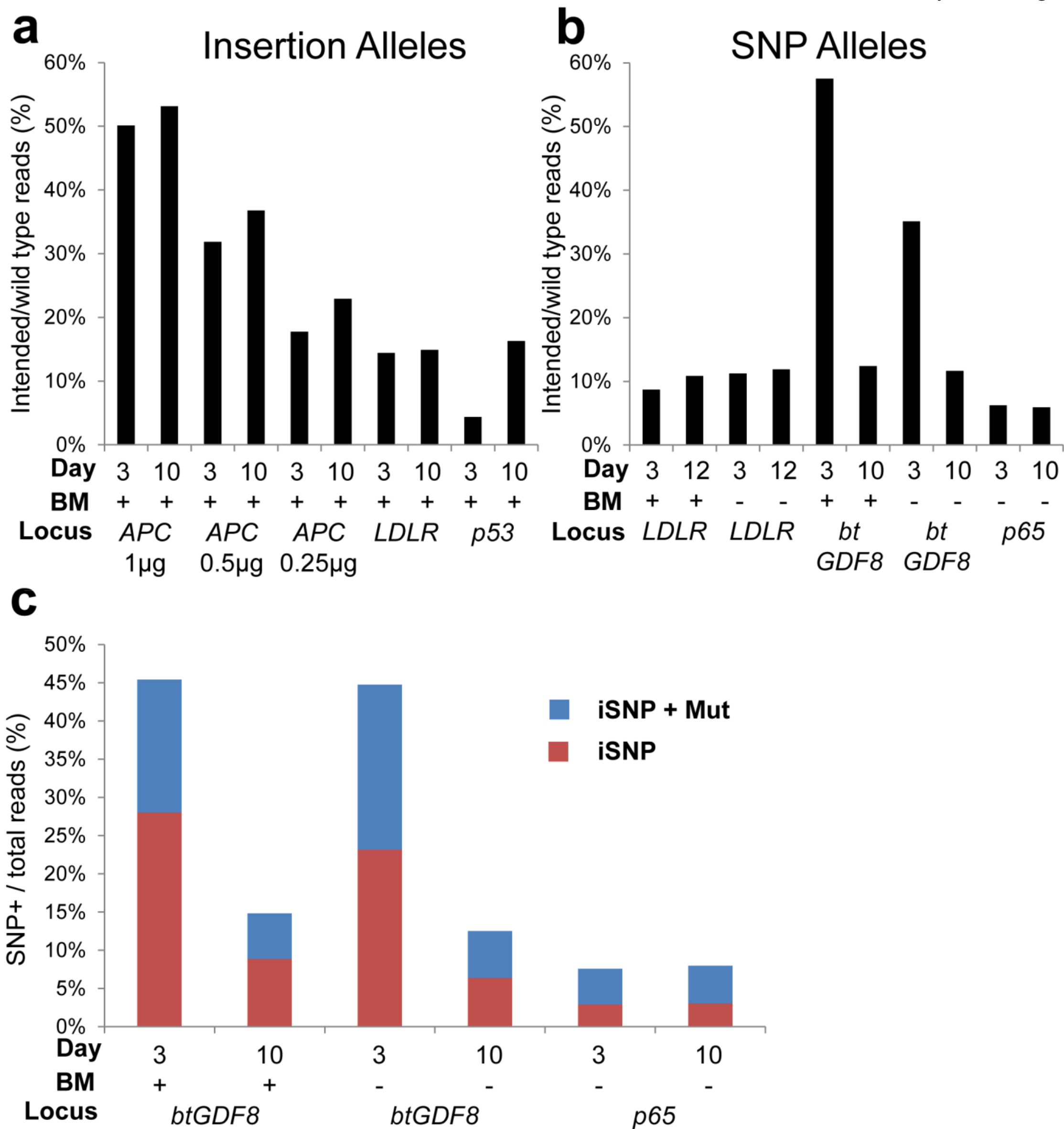


Figure 4. Sequence analysis of TALEN stimulated HDR alleles. PCR amplicons flanking the target site (200-250bp total) derived from TALEN mRNA and oligo transfected cell populations were sequenced by Illumina sequencing. Total read count ranged from 10,000 to 400,000 per sample. The count of perfect, intended HR reads versus the wild type reads is plotted for insertion (**a**) and SNP alleles (**b**). The target locus, time point and whether or not BMs were included in the oligo are indicated below. **c**). Reads from *btGDF8* and *p65* populations were sorted for incorporation of the target SNP and then classified into intended (iSNP only) versus those with the target SNP plus an additional mutation (iSNP+Mut) and plotted against the total number of reads.

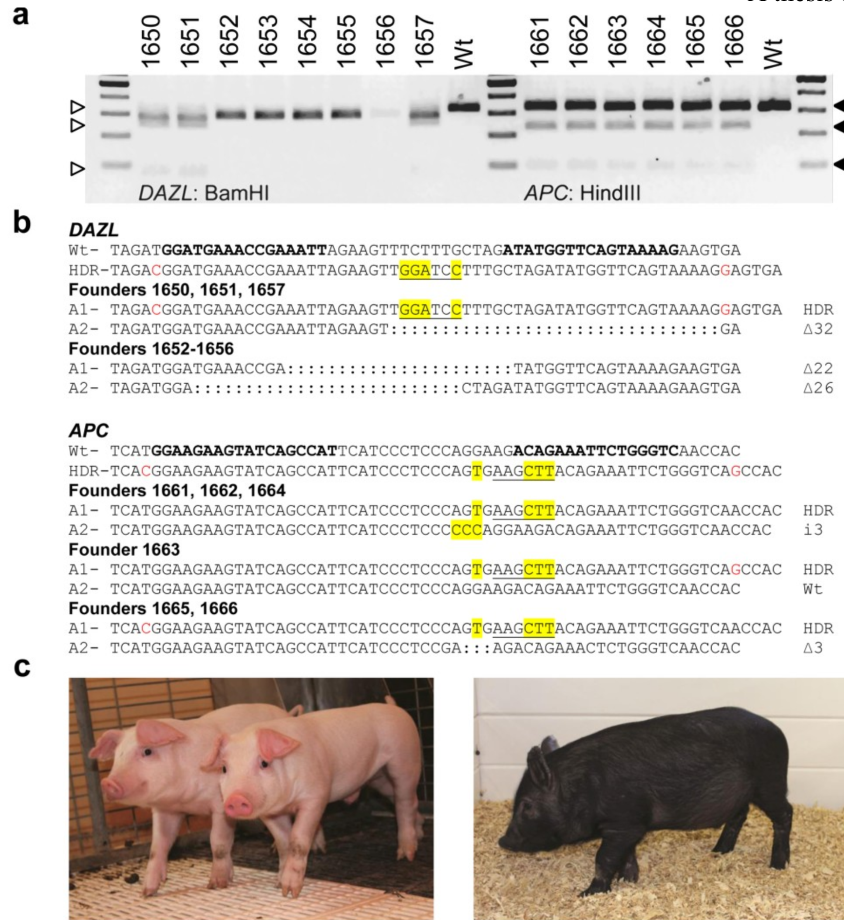
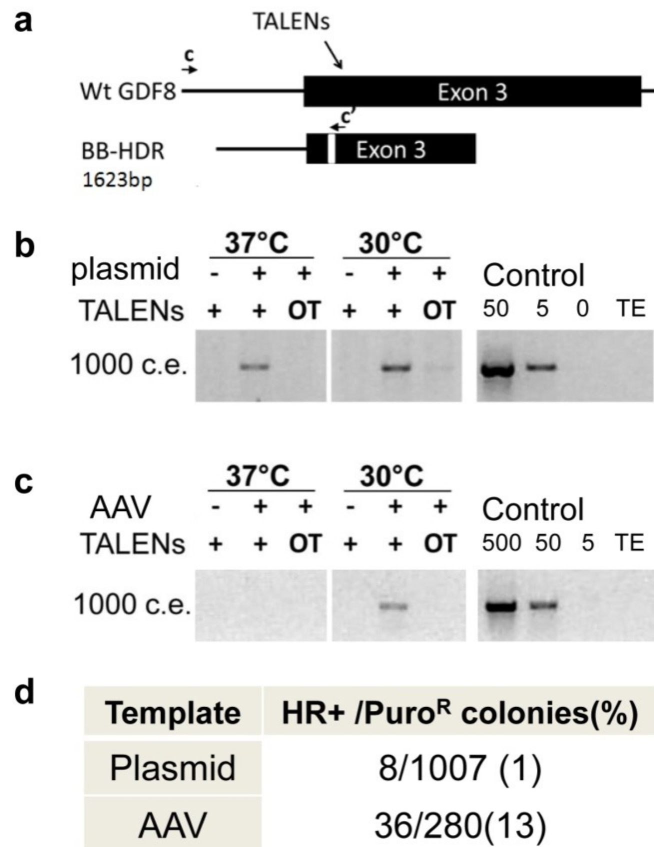
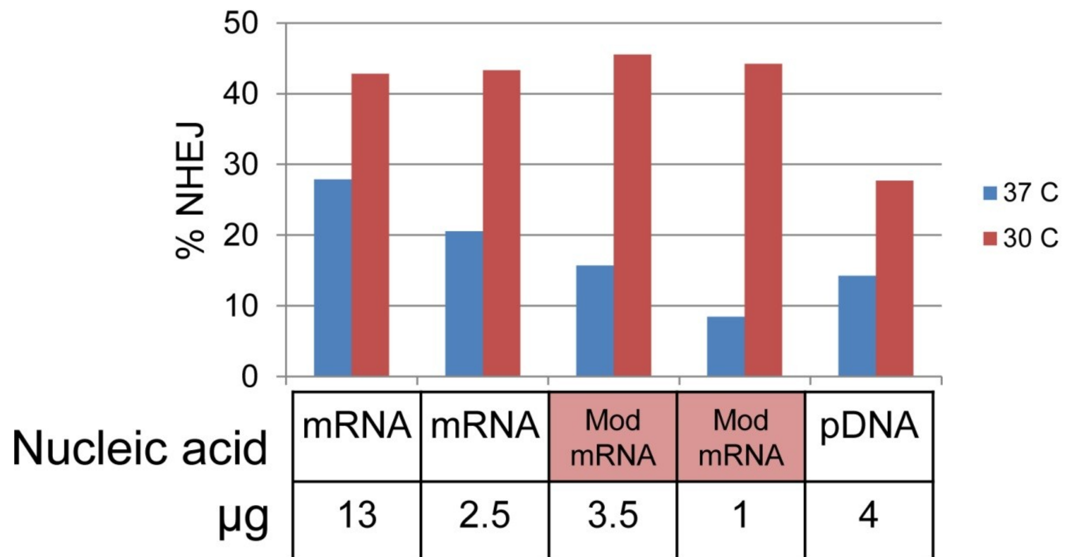


Figure 5.Cloned pigs with HDR alleles of *DAZL* and *APC*. **a**) RFLP analysis of cloned piglets derived from *DAZL* and *APC* modified landrace and Ossabaw fibroblasts respectively. Expected RFLP products for *DAZL* founders are 312, 242 and 70bp (open triangles) while expected products for *APC* are 310, 221 and 89bp (filled triangles). The difference in size of the 312bp band in wild type versus *DAZL* founders reflects the expected deletion alleles. **b**). Sequence analysis confirms the presence of the HDR allele in 3 of 8 *DAZL* founders, and 6 of 6 *APC* founders. Blocking mutations in the donor templates (HDR) are displayed in red and inserted bases are highlighted yellow. Bold text on the top Wt sequence indicates the TALEN binding sites. **c**). Photographs of *DAZL* (left) and *APC* (right) founder animals.

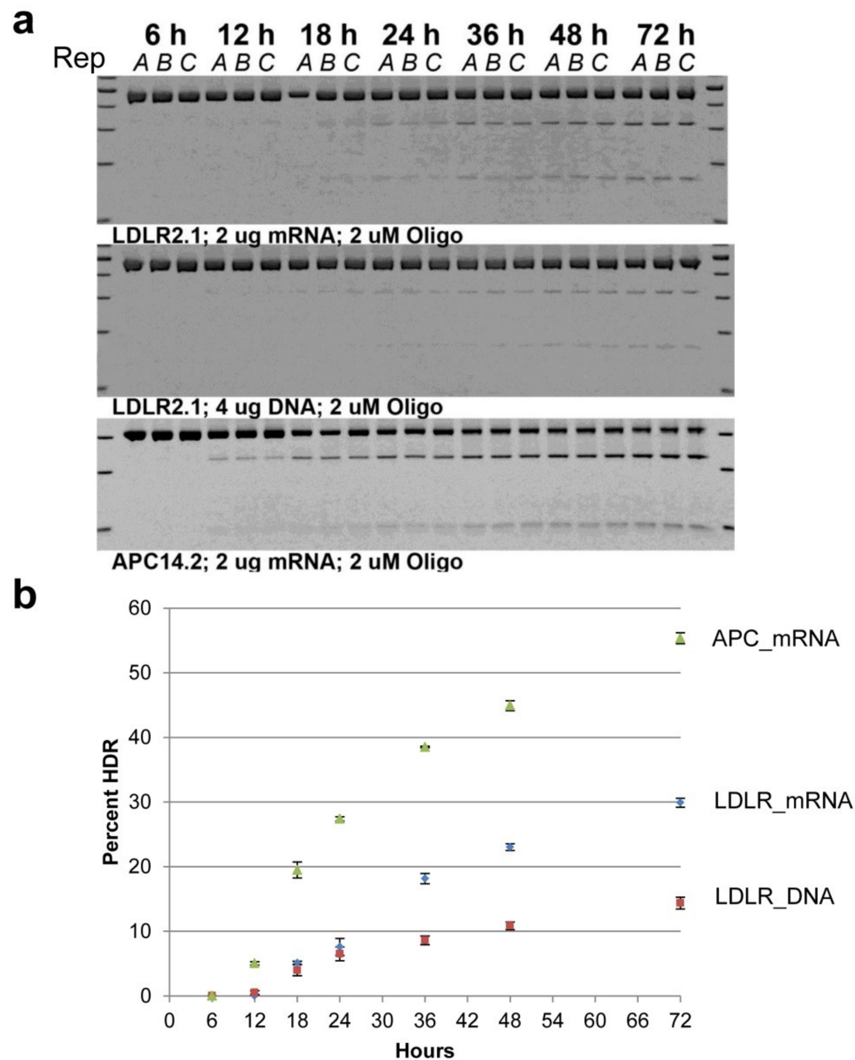


Template	HR+ /Puro^R colonies(%)
Plasmid	8/1007 (1)
AAV	36/280(13)

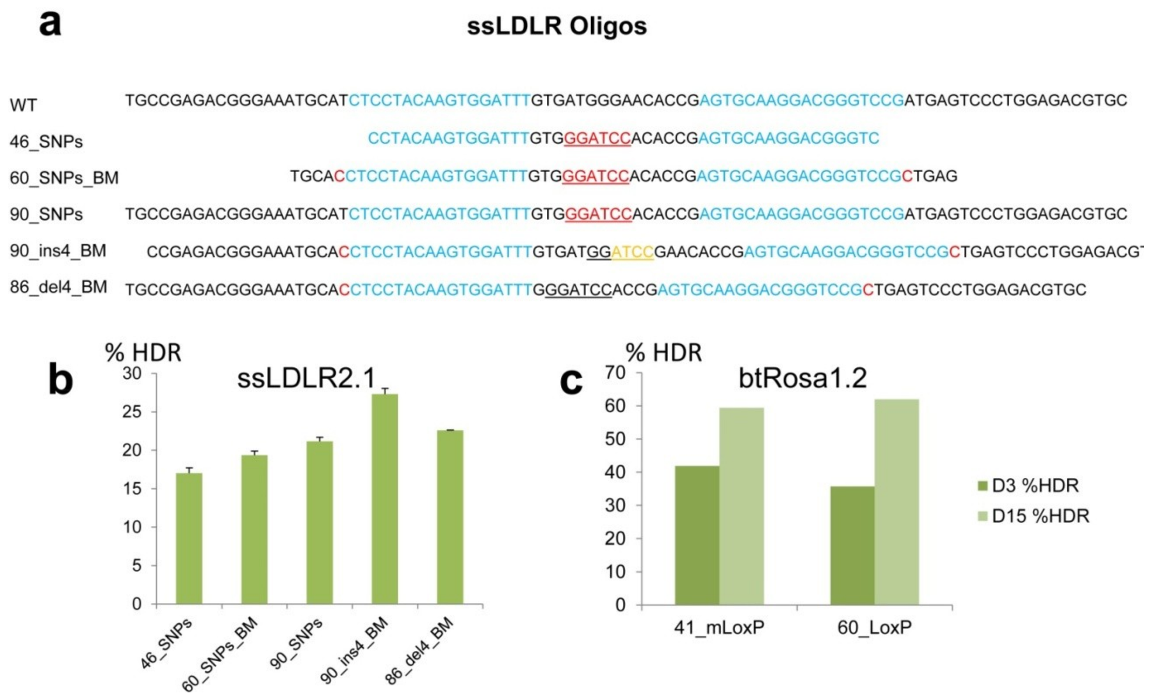
Supplementary Figure 1. TALEN stimulated allele transfer into Wagyu fibroblasts using plasmids or rAAV as repair templates. a) TALENs, btGDF83.1, and a homologous template (BB-HDR) were designed to introduce an 11bp deletion to exon 3 of bovine *GDF8* (Belgian Blue mutation) by TALEN stimulated homologous recombination. The homology template was prepared either as supercoiled plasmid DNA or packaged as a recombinant adeno-associated virus (rAAV) genome. Half of the binding site for the left TALEN is missing in the BB-HDR template due to the 11bp deletion, thus should be resistant to TALEN cleavage. **b, c**) Allele specific PCR demonstrates that HDR induction is dependent on co-transfection of TALENs and the BB-HDR template. The PCR assay was developed to specifically detect HDR modified *GDF8* alleles using primers c and c' (panel a). The 3' end of primer c' spans the 11 base pair deletion, and cannot amplify the wild type allele. Five hundred cell equivalents were included in each PCR reaction and positive controls consisted of 5-500 copies of a synthetic DNA corresponding to the outcome of homologous recombination. **d)** The allele specific PCR was conducted on individual puromycin resistant colonies derived from transposon co-transfected populations (30°C conditions only) as described previously(15). Candidate clones were confirmed by sequencing. Use of rAAV template resulted in a 16-fold enrichment in homologous recombination frequency in comparison to plasmid template.



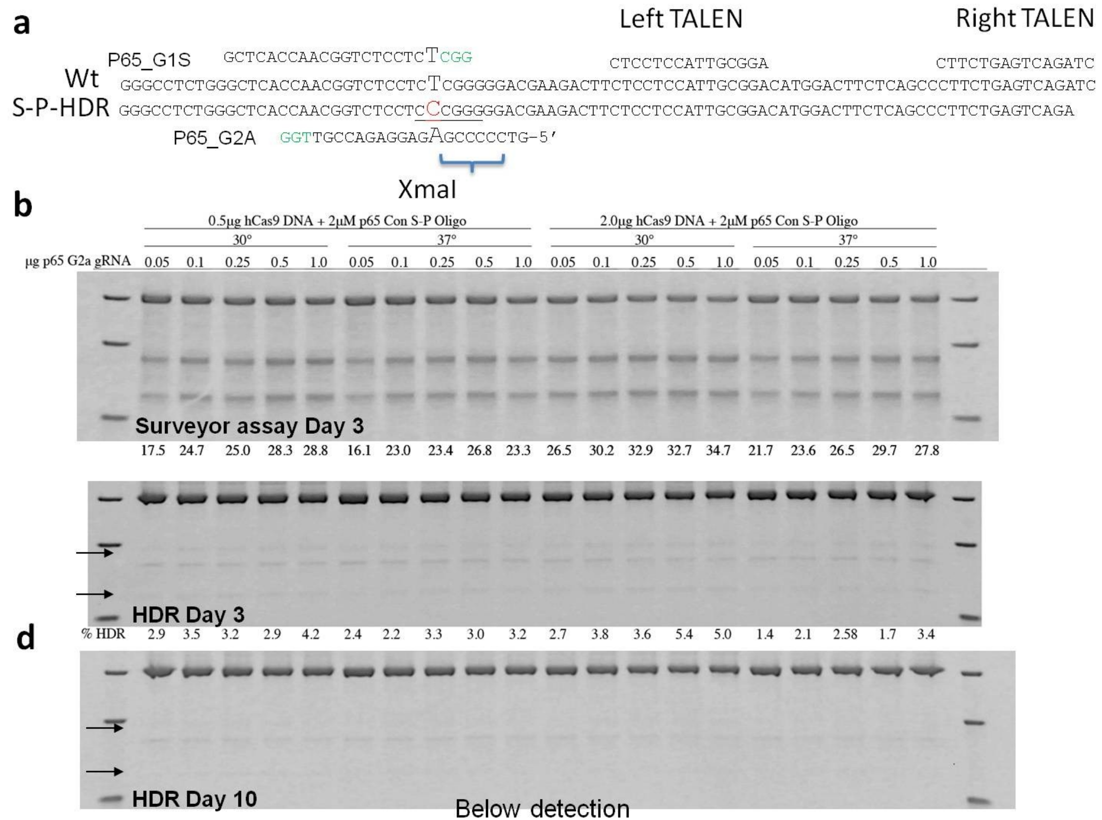
Supplementary Figure 2. Evaluation of transfected mRNA as a source of TALENs. The p65_11.1 TALENs were introduced into pig fibroblasts encoded by either unmodified mRNA, modified mRNA (mod mRNA) or plasmid DNA (pDNA). Two quantities of each TALEN preparation were transfected into cells by nucleofection (Lonza), cultured 3 days at 30°C or 37°C prior to analysis of indels. Percent NHEJ was similar for all mRNA transfections incubated at 30°C, while a dosage response could be observed for transfected cells incubated at 37°C. Notably, mRNA transfection in all groups incubated at 30°C significantly outperformed the TALENs transfected as plasmid DNA under the same conditions. There was little difference between modified and unmodified mRNA in this test.



Supplementary Figure 3. Kinetics of TALEN induced HDR with oligonucleotide templates. Porcine fibroblasts were transfected with either TALEN-encoding mRNA or plasmid DNA and oligos with 4 base pair insertions targeting *LDLR* or *APC* genes. Cells from each transfection were then evenly split into seven 24-well plate wells, cultured at 30°C and assayed by RFLP at the indicated time points. **a)** RFLP analysis on cell populations at indicated time points. **b)** Results from panel ***a*** were quantified by densitometry and the averages were plotted as a function of time with SEM (n = 3). HDR signal first appears 12 hours post-transfection and accumulates over time. The onset of HDR at *LDLR* was independent of TALEN source, but the rate of HDR between 24 and 72 hours was much higher when mRNA was used compared to plasmid DNA.



Supplementary Figure 4. Influence of mutation type on the frequency of HDR. a) The sequence of five oligos used to target ss*LDLR*. Oligos vary in length and the type of mutation they are intended to introduce. TALEN binding sites are indicated in blue text and the novel BamHI site is underlined. SNPs including BMs are in red while insertions are marked in orange. **b)** Cells were transfected with LDLR2.1 TALEN mRNA (1 µg) and oligos (2µM final). HDR at day 3 was determined by RFLP analysis and the average with SEM (n=3) was plotted. The results suggest that insertion alleles are more efficiently incorporated than SNPs or deletions, but that homology length from 46-90 bp has negligible influence on HDR efficiency. **c)** Cattle cells were transfected with btRosa1.2 TALEN mRNA and either 41_mloxP or 60_loxP oligos (2µM final). The numbers 41 and 60 refer to the number of homologous bases. Each oligo contains a 34bp *loxP* site, either a modified (*mloxP)* or wild type *(loxP)* version, in the center of the spacer. Densitometry at day 3 and 15 show that insertion of *loxP* sites is both efficient and stable.



Supplementary Figure 5. CRISPR/Cas9 mediated HDR to introgress the p65 S531P mutation from warthogs into conventional swine. a) The S531P missense mutation is caused by a T-C transition at nucleotide 1591 of porcine *p65* (*RELA*)(39). The S-P HDR template includes the causative T-C transition mutation (oversized text) which introduces a novel XmaI site and enables RFLP screening. Two gRNA sequences (P65_G1S and P65_G2A) are shown along with the p65.8 TALENs used in previous experiments. **b)** Landrace fibroblasts were transfected with S-P-HDR oligos (2µM), two quantities of plasmid encoding hCas9 (0.5 µg v.s. 2.0 µg); and five quantities of the G2A transcription plasmid (0.05 to 1.0 µg). Cells from each transfection were split 60:40 for culture at 30 and 37°C respectively for 3 days before prolonged culture at 37°C until day 10. Surveyor assay revealed activity ranging from 16-30%. **c and d)** RFLP analysis of cells sampled at days 3 and 10. Expected cleavage products of 191 and 118bp are indicated by black arrows. Despite close proximity of the DSB to the target SNP, CRISPR/Cas9 mediated HDR was less efficient than TALENs for introgression of S531P. Individual colonies were also analyzed using each gRNA sequence and are reported in Table 1.

a

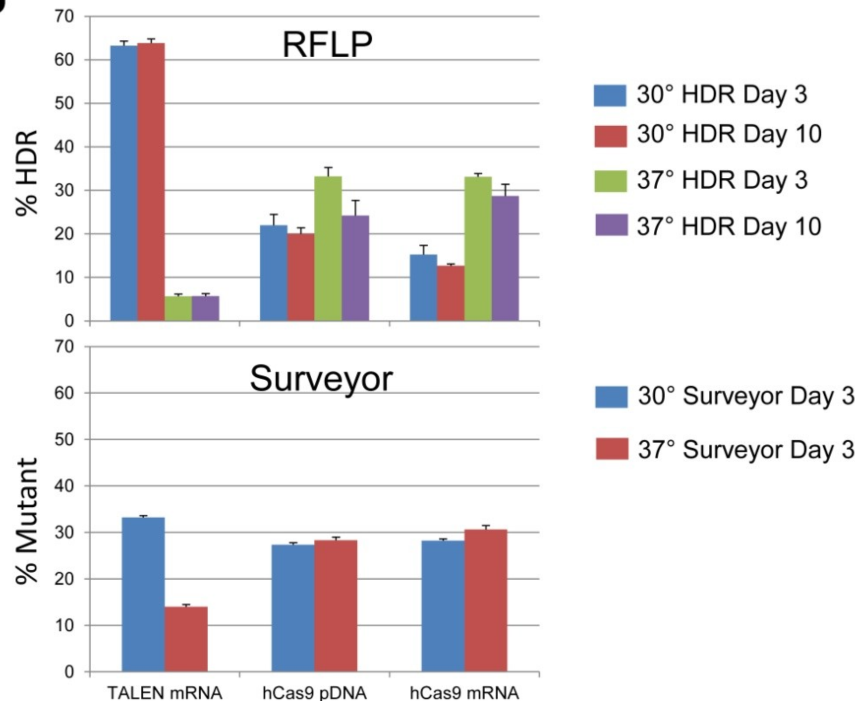
APC14.2 TALENs GGAAGAAGTATCAGCCAT ACAGAAATTCTGGGTC-ANTISENSE STRAND

Wt CCAGATCGCCAAAGTCATGGAAGAAGTATCAGCCATTCATCCCTCCCAGGAAGACAGAAATTCTGGGTCAACCACGGAGTTGCACT

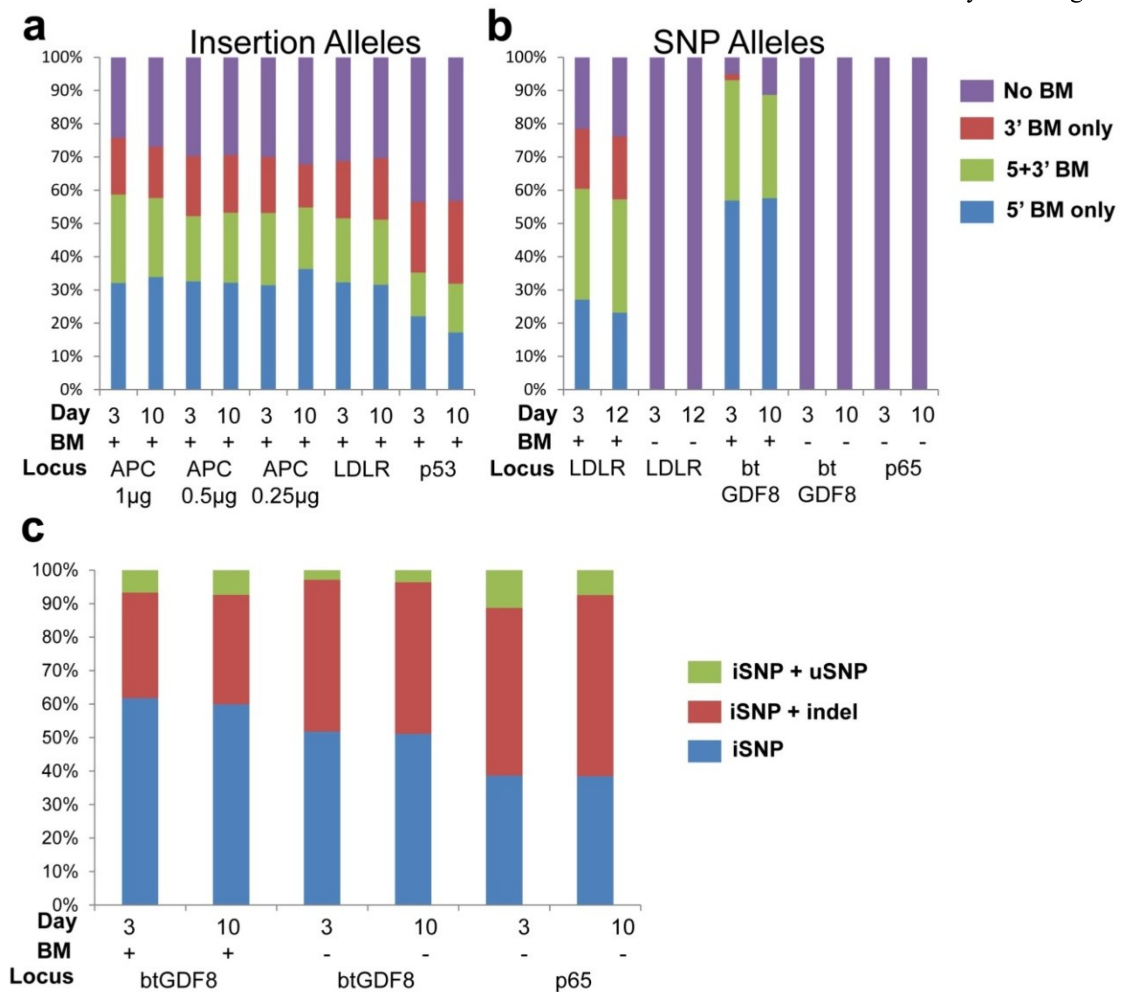
APC14.2 G1a GGGAGGGTCCTTCTGTCTTTAAG-5′

HDR CCAGATCGCCAAAGTCACGGAAGAAGTATCAGCCATTCATCCCTCCCAGTGAAGCTTACAGAAATTCTGGGTCGACCACGGAGTTGCACT

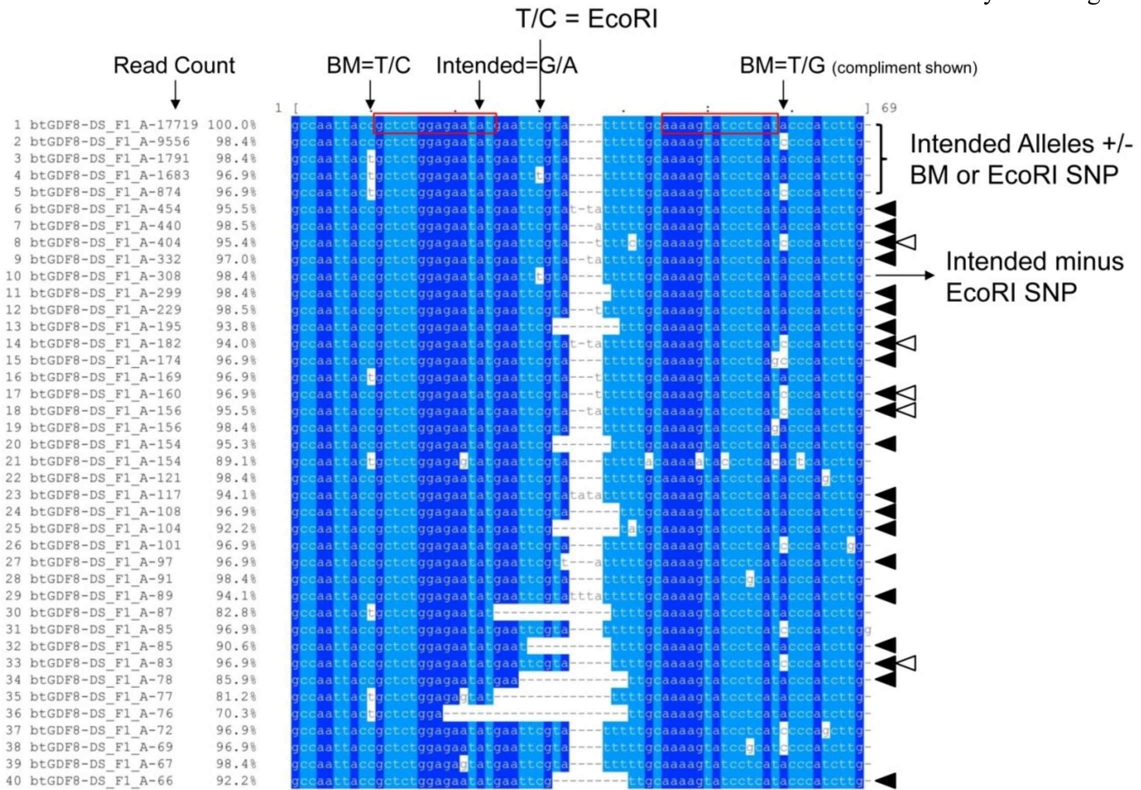
b



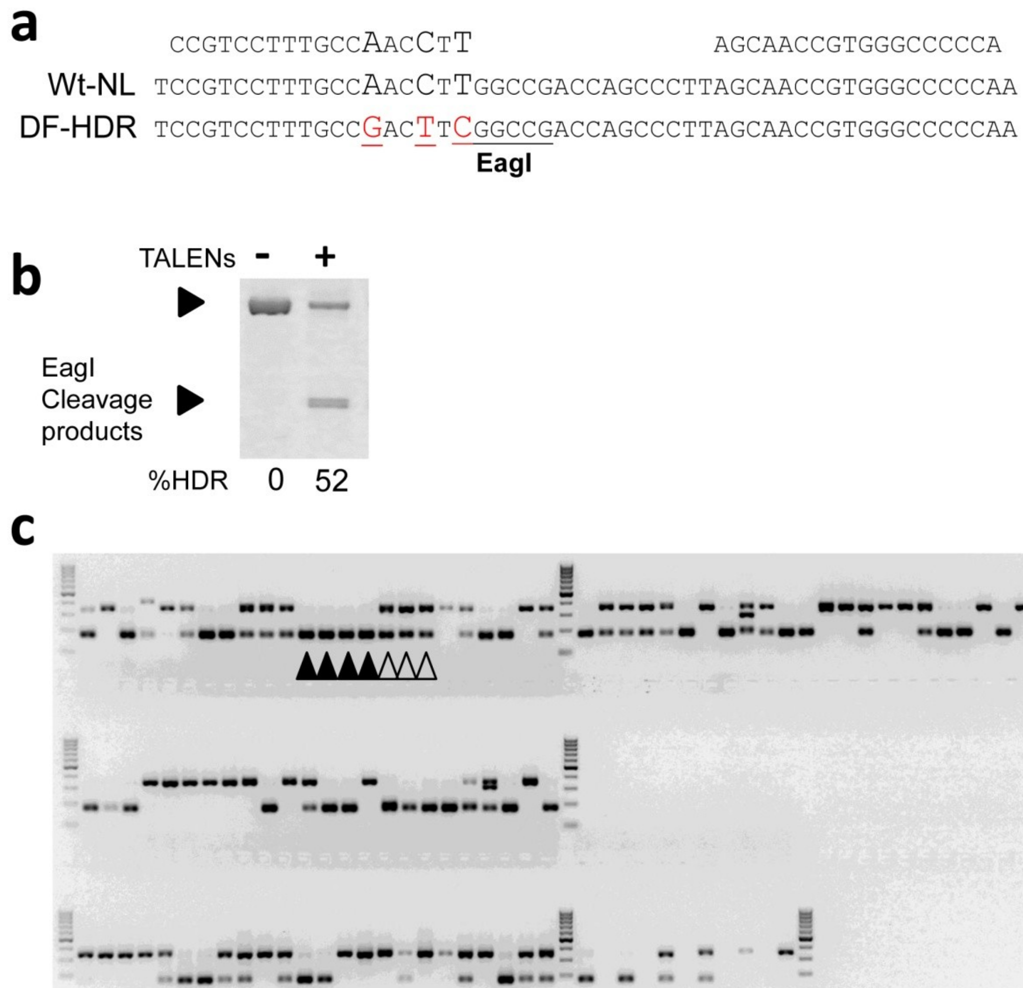
Supplementary Figure 6. Comparison of TALENs and CRISPR/Cas9 mediated HDR at porcine APC. a) APC14.2 TALENs and the gRNA sequence APC14.2 G1a are shown relative to the wild type *APC* sequence. Below, the HDR oligo is shown which delivers a 4bp insertion (orange text) resulting in a novel HindIII site. Pig fibroblasts transfected with 2µM of oligo HDR template, and either 1µg TALEN mRNA, 1 µg each plasmid DNA encoding hCas9 and the gRNA expression plasmid; or 1 µg mRNA encoding hCas9 and 0.5 µg of gRNA expression plasmid, were then split and cultured at either 30 or 37°C for 3 days before expansion at 37°C until day 10. **b)** Charts displaying RFLP and Surveyor assay results. As previously determined TALEN stimulated HDR was most efficient at 30°C, while CRISPR/Cas9 mediated HDR was most effective at 37°C. For this locus, TALENs were more effective than the CRISPR/Cas9 system for stimulation of HDR despite similar DNA cutting frequency measured by Surveyor assay. In contrast to TALENs, there was little difference in HDR when hCas9 was delivered as mRNA versus plasmid.



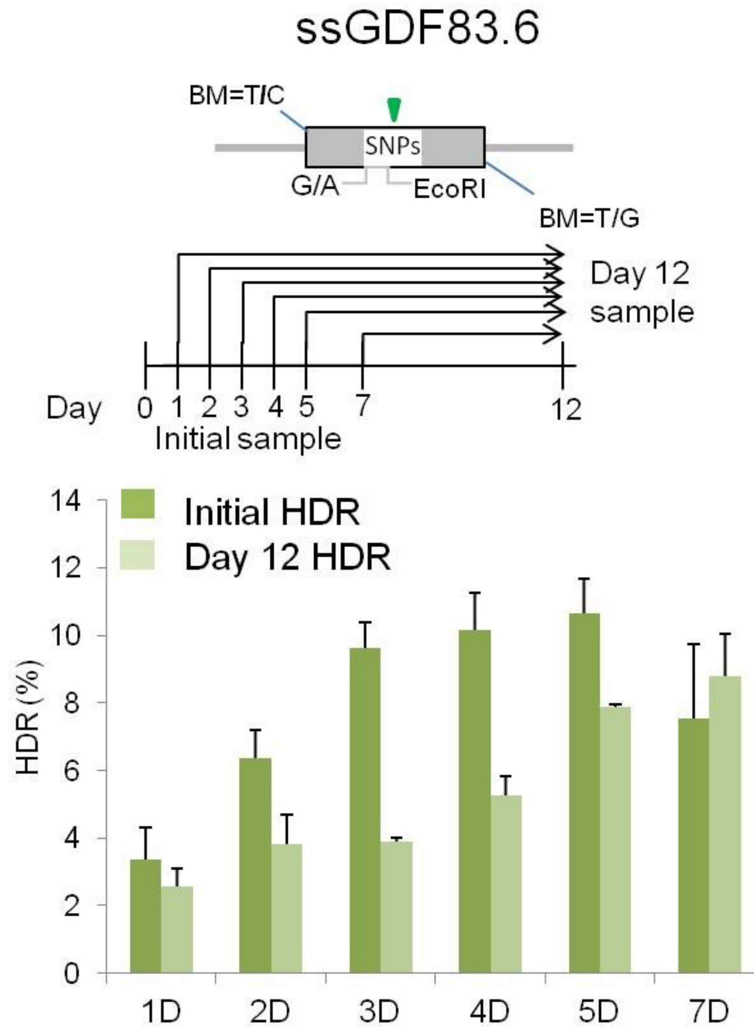
Supplementary Figure7. Sequence analysis of HDR alleles. Sequencing reads containing the correct insertion (**a**) or SNP allele (**b**) were analyzed for incorporation of BM. The target locus, time point and whether or not BMs were included in the oligo are indicated below each graph. In general, the 5' BM was incorporated most frequently into the HDR conversion tract, followed by inclusion of both BMs, or the 3' BM only. The distribution of BM is somewhat skewed towards incorporation of both BM when the intended mutation to *LDLR* is a SNP versus a 4 bp insertion allele. It is also interesting to note that the majority of intended reads for bt*GDF8* have incorporated at least one BM, but seldom have the 3' BM alone. Thus, while BM did not have a significant impact on the frequency of maintaining the intended SNP (iSNP) allele in culture, their enrichment relative to other loci suggests that they might have offered some protection from TALEN re-cleavage. **c**). The data of **Fig. 4c** was further classified by mutation type and compared. Some reads contained only the iSNP, others had a concomitant indel (iSNP + indel), or a concomitant unintended SNP (iSNP + uSNP). There appears to be some elevation in the frequency of iSNP + indel when BMs were not included in the template, and the majority of indels were located in the spacer region so are likely to be the result of re-cutting of already converted alleles.



Supplementary Figure 8. Sequencing reveals indels in HDR alleles with incorporated BM. An alignment of the top 40 reads sorted positive for the iSNP (G938A) are shown. These data are derived from day 10 btGDF83.6-G treated cells transfected with an oligo template that included BMs, an EcoRI site and the G938A SNP. Each of these sites and the TALEN binding sites (red boxes) are indicated. The number of reads counted for each line is indicated in the left margin. Reads displaying an intended outcome are shown; the 4bp gap is introduced by the alignment software to accommodate reads containing 4bp insertions. Several reads having the 5' BM (filled triangle), the 3' BM (open triangle), or both BMs (open and filled triangles) also have indels in the spacer region proving the TALENs can bind and cleave without the conserved 5' thymidine nucleotide. Several reads differ by only a single base mismatch with the intended allele; we suspect a small proportion of these are due to errors in oligo synthesis or DNA sequencing.



Supplementary Figure 9. Multiple SNPs in the TALEN DNA-binding site stabilize HDR alleles in the EIF4GI gene. a) A portion of wild type *EIF4GI* Wt-NL is shown. One pair of TALENs was designed to cut the wild type *EIF4GI* to stimulate homologous recombination. Also aligned to the Wt sequence is the core sequence of the donor oligo, DF-HDR, used to introduce three SNPs (red oversized letters) into the genome. The third SNP creates a novel EagI restriction site that was used for RFLP analysis. Pig fibroblasts were transfected with EIF4GI14.1 TALEN mRNA (2µg) and DF-HDR (2µM) and then cultured at 30°C for 3 days prior to analysis and colony propagation. **b)** RFLP analysis on population three days post transfection. Expected product sizes of 344, 177 and 167bp are indicated by filled triangles. **c)** RFLP assay on isolated cellular clones. Day 3 cells were used to derive monoclonal colonies through dilution cloning. An example of colonies with heterozygous (open triangles) or homozygous (filled triangles) HDR alleles are indicated.



Supplementary Figure 10. Extension of hypothermic treatment enhances maintenance of SNP HDR alleles. Pig fibroblasts were transfected with TALEN mRNA (1µg) and oligos (3µM). Cells from two independent transfections were pooled for each replicate and evenly distributed into six wells of a 6-well plate and cultured at 30°C. Samples were collected from these populations for RFLP analysis on days 1-7 (minus day 6, 1D to 7D along X-axis) post-transfection and the remaining cells were transferred to 37°C. Samples for each condition were collected again at day 12 for RFLP analysis. The average HDR and SEM (n = 3) is shown at the initial collection and once again at day 12. The frequency of HDR plateaus after 3 days of hypothermic treatment, however, longer hypothermic treatment enhances the stability of HDR alleles in culture.

Chapter 6 Summary and Future Directions

I. Background and aims

While *SB* transposons and recombinant AAV remain as convenient tools for transgene delivery, the next generation genome editing tools mature at a fast pace, revolutionizing many aspects of biomedical research. Owing to their efficiency and nucleotide-level precision, targetable nucleases especially ZFNs, TALENs and CRISPR/Cas9 are turning into major work force to knockout or alter a gene, for the purposes of gene discovery(1-3), disease modeling (3-5), or gene therapy(6-8). In the past decade, these endonucleases have edited numerous genes spanning 34 species; ~160 in human alone and > 70% of them was accomplished in the last three years(9, 10).

This reflects the paradigm shift towards a precision genetics era that is dominated by such site-specific nucleases. We have witnessed an exponential increase in the gene targeting efficiency between 10^{-6} and 10^{-2} with conventional methods (11, 12) to well above 10^{-1} regularly with these enzymes(1, 13, 14). Their prominence is built on the ability to be programmed and generate double strand lesions to a DNA sequence of choice, which is subsequently repaired by error-prone non-homologous end joining or homology directed repair with predictable outcomes (**Fig. 6**, Chapter 1). Recent progress in incorporating novel sequences at targeted sites with various types of homologous templates demonstrate the ability to achieve gene addition or correction in any gene at will in theory (1, 5, 15). Among the customable nucleases, TALENs and CRISPR/Cas9 are gaining favor due to their low cost and ease to devise.

Although large animals are the major livestock for meat production and possess a unique place in biomedical research, the adoption of these technological advances to enhance animal productivity, welfare and medical applications is lagging. Only seven pigs or cattle have been edited with these enzymes in the report up to date, including three pigs presented in Chapter 4 and 5 (16-20). However, to cope with a warming climate and sustain a growing population (21), it is crucial for animal research to assimilate such novel technologies, especially when a vast number of SNP markers has been annotated in the livestock gene pool and requires enrichment or elimination (22).

This thesis aims to contribute to such effort by applying current genome editing tools to improve large animal genomes in a straightforward way, instead of relying on crossbreeding alone (**Fig. 7-8**, Chapter 1). Specifically, it intends to evaluate the disruption of Myostatin in the pig, one of the major muscle growth factors, by means of transpositional transgenesis or gene targeting. With the interception of TALENs and CRISPRs, the thesis strives to utilize them to achieve non-meiotic allele introgression within and between livestock species, to rapidly

propagate SNPs or alleles that promote animal growth and welfare while eliminate those that have adverse effects. In addition, it attempts to manipulate genes responsible for diseases and to produce human disease models through nuclease or rAAV mediated gene knockout.

II. Progress

Chapter 2 illustrates the investigation on the influences of disrupted Myostatin over pig reproductive and growth performances. Pig fibroblasts harboring these transgenes were generated by *SB* transposition and led to the production of founder animals. Genotyping revealed integration of these transgenes in all animals with a strong bias towards tissue specific expression, suggesting an adverse effect on fetus survival when the transgenes were over-expressed. Unfortunately, this project had to terminate due to death of founder animals, and I was not able to collect data to draw further conclusions. Nevertheless, the existing data does claim that *SB* transposons are very efficient in delivering transgenes into the pig.

In Chapter 3, I describe efforts to knockout the *LDLR* gene and introgress dominant negative Myostatin in the pig. We were successful in achieving both goals using rAAV mediated gene targeting, with typical targeting efficiency compared to reported results from other groups (12, 23). We discovered that targeted does not equal SNP introgression, ~50% of Myostatin targeted colonies were absent of the intended SNP, indicating short conversion tracks or sister chromatin exchange. We initially had difficulty in isolating monoclonal targeted clones suitable for SCNT because the plating density of transduced cells on 96-well plates was set too high. We learnt that a ~35% confluency rate is optimal for the 96-well plate selection format to obtain targeted monoclonal clones according to the *Poisson* distribution.

Chapter 4 presents our work in applying TALENs to knock out genes in livestock and to generate Ossabaw pig models for atherosclerosis. Two thirds of the TALENs we created (23/36) demonstrated high activity in livestock primary cells and when injected into pronuclear bovine embryos, were able to induce both mono- and bi-allelic gene knockouts (KO) in up to three quarters of embryos analyzed. We applied a simple *SB* transposon based co-selection strategy to aid colony formation and enrich for modified colonies. With it, we were able to obtain mono- and bi-allelic modified fibroblast clones at rates up to 54% and 17% total colonies analyzed. Besides, cells treated with two pairs of TALENs targeting the same chromosome gave rise to colonies harboring large chromosomal deletions or inversions in 14% selected colonies.

TALEN modified PFFs containing mono- and bi-allelic mutations in the *LDLR* gene were effective nuclear donors for cloning and gave rise to miniature swine models of familial

hypercholesterolemia. This is the first example of TALEN modified large animal and our work has demonstrated that with such targetable endonucleases, one is able to knock out practically any gene of interest in livestock with very high efficiency, which was unthinkable prior to the onset of these nucleases.

In Chapter 5, we detail our work on utilizing TALEN and CRISPR/Cas9 induced homology-directed repair (HDR) to knock out or introduce defined mutations into specific livestock genes. By treating Holstein cells with TALEN mRNA and a plasmid template, we introgressed the bovine *POLLED* allele into horned dairy bull fibroblasts to achieve genetic dehorning. Using TALEN mRNA or CRISPR/Cas9 and oligonucleotide templates, we introduced single-nucleotide alterations, small insertions or deletions (indel) into fourteen additional genes in 10-50% of cells from pig, goat and cattle fibroblast populations, without any drug selection.

Up to 70% of colonies propagated from these populations harbored the intended edits of which over one half were homozygous. Several of the SNP-only edits with no other exogenous DNA replicate naturally occurring performance enhancing or disease resistance SNPs. This is the first demonstration of one-step non-meiotic introgression of natural SNPs between or within livestock species. We also generated pigs with knockout alleles in the *DAZL* and *APC* genes to model infertility and colon cancer through cloning using TALEN and oligo HDR edited fibroblast colonies as nucleus donors. We showed that nuclease induced HDR leads to versatile gene editing in livestock and has implications for novel breeding as well as disease modeling.

III. Future directions

For agricultural purposes

The quick implementation of site-specific nucleases provides unprecedented opportunities for the improvement of animal production and development in livestock derived medical applications. Supported by our initial work and others', I believe the following directions should be explored to fulfill the promises. One is to achieve embryo and germ cell based genetic modification. Due to the lack of embryonic stem cells, livestock research has been relying heavily on SCNT for transgenic animal production. However, this procedure is expensive, invasive, and health issues follow cloned animals. Studies in zebrafish and mice have shown that one can induce gene addition and alteration in early stage embryos by injecting TALENs or CRISPRs and oligo HDR templates (1, 15).

Since we have been able to knock out genes in bovine and porcine embryos, the next logical step is to co-inject nucleases with oligos to achieve HDR to introgress valuable alleles

directly in embryos. However, embryo transfer, the main factor that drives the cost, is still required. Perhaps the ultimate solution is to modify sperm progenitor cells directly, sperms matured *in vivo* or *in vitro* with the modifications could then inseminate recipient sows through artificial insemination, an established breeding routine on many farms, to quickly expand the engineered traits.

Another direction is to multiplex, to simultaneously knockout or edit multiple genes or loci in the same cells or even embryos; TALENs and CRISPR/Cas9 both possess this potential for livestock genome engineering. Several studies have used CRISPRs to multiplex in zebrafish and mouse by co-transfection or co-injection with multiple site-specific guide RNAs, with or without oligo repair templates (24, 25). We should expect to obtain similar results when multiple pairs of highly functional TALENs are introduced to the same cells or embryos. However, there is possibility that chromosomal abnormities might occur, such as translocations and big deletions, since multiple DSBs co-exist in the same cell.

This scenario could be true especially for CRISPR mediated work, in that it has been shown to exhibit broad off-targeting activity (26, 27). It seems as if this is not the case since viable animals have been produced from multiplex treated embryos, but it could simply be true that embryos with such abnormities did not survive early stages of development in the first place. It is important to learn how prevalent chromosome aberrations are in such situations because though not all are fatal, the survived animals might possess low value if they are unhealthy or infertile due to the abnormities and cannot transmit the edits through the germline. Again, multiplexing in embryos or sperm cells should be highly sought.

The majority of HDR presented in Chapter 5 was mediated by oligonucleotide templates. Oligos are single stranded, easy to synthesize and very concentrated; however, their wider application are constrained by the short size to deliver only small sequence changes such as SNPs or small indels. This limitation compels us to seek alternatives when bigger modifications are desired. For example, we had to use a plasmid template to introgress the *POLLED* allele that was only around ~200 bp. We also tested the efficiency to deliver changes with rAAV templates; in both situations, TALENs enhanced the targeting efficiency by at least 10-fold.

Other types of potential templates with even bigger capacities, including lentivirus and artificial chromosomes such as BACs and YACs, can be appealing and should be explored as well. One immediate application is direct transfer of quantitative trait loci or haplotypes of SNPs with small effects between breeds instead of crossbreeding. This not only avoids dilution of the current genetic merit from crossbreeding (**Fig. 7**, Chapter 1) but also saves time and cost from

arduous work to deal with individual SNPs, either by crossbreeding or nuclease stimulated HDR, since millions of SNPs with potential effects on animal performances have been identified by whole-genome sequencing (22).

For biomedical applications

Induced chromosomal aberrations might be unwanted in food animal production, they can be invaluable in biomedical research. Structural abnormities in chromosomes including translocations, large deletions, inversions and duplications have been found to cause various types of disorders, such as cancer, intellectual disabilities, and infertility. Large animals with such abnormities can be useful subjects to study the disease mechanisms, test therapeutic applications and attempt to reverse the genetic defects with nucleases in a similar way we create them.

To date, chromosomal inversions and deletions have been achieved in pigs (Chapter 4) and zebrafish (28, 29), all relying on spontaneous rearrangements of the chromosomal segments generated by the cutting of multiple pairs of TALENs. One hypothesis worth testing is we can promote the frequencies of these events by providing TALEN or CRISPR/Cas9 multiplexed cells with hybrid oligo repair templates that direct the movements of the resulting multiple chromosomal segments. Such an oligo should be designed with one-half of its sequence homologous to the broken end of a chromosomal segment derived from one DSB and the other half to the end of asegment derived from another DSB. We could also hypothesize that these hybrid oligos can guide translocation and other movements of the "free ranging" broken chromosome ends in addition to inversions and deletions.

The adaptation of multiplexing and alternative repair templates to the TALEN and CRISPR/Cas9 gene editing regimes could benefit livestock work in the biomedical field too. Instead of multiple rounds of gene targeting, expensive cloning and lengthy breeding to create animals with multiple edited genes, like the way the doubly homozygous *IGHM* and *PRNP* knockout cattle was created (30), the same goal should now be achievable in one step by combining multiplexing with oligo templates or high-capacity HDR vectors. This can have broad applications to humanize the pig for xenotransplantation or animal pharming by transferring a series of genes from human to livestock. In addition, high-capacity targeting vectors allow for gene stacking in safe harbors such as the Rosa26 locus, desirable for the construction of bioreactors.

IV. Summary

This thesis strived to apply the state-of-the-art technologies to large animal genetic engineering, for both agricultural and biomedical applications. It began with *SB* transposon mediated gene transfer, to AAV enhanced gene targeting, to TALEN and CRISPR/Cas9 induced gene knockout and editing. As the technologies advanced, our ability to modify livestock genomes evolved from random insertional transgenesis to gene specific alterations with nucleotide-level precision. The technological advances open up doors to numerous opportunities in the animal field; to harness the potential in full, imagination and asking the right questions is the key.

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