Efficient TALEN-mediated gene knockout in livestock


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Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved September 6, 2012 (received for review July 6, 2012)

Transcription activator-like effector nucleases (TALENs) are programmable nucleases that join FokI endonuclease with the modular DNA-binding domain of TALEs. Although zinc-finger nucleases enable a variety of genome modifications, their application to genetic engineering of 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 injections of TALEN mRNAs into livestock zygotes were capable of inducing gene KO in up to 75% of embryos analyzed, a portion of which harbored biallelic modification. We also developed a simple transposon coselection strategy for TALEN-mediated gene modification in primary fibroblasts that enabled both enrichment for modified cells and efficient isolation of modified colonies. Coselection after treatment with a single TALEN-pair enabled isolation of colonies with mono- and biallelic modification in up to 54% and 17% of colonies, respectively. Coselection 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 miniature swine containing mono- and biallelic mutations of the LDL 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.

The ability to knockout 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 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 homologous 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 complicate regulatory approval of engineered food products.

Zinc finger nucleases (ZFNs) offer an alternative platform for germ-line 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 germ-line 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, biallelic KO of the porcine GGTA1 gene using commercial ZFN reagents (9), wherein biallelic-null cells could be enriched by FACS for the absence of a GGTA1-dependent surface epitope. Double-strand DNA breaks dramatically enhance HR (10), leading to homology-dependent repair of ZFN lesions for precise gene alteration in human cells (11), mice, and rats (12, 13). Although 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). In vivo, custom-designed TALENs have been reported to induce indel frequencies between 2% and 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 biallelic KO of genes and large chromosomal rearrangements. Finally, toward 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 LDL receptor (LDLR) gene. To our knowledge, this report of genetic engineering and cloning of these valuable biomedical animals is unique, and is also a unique example of TALEN-mediated gene modification of livestock.

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 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 h postfertilization and were cultured in vitro until blastocyst stage (Table S1). Whole-genome amplification (WGA) and genotyping analysis revealed indels in one blastocyst injected with ACAN11 and five injected with ACAN12 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. (17) (Fig. L4) for use in subsequent experiments. An additional TALEN-pair, GoldyF83.1, was developed using the GT scaffold. Zygotes were injected with TALEN mRNA plus 2 ng/μL 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 (Fig. S1). Mutation frequency of GT-GDF83.1 TALENs significantly exceeded previous injections. Six of 14 blastocysts (43%) injected with a low mRNA dosage (2 ng/μL) displayed indels without a significant reduction in developmental rate (Table S1). Three of four blastocysts in the high-dosage group (10 ng/μL) displayed indels, with biallelic modification occurring in two of three mutant blastocysts (Fig. 1B).

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 20 ng/μL of TALEN mRNA along with 5 ng/μL 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+, WGA and PCR amplification was successful from 56 of the EGFP+ 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 biallelic 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 engineer alleles precisely led us to explore TALEN function in cultured livestock cells to meet two objectives: (i) to serve as a quality control measure for assembled TALEN-pairs before injection and (ii) 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. L4). 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, DMDE7.1 and ACAN12, displayed cleavage efficiencies of 38% and 25% (Fig. 2A). The TALEN scaffold had a significant effect on biallelic modification in fibroblast cell lines having four of six loci targeted with the GT scaffold cleaved at 3.5% or greater but only the DMDE7.1 TALEN-pair cleaved above 1% with the +231 scaffold (Fig. 2). As noted in previous studies (15, 22), a 72-h 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 Cotransfection.** 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. Although an average genomic modification level of 25% (i.e., ~44% of 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 of cells containing indels after an extended period in culture and tested an indel enrichment strategy based on transposon coselection (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 cotransfection with Mirus LT1, a commonly used cationic lipid transfection reagent. Whereas gene modification was below detection 14 d posttransfection without selection, genome modification levels in transposon-selected populations were 31%, 13%, and 20% for DMDE7.1, DMDE6, and LDLR2.1 TALEN-pairs, respectively (Fig. 3). Thus, despite low-transfection efficiency with cationic lipids (<5% for this experiment), efficient modification can be achieved using transposon coselection. We next applied transposon coselection to cells transfected by nucleofection where >90% transfection efficiency is routine. Without selection, the proportion of cells modified after 14 d in culture was 50–90% lower than levels measured at day 3, suggesting an attrition of modified cells. In contrast, transposon coselection was in all cases effective for maintenance of modified cells transfected by nucleofection but, with the exception of ACAN12, did not significantly increase the frequency of modified cells (Fig. 3C). Transposon coselection 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 Biallelic 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 transposon coselection (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 the percent NHEJ measured in the parent populations 3 d after transfection (Table 1). Biallelic 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 biallelic 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 biallelic 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 DMDE6 and DMDE7.1 because a high percentage of Duchenne 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 DMDE6 and 23% DMDE7.1), albeit slightly lower than when either TALEN-pair was transfected

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.1211446109)
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 6.5-kb 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 > 1,000) (Table S3) in colonies generated using the transposon coselection strategy. Deletion and inversion events occurred with remarkable fidelity, with 41 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 inversions in vitro, we next sought to examine the utility of TALEN-modified swine cells for animal production. Transposon coselected Ossabaw swine colonies with mono- and biallelic modification of the class A domain 1 of the LDLR gene were pooled disproportionately (pool A, four genotypes; pool B, three genotypes; pool C, five genotypes) and cloned by chromatin transfer (26). Pregnancy was established in seven of nine transfers (one of two for pool A, two of three for pool B, and four of four for pool C). Six pregnancies were maintained to term, resulting in 18 live-born piglets, including one stillborn from pool A, nine healthy and two stillborn piglets from pool B, and eight healthy, one stillborn, and one euthanized (because of a cloning defect) piglets from pool C (Fig. 5A). At 60+ days 17 piglets remained in good health. In total, four of eight colony genotypes from pools B and C were represented in cloned offspring. Ten of the 11 piglets from pool B carried biallelic modifications of LDLR (B1) (Fig. 5) derived from a colony harboring both an insertion-induced frame-shift (289_290ins34) in one allele and a 3-bp 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 five colony genotypes were represented in piglets from pool C, including 289_290del110 and 289_290insA, each resulting in frame-shift mutations expected to prematurely truncate the LDLR ORF.
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. Although 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 a result of technical or species-specific differences. Regardless, we speculate that selecting for EGFP+ embryos will be a valuable first-level method for enrichment of TALEN-modified embryos. Second, although 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, biallelic 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-btGDF83.1 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 fivefold 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.

Although 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 that the truncated 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 h 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 to 40%. Assuming a TALEN-pair introduces NHEJ at an average efficiency of 20%, about five 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 catonic lipids were used, enrichment for modified cells was likely a result of the elimination of nontransfected cells, >95% of total cells. However, coselection was also capable of preventing an observed 50–90% loss of TALEN-modified cells after 14 d of transfection. The basis of 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 toward 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 as cells in animal tissues (32–34), coselection might enrich for a subpopulation of cells that were amenable to NHEJ.

By whatever mechanism, transposon coselection was very effective for the clonal isolation of both mono- and biallelic 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 ZFN’s (11, 35, 36). Furthermore, approximately two-thirds of the biallelic 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 (30). The great advantage 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 phenotype 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 posttransfection (39), although inefficient isolation of modified primary cell clones (two of six, both monoaicelic indels) suggests low viability among sorted cells. In contrast, transposon coselection allowed efficient isolation of stable cells containing mono- and biallelic cells 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 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 cotransfection 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 coinduction 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 process could serve as a means to fix a desired trait or traits in livestock for agricultural purposes or to use nondisjunction to control the spread of genetically modified genomes.
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 biallelic 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 live-born clones, 8 contained mononuclear mutations and 10 contained biallelic modifications of the *LDLR* gene. These results demonstrate that TALENs can be used to generate animals with either mononuclear or biallelic 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, biallelic 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.

**Materials and Methods**

See *SI Materials and Methods* for more detail 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 for TALeffectors, with some modifications. Four new backbone plasmids were generated to replace pTA for the final step in TALEN assembly. The first pair of vectors, pC-TAL-231 and pC-GoldyTALEN, direct expression of the p-231 (19) and GoldyTALEN scaffolds from the mini Cagggs 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 D. F. Laboratory Golden Gate TALEN kit also available through Addgene (#1000000016). Intermediary arrays were created and joined into these vectors, as previously described (17).

TALEN mRNA was synthesized from Sall linearized RCIscript vectors using the HighMessage T73 Kit (Ambion), as previously described (44), and injected into the cytoplasm at specified concentrations. Fibroblasts were cultured and transfected using the Basic Fibroblast Nucleofection Kit (Amaka Biosystems/Lonza) or Mirus L1 reagent (Mirus), as previously described (44). TALEN activity was analyzed by Surveyor assay (Transgenomic) and measurements were performed as described in Guschin et al. (45). Indels were detected in embryos or individual colonies by Surveyor assay or direct sequencing. PCR assays described in *SI Materials and Methods* were developed to detect large deletions and inversions.

**Animal Husbandry/Cloning**. Pigs were cloned by CT under contract with Minutube of America under Recombinetics, Inc.*s* Institutional Animal Care and Use Committee Protocol 1103A97232 at the University of Minnesota.

**ACKNOWLEDGMENTS**. The authors thank Dr. Mark Westhusin, Gayle Williamson, Jane Pryor, and Ali Wilkerson from Texas A&M University for bovine zygote preparation and injection. This study was funded in part by a Small Business Technology Transfer program Grant 1R41HL108440-01 (to Recombinetics, Inc.); grants to C.B.A.W.; Biotechnology and Biological Sciences Research Council Institute Strategic Programme Grant BB/E/D07731442; and support from Genus plc (C.B.A.W.).