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Efficient nonmeiotic allele introgression in livestock using custom endonucleases

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We have expanded the livestock gene editing toolbox to include transcription activator-like (TAL) effector nuclease (TALEN)- and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-stimulated homology-directed repair (HDR) using plasmid, rAAV, and oligonucleotide templates. Toward the genetic dehorning of dairy cattle, we introgressed a bovine *POLLED* allele into horned bull fibroblasts. Single nucleotide alterations or small indels were introduced into 14 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 alteration of single base pairs. Up to 70% of the fibroblast colonies propagated without selection harbored the intended edits, of which more than 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 intraspecific and interspecific introgression of select alleles in livestock for agricultural and biomedical applications.

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 creation of the first transgenic pig (3), more than 180 trials have been recorded to genetically engineer livestock in various ways (2). Until recently, most were accomplished by random insertions of expression cassettes, which suffered from low efficiency and unpredictable expression, or homologous recombination (efficiency <1 in 10^4) with linked selection markers.

Three recent technologies—zinc finger nucleases (4), transcription activator-like (TAL) effector nucleases (TALENs) (5), and clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease cas9 (CRISPR/Cas9) (6–8)—have been used to disrupt gene function by introducing small insertions and/or deletions (indels) into genomes of species mediated by nonhomologous end-joining (NHEJ). In particular, 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 make screening for functionally disrupted clones arduous (15) and do not allow for 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 report precise, high-frequency editing of 15 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, nonmeiotic allele introgression. This work demonstrates that precise, high-efficiency gene editing can be achieved in commercially important loci in livestock for agricultural or biomedical purposes.

Results

Introgression of the Celtic *POLLED* Allele into the Genome of Horned Dairy Cattle Breeds. We previously reported 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 11-bp deletion or the Belgian Blue mutation (23–25) at position 821 (821del11) of growth differentiation factor 8 (*GDF8*) (*SI Appendix, Fig. S1*). Individual colonies with the intended mutation were recovered in 1% of colonies treated with the plasmid template and in 13% of colonies treated with the rAAV template (*SI Appendix, Fig. S1D*). These results inspired us to attempt introgression of more complex alleles into livestock.

To protect the welfare of dairy farm operators and cattle, horns are routinely manually removed from the majority of dairy cattle in the United States and Europe. Dehorning is painful, elicits a temporary elevation in animal stress, and adds expense to animal production (26). Moreover, despite the intent of protecting animals from subsequent injury, some view the practice as inhumane. Some beef breeds are naturally horn-free (e.g.,

Significance

Selective breeding has long been practiced to enrich for desirable DNA variation that influences livestock traits. We demonstrate that genetic variants can be directly introgressed into livestock genomes using a modified transcription activator-like effector nuclease system. The transient exposure of livestock cells to sequence-targeted editors stimulates homology-directed repair to levels that eliminate the need for transgene-dependent selection. Use of oligonucleotide template enables efficient single nucleotide changes to the genome and permits the transmission of both natural and novel DNA sequence variants into naïve livestock breeds and species. Gene editing offers a powerful method for accelerating the genetic improvement of livestock for food and also for developing swine as a resource for regenerative medicine and models of human disease.

Author contributions: W.T., D.F.C., C.A.L., and S.C.F. designed research; W.T., D.F.C., C.A.L., and D.A.W. performed research; W.T. and D.F.C. contributed new reagents/analytic tools; W.T., D.F.C., J.R.G., P.B.H., and S.C.F. analyzed data; and W.T., D.F.C., P.B.H., and S.C.F. wrote the paper.

Conflict of interest statement: D.F.C., C.A.L., D.A.W., P.B.H., and S.C.F. either have equity in and/or work for Recombinetics, Inc., a biotechnology company that receives funding from the National Institutes of Health to develop gene editing in commercial livestock.

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See Commentary on page 16295.

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Angus), a dominant trait referred to as *POLLED* (27). Two allelic variants conferring polledness have 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 owing to the admixture of unselected (inferior) alleles for net merit (i.e., milk production) into the population. We undertook the nonmeiotic introgression of the Celtic *POLLED* allele (duplication of 212 bp that replaces 10 bp), referred to as Pc, into fibroblasts derived from horned dairy bulls. We constructed a plasmid HDR template containing a 1,594-bp 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 as plasmid DNA (SI Appendix, 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 to confirm introgression of Pc (Fig. 1B). Three of the five clones were homozygous for Pc introgression and were confirmed by sequencing (SI Appendix, Table S1).

Reliable Oligonucleotide-Templated Allele Introgression in Pig, Goat, and Cattle Genomes. Although plasmid templates are effective for introgression of Pc and *GDF8* alleles, many desirable alleles correspond to single nucleotide polymorphisms (SNPs) or small indels that ideally do not rely on the use of double-stranded DNA templates that could randomly integrate into the genome. In addition, although introgression of the *GDF8* 821del11 allele with rAAV was efficient, viral methods are unlikely to be popular with consumers of animal protein. Recent studies have demonstrated that single-stranded oligonucleotides (oligos) 40–100 nt in length

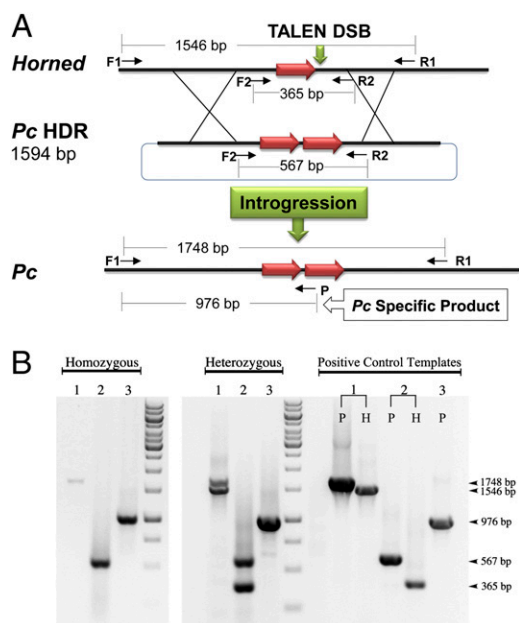


Fig. 1. TALEN-mediated introgression of *Polled*. (A) Strategy for introgressing the Pc allele into Holstein (*HORNED*) cells. The Pc allele is a tandem repeat of 212 bp (red arrow) with a 10-bp deletion (not shown). TALENs were developed to specifically target the *HORNED* allele (green vertical arrow), which could be repaired by homologous recombination using the Pc HDR plasmid. Primer sets used in B are depicted. (B) Representative images of colonies with homozygous or heterozygous introgression of Pc. Three primer sets, indicated by number, 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 a sequence-verified Pc 1,748-bp insert between primers F1 and R1; H, Holstein bull genomic DNA) are shown. The identity of the PCR products was confirmed by sequencing of F1+R1 amplicons.

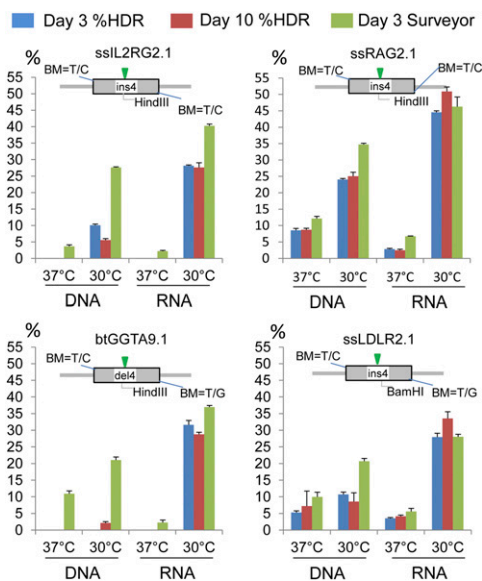


Fig. 2. An mRNA source of TALENs stimulates efficient and consistent HDR using an oligo donor. Each chart displays results of targeting a specific locus in fibroblasts (e.g., *sslL2RG*; “ss” for *Sus scrofa* and “bt” for *Bos taurus*) using oligo donor templates and TALENs delivered as plasmid DNA or mRNA. (Insets) Diagrams of the oligo templates, in which the shaded boxes represent the TALEN-binding site and the spacers are shown in white. Each oligo contains either a 4-bp insertion (ins4) or deletion (del4) that introduces a novel restriction site for RFLP analysis. Presumptive BMs replace the conserved –1 thymidine (relative to the TALEN-binding site) with the indicated nucleotide. Fibroblasts were transfected with either TALEN-encoding plasmids (3 μ g) or mRNA (1 μ g) along with 3 μ M of their cognate oligo-homologous template (SI Appendix, Table S4). Cells were then incubated at 37 °C or 30 °C for 3 d before expansion at 37 °C until day 10. TALEN activity was measured by the Surveyor assay at day 3 (Day3 Surveyor), and HDR was measured at days 3 and 10 by RFLP analysis (Day3 %HDR and Day10 %HDR). Each bar displays the average and SEM from three replicates.

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 oligo templates to overlap their cognate TALEN-binding sites and to introduce a 4-bp indel into the spacer region for restriction fragment length polymorphism (RFLP) analysis. Primary fibroblasts were transfected with plasmid- or mRNA-encoded TALENs plus oligo templates and incubated for 3 d at either 30 °C or 37 °C.

We found that TALENs delivered as mRNA consistently outperformed plasmid in cells incubated at 30 °C, as suggested by the Surveyor assay (Fig. 2). Despite appreciable levels of TALEN activity measured by Surveyor, HDR was consistently more than twofold higher when TALENs were delivered as mRNA rather than as plasmids. We speculated that this may have been a kinetic issue: TALENs from mRNA were translated more rapidly, allowing use of the template before oligo degradation. However, a time-course experiment showed little difference in the onset of HDR between TALENs encoded by plasmid versus those by mRNA (SI Appendix, Fig. S3). Among the 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, NHEJ-induced indels declined by 50–90% after extended culture without the aid of selection (15). In contrast, HDR levels at four loci were roughly equivalent when measured at day 3 and day 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 suggest that edited cells should be recoverable by dilution cloning without selective enrichment. Indeed, analysis of ~650 colonies for intended indel alleles in eight separate loci revealed a recovery rate of 10–64% (average, 45%), with up to 32% of the colonies homozygous for the 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. Both day 3 levels of HDR (7–18%) and the isolation of cellular clones with the intended SNP alleles (3–15%) in cattle and swine *GDF8* or pig *p65* were significantly lower compared with indel HDR (day 3 level, 10–53%; clone isolation, 10–64%) (Table 1). We hypothesized that indels likely were more stable than SNP because introduction of indels into the TALEN spacer region would be expected to reduce recombination of the locus, consistent with known constraints on TALEN spacer length (17). Accordingly, the introgression of large insertions (*loxP*) was extremely efficient and stable, and comparison of HDR frequencies with oligos within the same locus suggested that even a 4-bp insertion allele was more efficient than SNP alleles (*SI Appendix, Fig. S4*). Sequence analysis also revealed that nearly one-half of the isolated SNP-positive colonies for *GDF8* or pig *p65* harbored concomitant indels expected to change TALEN spacing (*SI Appendix, 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% level without any additional changes to the locus (Table 1 and *SI Appendix, Table S2*). We also introgressed 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.

For comparison, we designed CRISPR gRNAs that overlapped the T1591C site of *p65*, and we evaluated introgression using the two platforms. Despite efficient production of DSBs at the intended site, CRISPR/Cas9-mediated HDR was <6% at day 3 and below the limit of detection at day 10 (*SI Appendix, Fig. S5*). In addition, recovery of modified clones was lower with CRISPR-mediated HDR than with TALENs, even though the TALENs

cut 35 bp 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 (~30% vs. 60%; *SI Appendix, 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 [known as blocking mutations (BMs)] would inhibit recombination 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 suggests 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 effect in greater detail, we conducted Illumina next-generation sequencing of 200- to 250-bp 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 generally were 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 toward incorporation of BMs in SNP-edited alleles compared with insertional edits (*SI Appendix, 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 one-half of the reads with the iSNP had an additional mutation (iSNP+Mut) (Fig. 4B), the majority of which were indels (*SI Appendix, Figs. S7 and S8*). The majority of iSNP *btGDF8* reads with indels in the spacer also contained one or both BMs (*SI Appendix, Fig. S8*), demonstrating that modification of the conserved 5'-thymidine was not able to suppress recombination 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 recutting of the SNP edits is to design TALENs such that their binding sites overlap the target SNPs. We evaluated the influence of such repeat variable diresidue

Table 1. Frequencies of recovery of colonies with HDR alleles

Reagent	ID	Species	Mutation type	Nucleotide change	Amino acid change	Day3%HDR	HDR ⁺ (%)	Biallelic 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	ssAPC14.2†	Pig ♂	Ins/FS	2703 (ins4)	902ΔPTC	48	22/40 (55)	4/40 (10)
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 ♂	<i>Ins/mloxP</i>	Ins34	NA	45	14/22 (64)	7/22 (32)
TALEN	ssSRY3.2	Pig ♂	<i>Ins/mloxP</i>	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	ssEIF4G114.1	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 ^R	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	Extragenic	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	ssAPC14.2 G1a	Pig ♂	Ins/FS	2703 (ins4)	902ΔPTC	32	ND	ND

ss, *Sus scrofa*; bt, *Bos taurus*; ca, *Capra aegagrus*; FS, frame shift; NA, not applicable; ND, not determined; PTC, premature termination codon; R, replicate.

*Homozygous null *LDLR*^{-/-} could not be propagated in culture.

†HDR⁺ 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. A triprimer PCR combined with Sanger sequencing was used to identify positive colonies (*SI Appendix, Table S2*).

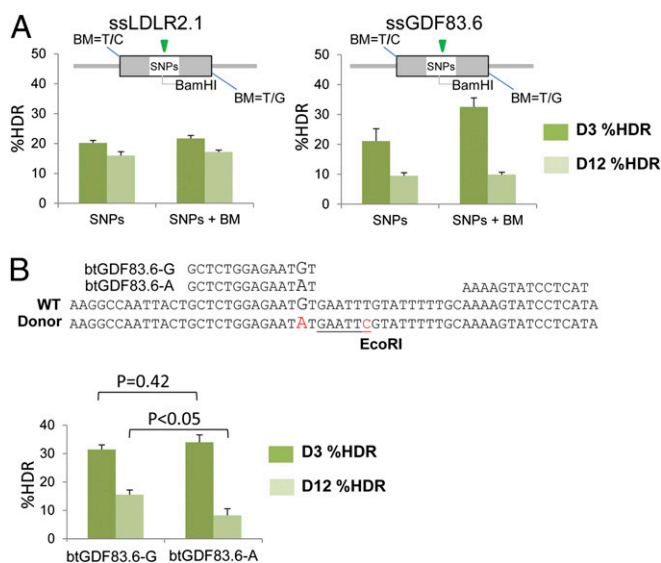


Fig. 3. SNP introgression using oligo donors. (A) The influence of BMs on maintenance of HDR alleles was evaluated. Each oligo was designed to introduce the same SNPs/restriction sites with or without BMs. HDR was quantified by RFLP assay in transfected populations initially cultured at 30 °C for 3 d and then maintained at 37 °C up to day 12. Values are averaged ($n = 3$). (B) Introgression of myostatin C313Y into Wagyu fibroblasts. The C313Y missense mutation is caused by a G-to-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 WT allele (Wt), and btGDF83.6-A targeting the mutant allele (C313Y). The two share a common right TALEN. Transfection, culture, and measurement were conducted as described above. The average and SEM values for btGDF83.6-G ($n = 30$) and btGDF83.6-A ($n = 5$) represent 12 and 3 biological replicates, respectively. A two-sided Student t test was used to compare averages between groups; P values are indicated.

(RVD)/nucleotide mismatches for introgression of G938A SNP into cattle *GDF8*. Two pairs of TALENs were generated, one pair that bound the WT “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 WT G allele, indicating that recleavage was more prevalent with btGDF83.6-A, which targets the repaired allele perfectly. Different RVD/nucleotide mismatches likely will have a greater influence on maintenance of HDR alleles, because the Asn-Asn (NN) RVD used for the WT 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; nearly 70% of isolated colonies contained an edited allele, more than half of which were homozygotes (Table 1 and *SI Appendix*, Fig. S9). Thus, the intentional alteration of the target locus to resist recleavage is an effective strategy for preserving edits.

Ultimately, gene editing is a dynamic process. TALEN cleavage and recleavage are in flux with repair by NHEJ, HDR with an oligo template, and HDR with the sister chromatid as a 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 (*SI Appendix*, Fig. S10).

Production of Biomedical Model Pigs with Gene-Edited Alleles. We chose two gene-edited loci in the porcine genome, deleted in azoospermia-like (*DAZL*) and adenomatous polyposis coli (*APC*),

to carry through to live animals. Colonies with HDR- or NHEJ-edited alleles of *DAZL* or *APC* were pooled for cloning by chromatin transfer (CT). 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 1 wk, and another died after 3 wk, leaving only founder 1661 alive. The lack of correlation between genotype and survival suggests that the early deaths were related to cloning rather than to gene edits (34). All six *APC* piglets were heterozygous for the intended HDR-edited allele, and all but one piglet had either an in-frame insertion or deletion of 3 bp on the second allele (Fig. 5A and B). The remaining animals are being raised for phenotypic analyses of spermatogenesis arrest (*DAZL*^{-/-} founders) or development of colon cancer (*APC*^{+/-} founders).

Discussion

The data presented in Table 1 demonstrate that combining mRNAs encoding TALENs and oligo templates for directing HDR achieves several key benchmarks for a precise genome-editing strategy: (i) Only target nucleotides were changed, and mRNA transfection avoids unintended integration of plasmid DNA; (ii) selection markers are unnecessary, an important factor in the acceptance of edited livestock for human consumption; (iii) gene edits were efficient, ranging from approximately 10% for SNPs to >50% for some larger alterations; and (iv) the method was highly reliable; at each locus tested, targeted alteration of 16 of 16 sites (15 genes)

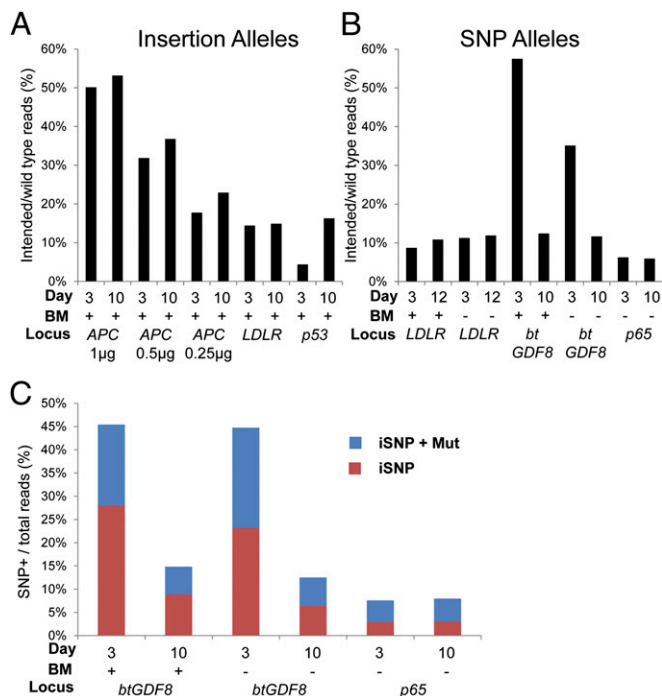


Fig. 4. Sequence analysis of TALEN-stimulated HDR alleles. PCR amplicons flanking the target site (200–250 bp total) derived from TALEN mRNA and oligo-transfected cell populations were sequenced by Illumina sequencing. The total read count ranged from 10,000 to 400,000 per sample. (A and B) The counts of perfect, intended HDR reads versus the WT reads are 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. (C) Reads from *btGDF8* and *p65* populations were sorted for incorporation of the target SNP and then classified into intended (iSNP only) vs. those with the target SNP plus an additional mutation (iSNP+Mut) and plotted against the total number of reads.

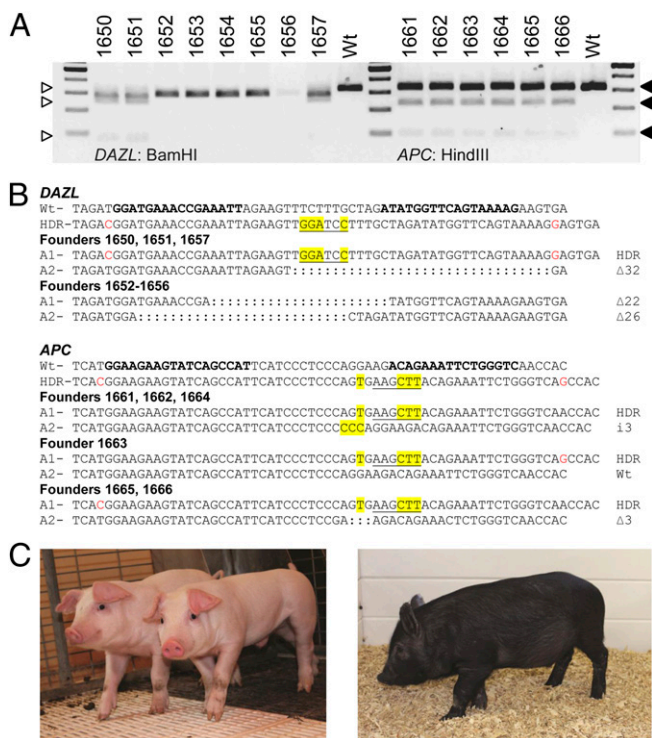


Fig. 5. Cloned pigs with HDR alleles of *DAZL* and *APC*. (A) RFLP analysis of cloned piglets derived from *DAZL*- and *APC*-modified landrace and Ossabaw fibroblasts, respectively. Expected RFLP products for *DAZL* founders are 312, 242, and 70 bp (open triangles), and those for *APC* are 310, 221, and 89 bp (filled triangles). The difference in size of the 312-bp band between WT and *DAZL* founders reflects the expected deletion alleles. (B) Sequence analysis confirming the presence of the HDR allele in three of eight *DAZL* founders, and in six of six *APC* founders. BMs in the donor templates (HDR) are displayed in red, and inserted bases are highlighted in yellow. The bold text in the top WT sequence indicates the TALEN-binding sites. (C) Photographs of *DAZL* (Left) and *APC* (Right) founder animals.

was achieved. The high efficiency and precision reported here are astonishing compared with what was feasible only a few years ago (2).

There are two concerns with gene editing: stabilizing the changes at the targeted site and avoiding modification of unintended sites. Regarding the first concern, we found evidence that HDR edits directing single bp 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 reexcision of introgressed alleles by introducing BMs; however, BMs did not prevent TALEN activity and reexcision of edited alleles (Fig. 3 and *SI Appendix*, Figs. S7 and S8). In contrast, introduction of multiple SNPs or additional sequences (Fig. 2 and *SI Appendix*, Fig. S9) resulted in more stable HDR edits. Extension of hypothermic culture also resulted in the stabilization of introgressed SNP alleles. Because hypothermia slows cell proliferation primarily by prolonging the G1 phase of the cell cycle (35), this treatment may differentially favor oligo-HDR versus sister chromatid-templated repair in a cell cycle-dependent manner. Regardless of the mechanism, this approach offers a straightforward strategy for recovering cells with precise introgression of SNP alleles. As to 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. Various objectives were achieved by precise gene editing (Table 1). Knockout of genes of biomedical relevance

was accomplished by interrupting coding sequences with 4-bp indels. This strategy was very reliable and generally resulted in HDR edits in approximately 40% of the clones (range, 26–60%), up to one-third of which 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 serve 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/knockin, a capability that we are currently exploiting in livestock and that was recently demonstrated in mice (38).

Of more immediate value, we achieved efficient nonmeiotic introgression of native alleles between species or breeds. Interspecies introgression is impossible by breeding, and intraspecies crossbreeding for allele introgression is either insufficient or deleterious to other performance features owing to the genetic admixture. Our nonmeiotic introgression includes the double-muscling mutations of *GDF8* [SNP G938A from Piedmontese cattle (23, 25) and 821del11 (23–25) from Belgian Blue cattle] 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-1B*) (40) and parent-of-origin dependent muscle hypertrophy (Callipyge) (41) into the goat genome. Up to now, nonmeiotic allele introgression has not been possible without selective enrichment, and our efficiencies are at least 10^5 -fold greater than those obtained previously with selection (42, 43). Such high levels of unselected single-allele introgression suggest the feasibility of altering 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 identifying specific alleles that affect livestock performance, and for locus-centric or 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. Nonmeiotic introgression provides a genetic method for precisely crossbreeding for large effect alleles without compromising the genetic merit of indigenous or purpose-bred populations by whole-genome admixtures (2). By allowing such single-generation introgression of select alleles, it will accelerate genetic improvement based on local ecologies and needs that may derive from climate change and emerging diseases.

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 producing genetically modified offspring by transfer of genetically modified germline stem cells, as has been demonstrated in pigs (45), goats (46), and rodents (47, 48). Gene-edited alleles of *APC* also could provide a size-relevant model of colon cancer for preclinical evaluation of therapeutics, surgical intervention, or detection modalities. These results, coupled with our previous findings (15), demonstrate relative groundbreaking 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 to serve as 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 that we have achieved

in altering either single nucleotides or small sequences in livestock fibroblasts is likely to be applicable to the correction of disease-causing mutations in patient-derived fibroblasts, a resource for conversion to induced pluripotent stem cells for use in cellular therapy (49), without the use of exogenous selection markers.

Materials and Methods

Detailed information is provided in *SI Appendix* and our previous paper (15). In brief, TALENs were assembled using the Golden Gate assembly protocol and library (50). TALEN-encoding mRNA was synthesized in vitro using the mMESSAGING mMACHINE T3 Kit (Ambion). The CRISPR/Cas9 endonucleases were generated based on the Church laboratory system and methods (8). The transfections were conducted using the Neon transfection system (Invitrogen) unless stated otherwise. 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 (Day3

Surveyor) was calculated by the Guschin method (51); %HDR was evaluated by RFLP or PCR. The colonies listed in Table 1 were obtained by dilution cloning from corresponding populations without drug selection. Population samples were sequenced on a Illumina MiSeq sequencer after PCR amplification. The RVD sequences of TALENs, oligo sequences for HDR, and PCR primers are listed in *SI Appendix, Tables S1, S3, S4, S5, and S6*. Pigs were cloned by CT under contract with Minitube of America under its Animal Welfare Assurance no. A4520/01.

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