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Original Article

Inclusion of homologous DNA in nuclease-mediated gene targeting facilitates a higher incidence of bi-allelically modified cells

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Abstract: Background: Recent advancements in gene editing techniques have increased in number and utility. These techniques are an attractive alternative to conventional gene targeting methods via homologous recombination due to the ease of use and the high efficiency of gene editing. We have previously produced cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*) knockout (KO) pigs in a Minnesota miniature pig genetic background. These pigs were generated using zinc-finger nucleases (ZFNs) in combination with donor DNA containing a total homology length of 1600 bp (800-bp homology on each arm). Our next aim was to introduce the targeted disruption of alpha-1,3-galactosyltransferase (*GGTA1*) in the *CMAH* KO genetic background and evaluate the effect of donor DNA homology length on meganuclease-mediated gene targeting.
Methods: Zinc-finger nucleases from a previous *CMAH* KO experiment were used as a proof of concept to identify a correlation between the length of donor DNA homology and targeting efficiency. Based on those results, experiments were designed to use transcription activator-like effector nucleases (TALENs) to generate bi-allelically modified *GGTA1* cells using donor DNAs carrying various lengths of homology. Donor DNA was designed to symmetrically flank the predicted cleavage sites in *CMAH* and *GGTA1* for both ZFN and TALEN cleavage sites, respectively. For both genes, the length of total homology ranged from 60 to 1799 bp. Sialyltransferase gene expression profiles were evaluated in *CMAH* and *GGTA1* double KO pig cells and were compared to wild-type and *CMAH* KO cells.
Results: Introduction of donor DNA with ZFNs demonstrated that small amounts of homology (60 bp) could facilitate homology-directed repair during ZFN-mediated targeting of *CMAH*; however, donor DNA with longer amounts of homology resulted in a higher frequency of homology-directed repair. For the *GGTA1* KO experiments that used TALENs and donor DNA, donor DNA alone did not result in detectable bi-allelic conversion of *GGTA1*. As the length of donor DNA increased, the bi-allelic disruption of *GGTA1* increased from 0.5% (TALENs alone, no donor DNA present) to a maximum of 3% (TALENs and donor DNA with total homology of 1799 bp). Inclusion of homologous donor DNA in TALEN-mediated gene targeting facilitated a higher incidence of bi-allelically modified cells. Using the generated cells, we were able to demonstrate the lack of *GGTA1* expression and the decrease in gene expression sialyltransferase-related genes.
Conclusions: The approach of using donor DNA in conjunction with a meganuclease can be used to increase the efficiency of gene targeting. The gene editing methods can be applied to other genes as well as other

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mammalian systems. Additionally, gene expression analysis further confirms that the *CMAH*/*GGTA1* double KO pigs can be a valuable source for the study of pig-to-human xenotransplantation.

Introduction

Advancements in genetic engineering technologies have reached a point where imagination, resources, and regulatory bodies are the only limitations. Conventional genetic engineering of cell lines and/or animal models has traditionally utilized random integration or low-efficiency gene targeting through homologous recombination (HR). The introduction of programmable meganucleases, specifically zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) have enabled high-efficiency modification of target genomic sequences. In principal, these tools induce double-strand breaks (DSB) at specific locations in the genome and result in either random mutations through non-homologous end joining (NHEJ) or the introduction of specific modifications through homology-directed repair (HDR) [1]. In the pig, the different types of nucleases have been effective in generating targeted mutations through NHEJ (ZFNs [2], TALENs [3], CRISPR/Cas9 [4]), as well as through HR in the presence of donor DNA [5,6].

As genetic engineering schemes become more elaborate, a need to generate precise modifications is required. Specifically, more precise modifications such as HDR will be of more interest rather than non-specific mutations, which can be generated through NHEJ. HR is a normal cellular mechanism that occurs to repair damaged sequence through a double crossover event with the sister chromatid. The efficiency of gene targeting through HR selection is relatively inefficient, ranging from 0.5% to 5.0% [7–11]. It should be noted that in order for HR to occur, genomic breakage of the target sequence is the rate-limiting factor [8,9,12–14]. Designed nucleases overcome this rate-limiting step by creating a targeted DSB.

Pigs are considered a potential resource as organ donors for humans because of their compatible organ size and physiology [15–18]. However, pig-to-human xenotransplantation has several immunological barriers to overcome. Rapid rejection of grafted organs is primarily due to hyperacute rejection (HAR) [19–21]. HAR is a response mediated by natural antibody reactivity followed by complement activation and cell lysis. In an attempt to overcome a major xeno-antigen, N-glycolylneuraminic acid (*Neu5Gc*), we have produced cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*) knockout (KO) pigs in a Minnesota miniature pig genetic background [5]. *Neu5Gc* is a sialic acid, which is synthesized by *CMAH*. A mutation in the human *CMAH* gene results in the absence of *Neu5Gc* in humans. The ablation of a second xeno-antigen gene (alpha 1,3 galactosyltransferase, *GGTA1*) was desired in the *CMAH* $-/-$ genetic background. *CMAH* and *GGTA1* are expressed on endothelial cells of most mammals, with the exception of humans [22–26]. Therefore, these epitopes are potential targets for human endogenous antibodies, and the ablation of these epitopes would prevent human preformed antibodies for these epitopes to initiate rejection of transplanted organs.

The objective of this project was to evaluate the effect of the length of homology of donor DNA with varying lengths of homology in TALEN-mediated gene targeting and to generate pigs that lack functional *GGTA1* and *CMAH* genes. A series of experiments were performed that used nucleases (ZFNs and TALENs) to characterize the effect of donor DNA homology length during nuclease-mediated gene targeting. In addition, gene expression analysis was performed on the pigs and the resulting expression data further adds to the value of this model and its application in xenotransplantation [27,28].

Materials and methods

ZFN design and construction

Design of custom ZFN plasmids targeting *CMAH* is described in Kwon et al. [5].

Production of CMAH small donor DNAs

To identify a minimum length of homology required to induce HR during ZFN-mediated targeting, a series of donor DNAs with varying lengths of homology were generated. First, two oligonucleotides containing short homology were annealed and then PCR was performed to extend the homology (Fig. 1). ZFN binding sites were modified, and an in-frame stop codon was introduced to the donor DNA: 35 (A)-, 85 (B)-, and 125 (C)-bp homology on each arm. All oligonucleotides used to generate the short donor DNAs are shown in Table S1.

Transfection of CMAH donor DNAs and ZFNs

Transfection conditions were a modification of Ross et al. [29]. For gene targeting, 1 million wild-type porcine fibroblast cells were co-transfected with a pair of ZFN plasmids and donor DNA (600 ng total; 1 : 1 : 1 mass ratio). The cells were electroporated at 490 V with 3 pulses at 1 millisecond per pulse with a square wave generator (BTX Electro Cell Manipulator, Harvard Apparatus, Holliston, MA). ZFNs alone served as a control.

DNA isolation and quantitative real-time PCR

To identify the rate of gene targeting induced by HR, DNA was isolated three days after transfections. Fifty nanograms of genomic DNA was used for PCR analysis. Quantitative real-time PCR was performed with IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The PCR primers are shown in Table S2. The PCR was performed on a MyiQ single color real-time thermal cycler (Bio-Rad). The program used for amplification included an initial temperature of 94 °C for 2 min followed by 40 cycles of 5 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Real-time fluorescence data were collected during the extension time. Differences in gene targeting were compared using ANOVA, and $P < 0.05$ was considered significant. Three biological replicates with two technical replicates were conducted.

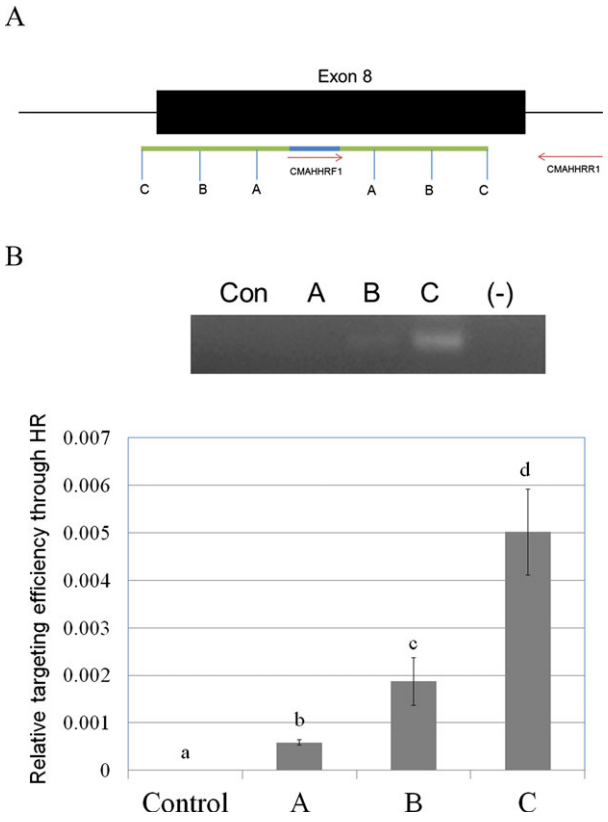

Fig. 1. Designed ZFN and comparison of donor DNA homology length. (A) Schematic design of ZFN-mediated targeting of *CMAH* using small donor DNAs. HR junction was amplified by PCR. Green indicates homology region. Blue indicates location of modified ZFN binding site with added in-frame termination codon. Three different donors were generated for the study: 35 (A)-, 85 (B)-, and 125 (C)-bp homology on each arm. (B) Efficiency of gene targeting through HR in ZFN-mediated targeting of *CMAH*. Donor with the longest homology resulted in the highest efficiency of targeting (donor C). Targeting through HR was detected from donor A using quantitative real-time PCR; however, we could not detect the PCR product on a gel. Different letters indicate statistical difference ($P < 0.05$).

TALEN design and construction

A single TALEN pair was designed to target exon 4 of the porcine *GGTA1* locus (NCBI: NM_213810) (Fig. 2). TALEN design and construction was completed by ToolGen, Incorporated (toolgen.com).

Targeting vector construction and production of small donor DNAs

A targeting construct was assembled from DNA isolated from female "Minnesota miniature" porcine fetal fibroblasts (Cell Line: 104824; National Swine Resource and Research Center [NSRRC]). A 2389-bp *GGTA1* genomic fragment, which includes most of exon 4, was generated by polymerase chain reaction (PCR) using specific primers (forward: 5′-TTCCACCCAGCATCCCTTCCCT CT-3′ and reverse: 5′-TGGCCAAAATGGTCCT GCTGGCTC-3′) and Q5 DNA polymerase (New England Biolabs, NEB, Ipswich, MA). PCR amplification was repeated for 30 cycles of 10 s at 98 °C for denaturation, 30 s at 72 °C for annealing, and 150 s at 72 °C for extension. The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen, Grand Island, NY, USA) to produce the plasmid pBB60. Sanger sequencing (University of Missouri DNA Core Facility, Columbia, MO, USA) and restriction enzyme diagnostics confirmed the sequence of the *GGTA1* fragment. Reverse PCR was performed to generate donor DNA that would contain a mutated TALEN cut site, stop codons in three reading frames, and unique restriction enzyme sites for future diagnostics. The PCR was performed on pBB60 using specific primers (forward: 5′-CTCGAGCCTGCAGGCAGGTAATT ATGAAAC-3′ and reverse: 5′-CTACCCTATC-TAGCATTGACAGAACCACTC-3′) and the Ta KaRa LA system (TaKaRa Bio Inc., Ohtsu, Japan). PCR amplification was repeated for 30 cycles of 30 s at 94 °C for denaturation, 30 s at 54 °C for annealing, 180 s at 68 °C and increased

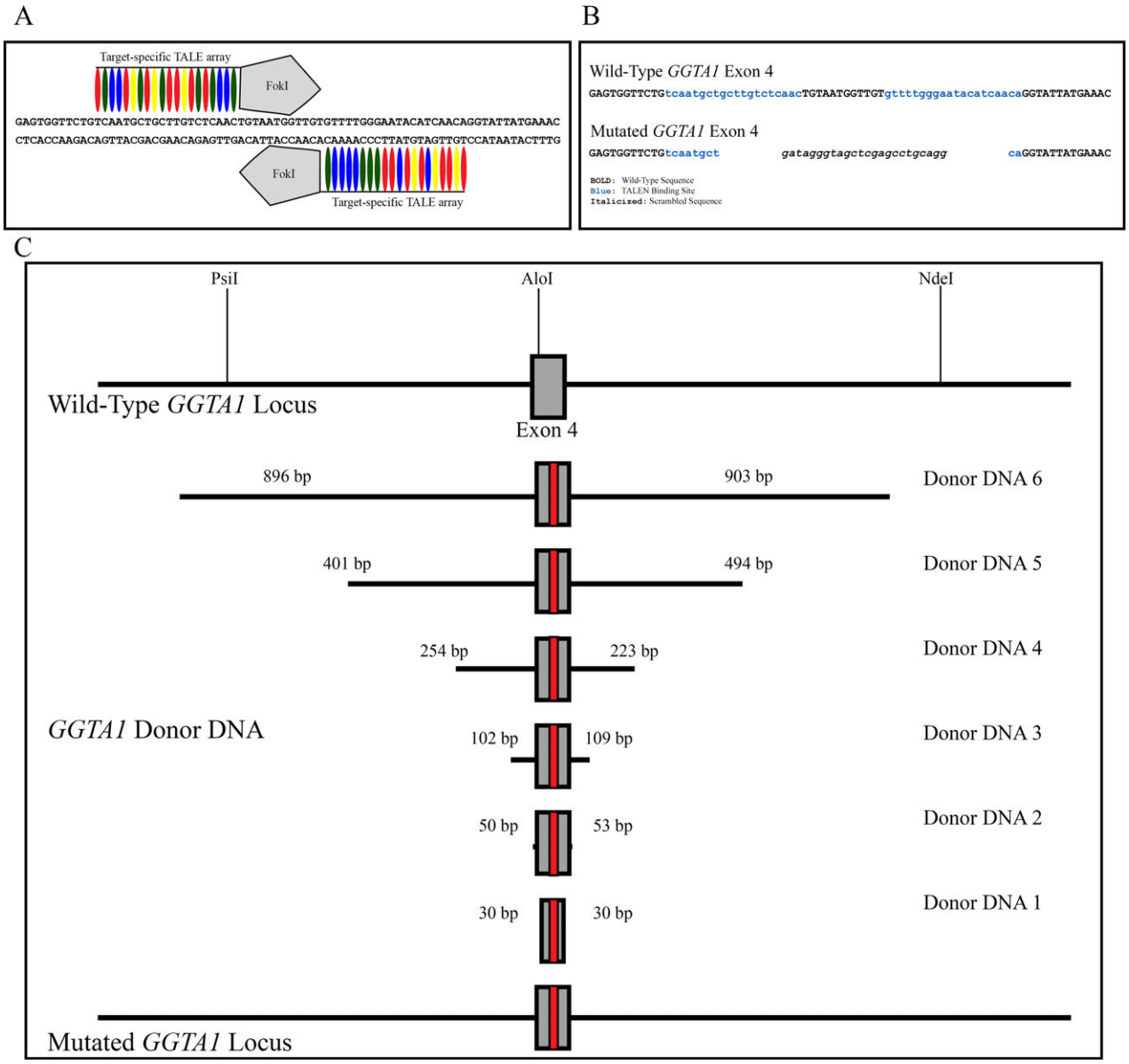

Fig. 2. Designed TALENs and comparison of homology length of donor DNA. (A) Designed TALENs to target porcine *GGTA1*. The TALENs were designed to recognize 20 bp with a 12-bp spacer between the left and right TALENs. The TALENs were designed to create a double-stranded break in exon 4 of *GGTA1*. (B) Targeted *GGTA1* exon 4 sequence and mutated *GGTA1* exon 4 donor DNA sequence. (C) Graphical representation of donor DNAs with 6 different lengths of homology.

by 6 s per cycle for extension, and with a final extension for 360 s at 72 °C. The PCR product was purified by ethanol precipitation; the 5′ and 3′ ends were phosphorylated (T4 PNK, NEB) and ligated (T4 ligase, NEB) prior to *E. coli* transformation (5α Competent, NEB). Sanger sequencing (DNA Core Facility) confirmed the mutated sequence. The targeting and mutated targeting constructs are graphically depicted, and sequence differences are represented in Fig. 2.

Small donor DNAs with minimal homology sequence (total length of homology: 60, 103, 211, 477, 895, and 1799 bp) were amplified by PCR (EconoTaq DNA Polymerase, Lucigen, Madison, WI, USA) from primer sets shown in Table S3. PCR amplification was repeated for 30 cycles of 15 s at 94 °C for denaturation, 15 s at 56 °C for annealing, 30 s (short donor DNA: 86 bp, 129 bp, 237 bp, 495 bp) or 120 s (long donor DNA: 921 bp, 1825 bp) at 72 °C for extension, and with a final extension for 120 s at 72 °C. The PCR product was visualized on a 0.8% agarose gel, isolated, and purified from agarose using β-agarase I (NEB), followed by ethanol precipitation.

Preparation of porcine fibroblast cells and culture conditions

Ear tissue was collected and minced from newborn piglets to create ear fibroblast cultures from female *CMAH* KO Minnesota miniature pig genetic background [5]. The minced tissue was digested in 20 ml of digestion medium (Dulbecco's modified Eagle's medium containing 2.77 mM glucose, 1.99 mM L-glutamine, 0.5 mM sodium pyruvate (Cellgro, Manassas, VA, USA) supplemented with 200 units/ml collagenase and 25 Kunitz units/ml DNaseI) for 5 h at 38.5 °C. After digestion, ear fibroblast cells were washed and cultured with DMEM, 15% fetal bovine serum (FBS, Cellgro) with 40 μg/ml gentamicin (Cellgro). After overnight culture, cells were trypsinized and frozen at -80 °C in aliquots of FBS with 10% dimethyl sulfide (DMSO, Sigma, St. Louis, MO, USA) and then stored in liquid nitrogen.

Transfection of donor DNA and TALEN plasmids

Transfection conditions were a modification of Ross et al. [29] and as previously reported [30]. Four replicates of eight transfections were conducted with porcine ear fibroblasts from a female *CMAH* KO Minnesota miniature pig [5]. The fibroblast cells were cultured for 72 h in complete medium (DMEM and 12% FBS) at 38.5 °C in 5.5% CO_2, 5% O_2, and 89.5% N_2 balanced humidified air. Fibroblasts were harvested using trypsin and resuspended in complete medium at a density of 57 000 cells/ml. Cells were plated at a density of 28 500 cells (0.5 ml of suspension) per well in eight wells (seven treatments and one control) of a 24-well plate. Approximately 24 h post-plating of the cells they reached 50 to 60% confluency and were ready to be transfected. Left TALEN, right TALEN, and the donor DNA were cotransfected (600 ng total DNA; 1 : 1 : 1 mass ratio) using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). Transfected cells were cultured in complete medium for 2 days, passaged, and cultured for another 3 days.

Screening and analysis of knockout cells and pigs

Five days after transfection, cells were analyzed by fluorescence-activated cell sorting (FACS). Cells were trypsinized, washed with PBS, and centrifuged. Cells were resuspended in 1 ml PBS + 3% FBS and incubated with 10 μg isolectin-B4-FITC (IB4-FITC) (Sigma) for 1 h at 4 °C. As the α-1, 3-galactose (Gal) epitope is recognized by IB4-FITC, *GGTA1*-null cells would not fluoresce green and thus were counter-selected by using flow cytometry (MoFlo XDP, Beckman Coulter; University of Missouri Cell & Immunobiology Core Facility, Columbia, MO, USA). Three biological replicates were performed for each treatment. Flow sorted cells were pooled and cultured for 7 days. After 7 days, the cells were harvested and resuspended in 30 μl of lysis buffer (40 mM Tris, pH 8.9; 0.9% Triton X-100; 0.9% Nonidet P-40; 0.4 mg/ml proteinase K, [Sigma]), incubated at 65 °C for 15 min to disrupt the cells, and then heated to 95 °C for 10 min to inactivate the proteinase K. Using Q5 DNA polymerase (NEB), a single primer set was used for PCR analysis (Table S4). The PCR was designed to amplify porcine *GGTA1* sequence located outside of the recombination region homologous with the donor DNA template resulting in an amplimer of 2389 bp. PCR amplification was repeated for 30 cycles of 10 s at 98 °C for denaturation, 30 s at 72 °C for annealing, and 150 s at 72 °C for extension. To test whether there was a statistical significance across the entire dataset, a pairwise chi-squared analysis was performed and showed significance at $P < 0.05$. To test between treatment means, count data were analyzed using the Student's *t*-test.

One replicate of *GGTA1*-null cells were sorted and plated at clonal density to later perform analysis on single cell populations. After 10 days, half of the cells were used for genotyping and half were expanded to be cryopreserved for long-term preservation. Upon expansion of *GGTA1*-null cells, the lack of cell surface expression of *GGTA1* was determined. Fibroblast cells were washed with PBS, and stained with a 1 : 500 dilution of 1 mg/ml isolectin B4 (IB4)-FITC in PBS for 30 min at 4 °C.

Somatic cell nuclear transfer

For somatic cell nuclear transfer (SCNT), sow-derived oocytes were purchased from ART (Madison, WI). Cumulous oocyte complexes were aspirated from sow ovaries and then shipped overnight in maturation medium (TCM199 with 2.9 mM Hepes, 5 mg/ml insulin, 10 ng/ml EGF, 0.5 mg/ml p-FSH, 0.91 mM pyruvate, 0.5 mM cysteine, 10% porcine follicular fluid, 25 ng/ml gentamicin). Upon arrival, the oocytes were transferred into fresh medium and cultured for additional 20 to 24 h. After 40 to 42 h of maturation, cumulus cells were removed from the oocytes by vortexing for 3 min in the presence of 0.1% hyaluronidase. The denuded oocytes were manipulated in the maturation medium supplemented with 7.0 μg/ml cytochalasin B. The polar body along with a portion of the adjacent cytoplasm was

removed, and a donor cell was placed in the perivitelline space as described previously [15]. A combination of two donor cell lines carrying bi-allelic modifications of *GGTA1* and *CMAH* was used. The reconstructed embryos were then fused in a fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM Hepes) using two DC pulses (1-sec interval) at 1.2 kV/cm for 30 μs (using a BTX Electro Cell Manipulator, Harvard Apparatus, Holliston, MA). After fusion, fused embryos were fully activated with 200 mM thimerosal for 10 min in the dark followed by 8 mM dithiothreitol for 30 min [31]. Embryos were then incubated in PZM3 [32] with 0.5 mM scriptaid, a histone deacetylase inhibitor, for 14 to 16 h [33,34]. The following day, the SCNT embryos were surgically transferred into the ampullary–isthmic junction of a surrogate at 0 or 1 day after observed estrus.

RNA isolation and real-time RT-PCR

Total RNA was extracted from WT-, *CMAH* KO-, and *CMAH*/*GGTA1* double KO (DKO)-null pig fibroblast using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was conducted using an ABI ViiA™ 7 system (Applied Biosystems, Foster City, CA, USA) and SYBR Green as the double-stranded DNA-specific fluorescent dye (Bio-Rad, Hercules, CA, USA) (see Table S5 for RT-PCR primer sets). The pig *ACTB* gene was used as an internal control to normalize the RT-PCR efficiency and to quantify the expression of the genes in WT pig, homozygous *CMAH* KO, and homozygous *CMAH*/*GGTA1* DKO pig-derived mRNA. After normalization with *ACTB* mRNA, the relative expression of each mRNA was compared in the homozygous *CMAH* and homozygous *CMAH*/*GGTA1* DKO pig-derived genes with those of the controls. We performed RT-PCR on each sample independently and in triplicate.

Results

Effects of the homology length of donor DNA in ZFN-mediated gene targeting

When ZFNs were used to target *CMAH* using various lengths of donor DNAs, the highest targeting efficiency through HDR was observed when donor DNA with 125-bp homology on each arm was used; the efficiency detected by quantitative real-time PCR was 10 times higher compared to the donor DNA with 35-bp homology (Fig. 1). No recombination event was detected in the negative control, as was expected. When the PCR products were examined by gel electrophoresis, we could not detect a visible PCR product from the 35-bp homology group. However, the highest intensity of PCR product was detected in the presence of donor DNA with the longest homology (donor C).

Effects of the homology length of donor DNA in TALEN-mediated bi-allelic modifications

To identify the minimal homology length of donor DNA that can induce HR at the targeting locus, a series of DNA fragments of various lengths homologous to *GGTA1* exon 4 were generated (Fig. 2). Each donor DNA was transiently transfected with TALENs into fibroblast cells that lack functional *CMAH*. Fibroblast cells were recovered 5 days after transfection and analyzed by FACS. Bi-allelic modifications to the *GGTA1* gene were analyzed by counter-selection with IB4-FITC. All treatments that included donor DNA resulted in higher numbers of *GGTA1*-null cells (Fig. 3), with the largest quantity of *GGTA1*-null cells resulting from donor DNA with the longest length of homology. Based upon our results, all treatments that contained TALEN expression vectors resulted in significantly more mutations within *GGTA1* as compared to donor DNA alone. Additionally, treatments that contained TALEN expression vector DNA and donor DNAs with the lengths associated with donor DNAs 2, 3, and 4 resulted in significantly more mutations within *GGTA1* as compared to TALEN expression vector alone. However, there was not a significant difference between the treatments that contained both donor DNA and TALEN expression vectors.

Disruption of GGTA1 gene

When TALENs designed for the *GGTA1* gene were introduced into female *CMAH*-null fibroblast cells with donor DNA, *GGTA1*-null cells were detected. After transfection and sorting of cells that lacked the Gal epitope, the Gal-negative cells were plated at clonal density. DNA was isolated from 66 cell colonies, and then, the DNA from each colony was used to determine the type of mutations within *GGTA1* by PCR (data not shown). Zero colonies showed targeted mutation of the *GGTA1* gene via homologous recombination, and six colonies showed targeted bi-allelic mutation of the *GGTA1* gene via NHEJ with major deletions (395 to 716 bp). A handful of colonies that did not have major deletions were also sequenced. All contained

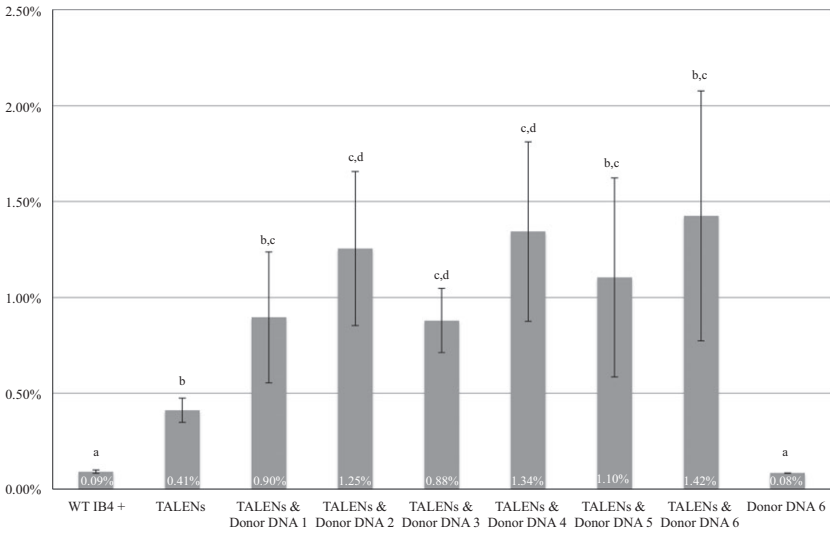

Fig. 3. Targeting of *GGTA1* locus via TALENs and varying lengths of donor DNA. Average IB4-negative cells are represented in each column along with the standard error of means (4 replicates). *GGTA1*-null cells were counter-selected using flow cytometry post-TALEN transfection: including controls wild-type Minnesota miniature (WT IB4+) cells, homozygous KO *GGTA1* (D.KO IB4−) cells (NSRRC) (data not shown), WT cells transfected with TALENs alone, and WT cells transfected with longest donor DNA alone (Donor DNA 6). All treatments are compared to WT control, WT control transfected with TALENs alone, and longest donor DNA. Different letters indicate statistical difference ($P < 0.05$).

a single-bp deletion near the right TALEN recognition site.

Production of bi-allelic GGTA1 knockout miniature pigs in a CMAH-null background

Upon screening for *GGTA1* KO events by both flow cytometry and PCR, three cell colonies were used to produce *CMAH*/*GGTA1* DKO pigs. Two colonies (26 and 30) that harbored the same homozygous deletions of 395 bp and the colony (22) that harbored a homozygous deletion of 719 bp of *GGTA1* were used to produce the *CMAH*/*GGAT1* DKO pigs. A single day of SCNT was performed resulting in 183 reconstructed embryos that were transferred into a single surrogate and produced 3 piglets (Fig. 4). Genotyping from the cloned pigs showed the expected deletions of the *GGTA1* and *CMAH* genes (Fig. 4). Two piglets harbored a 395-bp deletion, one piglet had a 716-bp deletion, and for all three piglets, the genotypes appeared to be bi-allelic modifications for the respective deletions (Fig. 4).

As the IB4 lectin from *Bandeiraea simplicifolia* binds the Gal epitope [35], a FITC-conjugated IB4 lectin was applied to fibroblasts that were isolated from the tail of the live piglets. In contrast to wild-type cells, a fluorescence signal for IB4 could not be detected on cells from any of the three piglets (Fig. 4), indicating the lack of Gal epitope and implying a loss of function of *GGTA1*.

Decreased transcript level of H-D and Tn-related genes in CMAH and GGTA1 DKO pig cells

The Hanganutziu–Deicher antigen (H-D) plays a pivotal role in acute immune rejection of pig xenografts [36–38]. Generally, H-D antigen families are classified as 2 different subfamilies; *ST3Gal* (*ST3Gal1*, *ST3Gal2*, *ST3Gal3*, *ST3Gal4*, *ST3Gal5*, and *ST3Gal6*) and *ST6Gal* (*ST6Gal1* and *ST6 Gal2*), according to the carbohydrate linkages synthesized [39]. To normalize the mRNA level of WT, *CMAH* bi-allelic KO, and *CMAH*/*GGTA1* bi-allelic DKO pigs, *ACTB* mRNA was used as an internal standard. After normalization with *ACTB* mRNA, *ST3GAL1*, *ST3GAL3*, *ST3GAL4*, *ST6G AL1*, and *ST6GAL2* gene expression in *CMAH*/*GGTA1* bi-allelic DKO pigs were observed to be significantly downregulated, whereas *ST3GAL2* was upregulated, compared to those of WT and *CMAH* bi-allelic KO pigs, respectively (Fig. 5). This observation suggested that downregulated *ST3GAL1*, *ST3GAL3*, *ST3GAL4*, *ST6GAL1*, and *ST6GAL2* in *CMAH*/*GGTA1* bi-allelic DKO pigs potentially should result in decreased Siaα2, 3Galβ1,3GalNAc-R, Siaα2,3Galβ1,4GlcNAc-R, and Siaα2,6Galβ1,4GlcNAc-R expression, but increased Siaα2,3Galβ1,3GalNAc-R, Sialyl Lew X: Siaα2,3Galβ1, 4(Fucα1,3)GalNAc-R, and Sia α2,4Galβ1,4GlcNAc-R expression, respectively, that act as an immune antigen within transplanted recipients. Also, *ST6GALNAC3* expression for Sialyl-Tn antigen, and *GALNT2, GALNT3, GALNT4,* and *GALNT7* expression for Tn antigen in bi-allelic *CMAH* KO pigs were significantly downregulated compared with *CMAH* bi-allelic KO pigs (Fig. 5).

Discussion

Gene targeting allows precise modifications to be made in cells for the functional analysis of a specific gene. However, the fairly low efficiency of

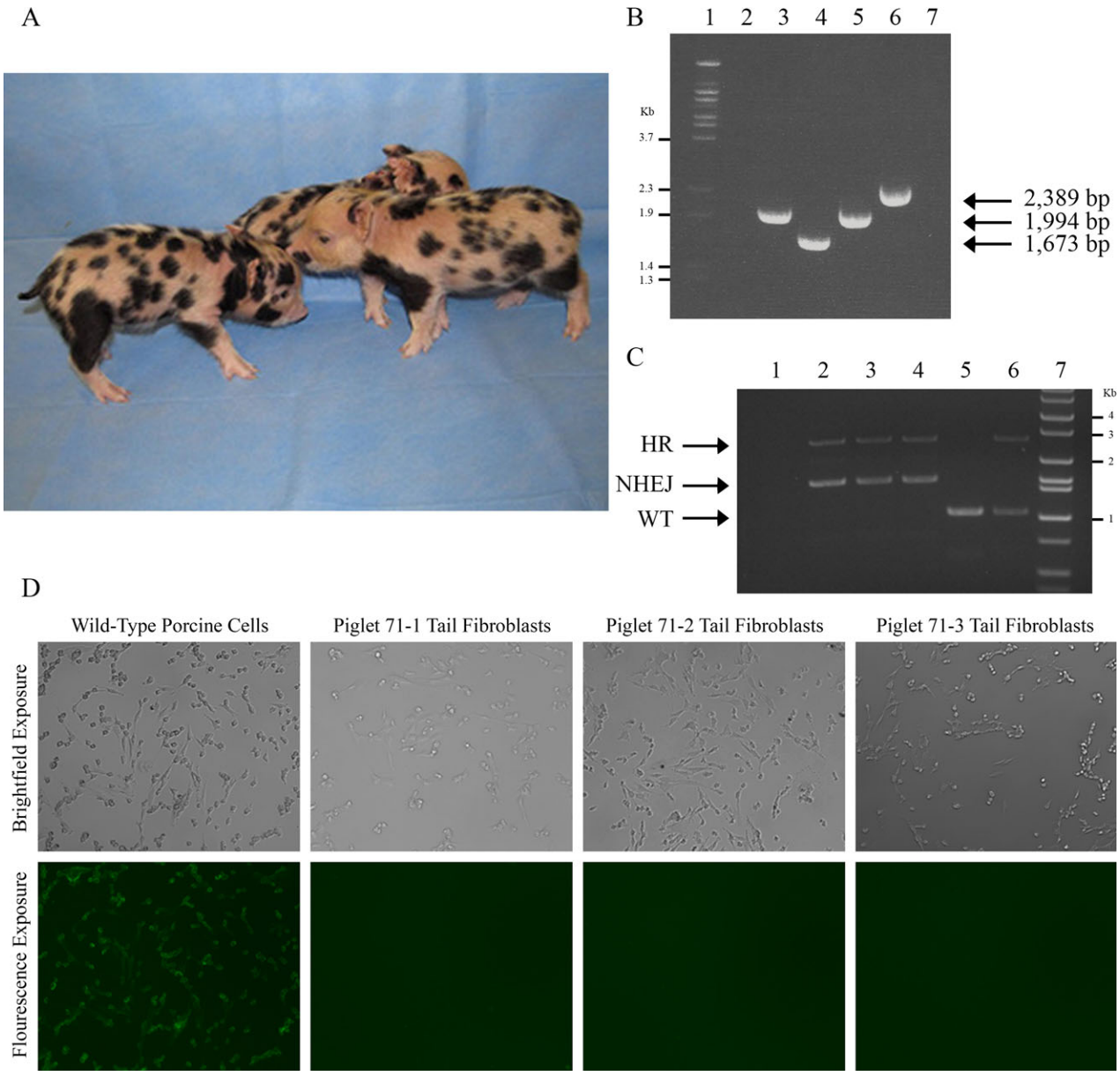

Fig. 4. CMAH/GGTA1 DKO Pigs. (A) Image of *CMAH/GGTA1* DKO pigs. (B) Genotyping of DKO Pigs, *GGTA1* locus. For genotyping, PCR was performed to verify KO of *GGTA1* by amplifying the entire exon 4 junction (2389 bp for the endogenous gene). Lane 1: DNA ladder, lambda genome digested with BstEII (NEB), lane 2: empty lane, lane 3: piglet 71-1, lane 4: piglet 71-2, lane 5: piglet 71-3, lane 6: wild-type (WT) control, and lane 7: H_2O negative control. PCR products from piglets 71-1 and 71-3 indicate that both alleles have a deletion of 395 bp (lanes 3 and 5, respectively). PCR product from piglet 71-2 indicates that both alleles have a deletion of 716 bp (lane 4). (C) Genotyping of DKO Pigs, *CMAH* locus. For genotyping, a PCR was run to verify KO of *CMAH*. A left arm PCR was performed to amplify the left HR junction (1.2 kb for endogenous WT, a 3.2 kb for KO by HR, and 1.7 kb for partial insertion of selectable marker) using ScS5 and *CMAH* R [5]. PCR products from bi-allelic *CMAH* KO pigs confirmed one allele was targeted through HR and the other allele was targeted through NHEJ; lane 1: H_2O negative control, lane 2 to 4: piglets 71-1, 2, and 3, lane 5: WT control, lane 6: heterozygous targeted *CMAH* control pig, and lane 7: DNA ladder. (D) Cell surface expression of the Gal epitope using IB4 lectin-FITC. The WT cells clearly have lectin binding, thus *GGTA1* expression. All three *CMAH/GGTA1* DKO pigs clearly do not have lectin binding, thus are lacking *GGTA1* expression. Cells were imaged at 10× magnification, with a 5-s exposure.

traditional gene targeting has limited the utility of gene targeting in the study of gene function. Therefore, an increase in the efficiency of gene targeting will have an impact on the production of genetically engineered pigs.

The application of programmable meganucleases has been used to edit the genomes of a variety of species. To date, three different types of programmable nucleases, ZFNs [40], TALENs [41], and CRISPR/Cas9 [42], have been used for

genome editing. These tools facilitate the induction of DSBs in a target-specific manner. Upon genomic breakage, cells can repair the damage through one of two major DNA repair systems, NHEJ or HR. If the break is repaired by NHEJ, repaired sequences frequently harbor insertions or deletions. The mechanism of HR enables a precise repair of the genomic break, but operates at a much lower frequency [43]. Traditional gene targeting approaches, which utilize HR machinery in the cell, have typically used targeting arms carrying homology sequence to the target locus in the range of 1 to 8 kb [8] although successful HR has also been reported using less than 0.5-kb homologous arms [44]. In general, the length of contiguous sequence identity is the major determinant of HR efficiency [45]. In addition, Smih et al. [10] demonstrated that gene targeting through HR could be stimulated by genomic DSBs introduced by a restriction enzyme. Customized meganucleases provide a means to generate DSB at specific locations within the genome, thereby increasing the efficiency of gene targeting.

When we used a pair of functional ZFNs [5] in conjunction with three different sizes of donor DNAs, we demonstrated that small repair template (donor DNA) can facilitate HDR. We detected recombination events with all lengths of homology arms (as small as 35 bp and as long as 125 bp), indicating that activation of HDR does not require long amounts of homology. However, an increase in efficiency of HDR with the donor with longer homology suggests that the length of homology is a critical element in successful HDR.

Given our ability to facilitate HDR with small donor DNA in vitro, we chose to investigate the

A

Antigen	Gene	WT	*CMAH* KO	Double KO
H-D antigen	*ST3Gal1*	1.049±0.048	0.440±0.020**	0.035±0.001***
	ST3Gal2	1.031±0.033	0.150±0.009***	0.302±0.119*
	ST3Gal3	1.038±0.037	0.290±0.012***	0.178±0.005***
	ST3Gal4	1.040±0.035	0.562±0.037**	0.311±0.006***
	ST6Gal1	1.046±0.067	0.336±0.004*	0.018±0.001***
	ST6Gal2	1.038±0.053	0.638±0.055*	0.001±0.000***
Sialyl Tn antigen	*ST6GalNac2*	1.087±0.076	0.604±0.017*	1.589±0.115*
	ST6GalNac3	1.079±0.112	0.074±0.033*	0.003±0.002*
	ST6GalNac6	1.048±0.046	0.196±0.009***	0.234±0.008***
Tn antigen	*GalNT1*	1.015±0.026	0.463±0.022***	0.491±0.007**
	GalNT2	1.046±0.066	1.311±0.031	0.582±0.094*
	GalNT3	1.042±0.056	1.413±0.104	0.249±0.023**
	GalNT4	1.052±0.023	0.621±0.023*	0.414±0.018**
	GalNT7	1.053±0.039	0.801±0.039	0.336±0.026*

*P=<0.005, **P<0.0001, ***P<0.00001

B

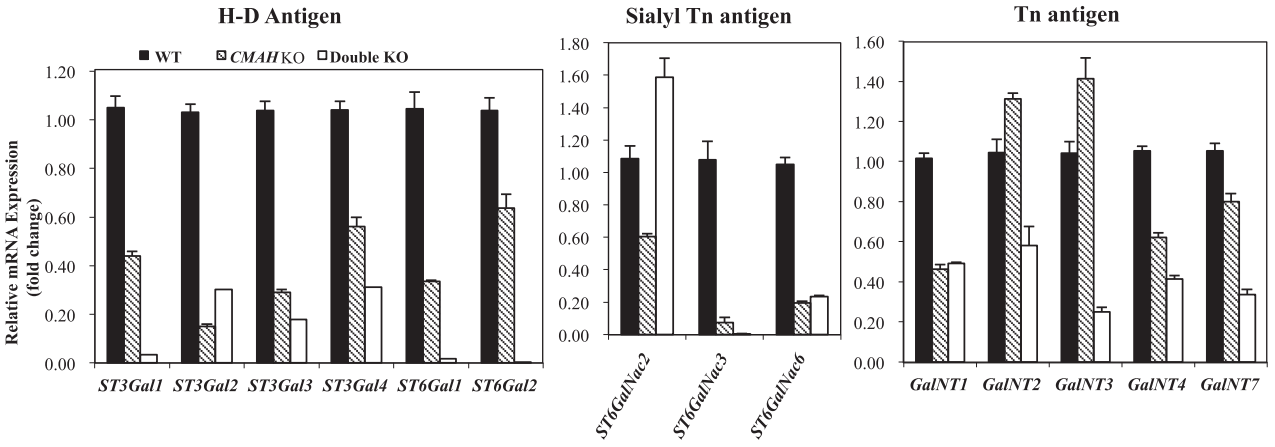

Fig. 5. Sialyltransferase gene expression levels in control, bi-allelic *CMAH* KO, and bi-allelic *CMAH* and *GGTA1* DKO pigs. (A) Comparison of sialyltransferase gene expression in control-, *CMAH* KO-, and *CMAH*/*GGTA1* DKO-derived pig fibroblast cells using real-time RT-PCR. (B) Quantification of real-time RT-PCR analysis in control, *CMAH* KO, and *CMAH*/*GGTA1* DKO pig fibroblast cells. All RT-PCRs were conducted in triplicate and normalized with pig actin mRNA. Each of *CMAH* KO and *CMAH*/*GGTA1* DKO pig relative values is presented as an n-fold expression difference compared to the control pig, which was set as 1. *P < 0.05, **P < 0.001, and ***P < 0.00001.

lengths of donor DNA and their effects with TALEN-mediated gene targeting of the porcine *GGTA1* gene. In an attempt to determine the minimum length of homology utilizing TALEN-mediated gene targeting, we demonstrated that the addition of donor DNA increases the bi-allelic modifications when compared to TALEN-mediated gene targeting without donor DNA. As our primary goal was to identify homozygous *GGTA1* KO cells, *GGTA1*-null cells were counter-selected using flow cytometry to detect the Gal-deficient cells. The introduction of any sized donor DNA resulted in a higher amount of bi-allelic modifications. However, the modifications were point mutations. The normal cellular repair pathway of NHEJ likely introduced these mutations. The mechanism of donor DNA stimulation of NHEJ remains unknown.

When comparing the two methods of nuclease-mediated gene targeting (ZFN vs. TALEN), the differences observed are likely due to: (i) experimental variation; (ii) the higher fidelity of the PCR detection methods (ZFN experiment); and/or (iii) the donor DNA may have an effect on the NHEJ pathway rather than through the HR repair mechanism (TALEN experiment). In the ZFN/*CMAH* study, we exclusively measured the efficiency of HR, while in the TALEN/*GGTA1* experiments a lectin-based approach screened for the phenotypic changes of the Gal epitope after bi-allelic modifications to the *GGTA1* gene. Even though our ZFN/*CMAH* results demonstrated that HR was occurring, HR did not create bi-allelic modifications in the TALEN/*GGTA1* results. However, we did observe an increase in *GGTA1*-null cells when both donor DNA and TALENs were applied. There are two potential explanations for this. The first is that our study only assessed for bi-allelic modifications of *GGAT1* (KO cells); we may have unintentionally created a bias to observe cells that were modified by the NHEJ pathway. The second is that the donor DNA may actually play a role in NHEJ and not just HR. As previously reported [5,6], we have observed an increase in targeting efficiency in the presence of donor DNA, but NHEJ and not HR is observed. A potential explanation is that HR and NHEJ pathways are not independent, but can, or potentially, do act together in the repair process.

The Gal epitope has been identified as the primary target for natural human antibodies, and the successful KO of the *GGTA1* gene was an important step toward overcoming the major immunological barrier in pig-to-human xenotransplantation [16,18]. The *CMAH* gene has also been identified as another potential major xeno-antigen [23,24]. As previously reported by Kwon et al. [5], the ablation of the *CMAH* gene resulted in the reduction in expression of Hanganutziu–Deicher (H-D), Thomsen–Friedenreich (TF), Tn, and sialyl-Tn. It is known that the enzymes from all four gene families play a pivotal role in acute immune rejection of pig xenografts [38]. In this study, we were able to demonstrate that the gene expression in most instances was drastically reduced in the *CMAH*/*GGTA1* DKO cells compared to wild-type cells and even further downregulated compared to *CMAH*-null cells.

In conclusion, the methods described in this study allow for an approach to generate KO cells and pigs with an increased targeting efficiency. The gene editing methods can be applied to other genes as well as other mammalian systems. Additionally, we predict that the utility of the *CMAH*/*GGTA1* DKO pigs can be a valuable source for the study of pig-to-human xenotransplantation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sets used to generate small *CMAH* donor DNA with minimal homology length in ZFN-mediated gene targeting.

Table S2. Primer sets used for genotyping *CMAH* KO cells and pigs.

Table S3. Primer sets used for donor DNA with minimal homology length in the TALEN mediated gene targeting.

Table S4. Primer sets used for screening and genotyping *GGTA1* KO cells and pigs.

Table S5. Primer sets used for detection of sialyltransferases mRNA expression.