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

Beaton BP, Kwon D-N, Choi Y-J, Kim J-H, Samuel MS, Benne JA, Wells KD, Lee K, Kim J-H, Prather RS. Inclusion of homologous DNA in nuclease-mediated gene targeting facilitates a higher incidence of bi-allelically modified cells. Xenotransplantation 2015: 22: 379–390. © 2015 The Authors. Xenotransplantation Published by John Wiley & Sons Ltd.

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

Key words: cytidine monophosphate-N-acetylneuraminic acid hydroxylase – genetic engineering – alpha-1,3-galactosyltransferase – knockout – porcine – transcription activator-like effector nuclease – zinc-finger nuclease

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Received 12 June 2015; Accepted 17 August 2015
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.

TALEN design and construction

A single TALEN pair was designed to target exon 4 of the porcine GGTAl1 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 GGTAl1 genomic fragment, which includes most of exon 4, was generated by polymerase chain reaction (PCR) using specific primers (forward: 5'-TTCCACCCAGCATCCCTTCCT-3' and reverse: 5'-TGGCCAAAATGGTCCTGCTGGCTC-3'). PCR amplification was repeated for 30 cycles of 10 s at 98 °C for denaturation, 30 s at 54 °C for annealing, and 180 s at 68 °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 GGTAl1 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'-CTCGAGCCTGCAGGTAATTAGAACCACGATACTC-3' and reverse: 5'-CTACCCCTATCATCCTGCTGCT-3').

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).
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, 300 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 sulfoxide (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% CO2, 5% O2, and 89.5% N2 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 isoleucin-B4-FITC (IB4-FITC) (Sigma) for 1 h at 4 °C. As the α-1, 3-galactose (Gal) epitope is recognized by IB4-FITC, GGT1A1-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 GGT1A 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 GGT1A1-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 GGT1A1-null cells, the lack of cell surface expression of GGT1A1 was determined. Fibroblast cells were washed with PBS, and stained with a 1 : 500 dilution of 1 mg/ml isolec tin 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

Donor DNA in nuclease-mediated targeting

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, 300 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 sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and then stored in liquid nitrogen.
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 CaCl2, 0.1 mM MgCl2, 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 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 deacetylation inhibitor, for 14 to 16 h [33,34]. The following day, the SCNT embryos were surgically transferred into the amnioserosal 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 quantitatively determine 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
implying a loss of function of (Fig. 4), indicating the lack of Gal epitope and be detected on cells from any of the three piglets type cells, a fluorescence signal for IB4 could not from the tail of the live piglets. In contrast to wild-lectin was applied to fibroblasts that were isolated binds the Gal epitope [35], a FITC-conjugated IB4

The Hanganutziu–Deicher antigen (H-D) plays a pivotal role in acute immune rejection of pig

Production of bi-allelic GGT1 knockout miniature pigs in a CMAH-null background

Upon screening for GGT1 KO events by both flow cytometry and PCR, three cell colonies were used to produce CMAH/GGT1 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 GGT1 were used to produce the CMAH/GGT1 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 GGT1 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 GGT1.

Decreased transcript level of H-D and Tn-related genes in CMAH and GGT1 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/ GGT1 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/ GGT1 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/GGT1 bi-allelic DKO pigs potentially should result in decreased Sia

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

Donor DNA in nuclease-mediated targeting

| Donor DNA | TALEN & | TALEN & | TALEN & | TALEN & | TALEN & | TALEN & | TALEN & | TALEN & |
|-----------|---------|---------|---------|---------|---------|---------|---------|---------|
| WT IB4    | 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |
| TALENs    | 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |
| Donor DNA 1| 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |
| Donor DNA 2| 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |
| Donor DNA 3| 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |
| Donor DNA 4| 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |
| Donor DNA 5| 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |
| Donor DNA 6| 1.00%   | 0.50%   | 1.50%   | 2.00%   | 2.50%   | 3.00%   | 3.50%   | 4.00%   |

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

A single-bp deletion near the right TALEN recognition site.
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, Smith 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

| 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** | 0.31±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 | 1.31±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

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 GGTA1 (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 sia-lyl-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.

Acknowledgments

The authors would like to thank those who assisted with all facets related to the project including: Lee Spate, Keith Giroux, Tricia Meyer, Jason Dowell, and Lonnie Dowell. Woo Jung-Choon Project (PJ007849) from the Rural Development Administration (RDA), Republic of Korea, and the University of Missouri Food for the 21st Century.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:
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**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 sialyl-transferases mRNA expression.