

Production of α 1,3-galactosyltransferase and cytidine monophosphate-N-acetylneuraminic acid hydroxylase gene double-deficient pigs by CRISPR/Cas9 and handmade cloning

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Abstract. Gene-knockout pigs hold great promise as a solution to the shortage of organs from donor animals for xenotransplantation. Several groups have generated gene-knockout pigs via clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) and somatic cell nuclear transfer (SCNT). Herein, we adopted a simple and micromanipulator-free method, handmade cloning (HMC) instead of SCNT, to generate double gene-knockout pigs. First, we applied the CRISPR/Cas9 system to target α 1,3-galactosyltransferase (GGTA1) and cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) genes simultaneously in porcine fetal fibroblast cells (PFFs), which were derived from wild-type Chinese domestic miniature Wuzhishan pigs. Cell colonies were obtained by screening and were identified by Surveyor assay and sequencing. Next, we chose the *GGTA1/CMAH* double-knockout (DKO) cells for HMC to produce piglets. As a result, we obtained 11 live bi-allelic *GGTA1/CMAH* DKO piglets with the identical phenotype. Compared to cells from *GGTA1*-knockout pigs, human antibody binding and antibody-mediated complement-dependent cytotoxicity were significantly reduced in cells from *GGTA1/CMAH* DKO pigs, which demonstrated that our pigs would exhibit reduced humoral rejection in xenotransplantation. These data suggested that the combination of CRISPR/Cas9 and HMC technology provided an efficient and new strategy for producing pigs with multiple genetic modifications.

Key words: CRISPR/Cas9, Double-knockout pigs, Handmade cloning, Xenotransplantation

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Gene-knockout pigs have great promise for clinical xenotransplantation as sources of organs and cells [1]. Because of the lack of identifiable embryonic stem cells, the generation of gene-knockout pigs has proved difficult and slow [2].

Zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) gene-editing technologies have made the creation of gene-knockout pigs easier and faster. To date, several gene-knockout pigs have been generated by ZFNs and TALENs [3–7]. However, both of these systems are expensive and complex

to design. Additionally, TALENs cannot easily target some parts of the genome [2].

Recently, some problems have been solved by the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system, which has been developed as a new gene-editing technology [8–10]. This efficient and powerful system has revolutionized genome engineering. The CRISPR/Cas9 system has been applied in various animals, such as mice [10], rats [9], sheep [11], chickens [12], and pigs [2, 13, 14].

The somatic cell nuclear transfer (SCNT) technique, which was invented in 1997, was a great scientific innovation in the animal cloning field. Based on SCNT, handmade cloning (HMC), which was invented by Vajta *et al.*, is not only a promising and simple version of SCNT but also a hand-guided technique or zona-free cloning without the use of a micromanipulator [15, 16]. In the HMC process, the oocytes are enucleated using a microblade under a stereomicroscope. Then, a single donor cell is placed in the middle of two enucleated cytoplasts and fused by an appropriate electric shock. The constructed

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embryos are cultured *in vitro* and D6 blastocysts are transferred to uterine horns of surrogates. Finally, the cloned piglets are delivered approximately 114 days later. HMC has revolutionized the field of embryology without the use of expensive micromanipulators and skilled expertise [17, 18].

In the present study, we attempted to generate two gene-knockout pigs rapidly by combining CRISPR/Cas9 and HMC. The α 1,3-galactosyltransferase (GGTA1) and cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) genes were chosen as targets, as these are responsible for the synthesis of Gal α -1,3-Gal (Gal) and N-glycolylneuraminic acid (Neu5Gc), respectively—two carbohydrate xenoantigens that are important for xenotransplantation. Deletion of the two genes together may further reduce antibody-mediated rejection after xenotransplantation. In fact, *GGTA1/CMAH* double-knockout (DKO) pigs have been generated by ZFNs [5], a combination of ZFNs and TALENs [6], and the CRISPR/Cas9 system [19, 20]. However, in this study, we generated the DKO pigs with a novel strategy that combined the CRISPR/Cas9 system and HMC. We disrupted these two genes simultaneously in the same somatic cell using the CRISPR/Cas9 system, followed by HMC to produce the gene DKO pigs. DKO pigs were obtained within 6 months. Our study provided a new strategy to generate pigs with more than one gene modification.

Materials and Methods

Animals care and chemicals

All the animal experiments were approved by the Institutional Review Board on Bioethics and Biosafety of Beijing Genomics Institute (BGI-IRB). All surgical procedures were performed under full anesthesia, and all efforts were made to minimize animal suffering. All chemicals were purchased from Sigma Chemical Co., except where otherwise indicated.

sgRNA design and vector construction

Single guide RNAs (sgRNAs) targeting pig *GGTA1* and *CMAH* genes were designed following protocols described previously [21]. The *GGTA1* sgRNA sequence was 5'-GCAAATACATACTTCATGGTTGG-3', which targets exon 6, and the *CMAH* sgRNA sequence was 5'-GAAGCTGCCAATCTCAAGGAAGG-3', which targets exon 1. The cas9-coding DNA fragment was synthesized and cloned into the pMD-18T vector (Takara, Dalian, Liaoning, China). A cytomegalovirus (CMV) promoter was used to drive transcription of Cas9 in the vector. The U6-sgRNA fragment was synthesized and cloned into the pMD-18T vector. Two *BsaI* restriction sites were introduced into the region between the U6 promoter and sgRNA tail. For sgRNA vector construction, two complementary oligo DNAs were synthesized and then annealed to a double-strand DNA, ligated to the *BsaI* site of the U6-sgRNA vector to form an integral sgRNA-expressing frame.

sgRNA cleavage activity validation

For sgRNA activity validation, PK15 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco) in a 24-well plate. When the cells grew

to 50–60% confluence, sgRNAs and cas9 vectors were mixed at a weight ratio of 3:1 and co-transfected into PK15 cells using the lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. To enhance the transfection efficiency and increase the sensitivity of the subsequent Surveyor assay, the transfection was completed twice. The second transfection was performed on the same cell well 12–24 h after the first, and all the cells were collected 48 h later. Cells transfected with the pN1-EGFP vector (Clontech, Palo Alto, CA, USA) acted as a control group.

Subsequently, the Surveyor assay was performed to identify sgRNA cleavage activity, as described previously [22]. Briefly, PCR was performed on DNA extracted from transfected cells using specific primers, which amplified exon 6 of pig *GGTA1* and exon 1 of pig *CMAH*. For the *GGTA1* gene, the forward primer was 5'-AGCCACCCTGCCAGAATCAC-3' and the reverse primer was 5'-GGAGGATTCCTTGAAGCACTC-3'. For the *CMAH* gene, the forward primer was 5'-GCATCTAAAAGCGAGGTAGTAG-3' and the reverse primer was 5'-ATCCAAAAGAGGCTTGAGTC-3'. The PCR conditions were 94°C for 5 min, 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, for 35 cycles, 72°C for 5 min, and held at 16°C. Subsequently, a mixture containing 18 μ l PCR product and 2 μ l 10 \times NEB buffer 2 was incubated at 95°C for 10 min and then incubated at room temperature for 30 min. After the annealing reaction, 0.2 μ l of T7 endonuclease I (T7E1) (NEB, Ipswich, MA, USA) was added to the sample and the mixture was incubated at 37°C for 1 h. Finally, the digested product was electrophoresed on a 2% agarose gel and the electropherogram was visually analyzed using Tanon 1.1 (Tanon, Shanghai, China).

Preparation of Wuzhishan porcine fetal fibroblasts (PFFs)

PFFs used throughout this study were primary cells isolated from the Chinese Wuzhishan male fetuses at 40 days after insemination, as described previously [23]. The PFFs were cultured in Dulbecco's Minimum Essential Medium (DMEM; Gibco) supplemented with 15% (vol/vol) FBS, 1% non-essential amino acid (NEAA; Invitrogen), and 1% L-glutamine (Invitrogen) at 38°C in 5% CO₂ in air.

Selection of GGTA1 and CMAH double gene bi-allelic knockout cells

Before nucleofection, PFF cells were thawed and cultured until 70–90% confluent. Approximately 1 \times 10⁶ cells were subjected to nucleofection using the Basic Nucleofector® Kit for Primary Mammalian Fibroblasts (Lonza, Allendale, NJ, USA) following the manufacturer's protocols. Briefly, the required number of cells were suspended in 100 μ l Nucleofector® Solution at room temperature. Cyclic vectors of *GGTA1*-sgRNA, *CMAH*-sgRNA, Cas9, and linearized pcDNA3.1(+) were mixed in advance at a weight ratio of 3:3:2:0.4. A total of 5 μ g of DNA was mixed with the cell resuspension. Nucleofection was performed on a Nucleofector™ 2b Device (Lonza) by running the U-023 program. After 24 h recovery, the cells were selected with G418 at a concentration of 450 μ g/ml for 8–10 days. Individual cell colonies were selected and genotyped.

The cell colonies were subjected to two rounds of T7E1 digestion. In the first round, PCR products were digested immediately after the annealing reaction for identification of heterozygotes. In the

second round, PCR products of the candidate homozygotes and wild-types were mixed at a volume ratio of 1:1 and the T7E1 assay was performed. Positive digestion during the second round was considered indication of probable homozygotes. Then *GGTA1* and *CMAH* loci of the positive cell colonies were genotyped by Sanger sequencing.

Oocyte maturation

Slaughterhouse-derived sow ovaries were collected in physiological saline solution and transported to the laboratory at 32°C within 4 h. Cumulus-oocyte complexes (COCs) were recovered from 3–5 mm follicles. The compact COCs were selected in groups of 50. Each group was matured in 400 µl of bicarbonate buffered TCM-199 (Gibco) supplemented with 10% (v/v) pig follicular fluid, 10% cattle serum (CS; Gibco), 5 IU/ml hCG and 10 IU/ml eCG at 38.5°C in a humidified atmosphere of 5% (v/v) CO₂ for 41–43 h. Subsequently, COCs were treated with 1 mg/ml hyaluronidase dissolved in HEPES-buffered TCM-199 to remove the cumulus cells.

Handmade cloning (HMC)

For HMC, cells of passages 8–12 (total passage) were used as nuclear donors. Confluent cell monolayers were harvested by digestion with 0.05% trypsin-EDTA (Gibco) and the cell suspension was placed at room temperature for 0.5–1 h before fusion.

HMC was performed, as previously described [17]. Briefly, zona pellucida of oocytes were partially digested by 3 mg/ml pronase dissolved in T33 (T means HEPES-buffered TCM 199 medium; the number for percentage (v/v) of calf serum supplement). Then, enucleation was performed with a microblade (AB Technology, Pullman, WA, USA) under a stereo microscope. The cytoplasm was washed in T2 and T20 drops (to wash out the zona pellucida fragments), and collected in a T10 drop (for buffering before fusion with the donor cells).

Fusion was performed in two steps in which the second step included the initiation of activation. For the first step, each cytoplasm was transferred to 20 µl T0 containing 1 mg/ml of phytohemagglutinin (PHA) for 1 to 3 sec and subsequently attached to a single donor cell. Then, cytoplasm-fibroblast pairs were fused with a single 100 V direct current (DC) impulse of 2.0 kV/cm for 9 µs (BLS CF-150/B fusion machine) in fusion medium (0.3 M mannitol and 0.01% PVA). Approximately 1 h later, each fused pair was fused with another putative cytoplasm in activation medium (0.3 M mannitol, 0.1 mM MgSO₄, 0.1 mM CaCl₂, and 0.01% PVA) with a single DC pulse of 0.86 KV/cm for 80 µs. Reconstructed embryos were treated with 10 mg/ml cycloheximide and 5 mg/ml cytochalasin B dissolved in PZM-3 at 38.5°C in 5% CO₂ and 5% O₂ for 4 to 6 h.

Embryo culture, transfer, and cloned piglet genotype assay

Zona-free embryos produced from HMC were cultured in groups of 20 to 30 in 400 µl PZM-3 supplemented with 4 mg/ml bovine serum albumin (BSA) in a modified 'Well of the Well' (WOW) system [24]. Day 6 (D6) blastocysts produced from HMC were surgically transferred to uterine horns of surrogates. Pregnancies were confirmed by B ultrasound scanning on day 28 and monitored every 2 weeks thereafter. The cloned piglets were delivered by natural birth. The genomic DNA extracted from an ear skin biopsy

of the newborn cloned piglets was assessed for mutagenesis at the targeted site by PCR-based assays.

Preparation of peripheral blood mononuclear cells (PBMCs), red blood cells (RBCs), and artery endothelial cells (AECs)

PBMCs and RBCs from wild-type, *GGTA1/CMAH* DKO, and *GGTA1*-KO pigs (which were kindly supplied by Dr. Dengke Pan) were isolated as previously described [25, 26]. AECs were isolated, cultured, and passaged as previously described [27, 28].

Immunofluorescence staining

Staining for Gal and Neu5Gc was performed as previously described [29]. Gal was stained with fluorescein isothiocyanate (FITC)-conjugated BS-IB4 lectin (Sigma-Aldrich). Neu5Gc was stained with an anti-Neu5Gc antibody kit (Biolegend, San Diego, CA, USA, secondary antibody and tertiary antibody from Jackson ImmunoResearch, West Grove, PA, USA), following the manufacturer's protocols. The picture was obtained using a Leica DMIL fluorescent inverted microscope (Leica Microsystems) provided with an ebq 50 ac-L lamp (Leistungselektronik Jena GmbH, Jena, Germany), and UV filter.

Expression of Gal and Neu5Gc on PBMCs and AECs

Flow cytometry analysis of Gal and Neu5Gc expression in cells was previously described [26, 28]. Briefly, for Gal staining, cells were stained with FITC-conjugated BS-IB4 lectin for 30 min at 4°C; unstained cells were used as a negative control. For Neu5Gc staining, cells were stained with an anti-Neu5Gc antibody for 30 min at 4°C; an isotype-matched antibody was used as a negative control. After washing with PBS, the cells were stained with a secondary antibody (Jackson ImmunoResearch) for 30 min at 4°C, washed again, and incubated with a tertiary antibody (Biolegend) for 30 min at 4°C in the dark. After washing, the treated cells were analyzed by flow cytometry.

Human antibody binding to RBCs and PBMCs

IgG and IgM binding assays were performed as previously described [26, 27]. In brief, serum from several healthy volunteers (n = 20, including all ABO types) were mixed and heat-inactivated at 56°C for 30 min. Cells (1 × 10⁶ RBCs or 1 × 10⁵ PBMCs) were incubated with 2.5% (RBCs) or 13% (PBMCs) heat-inactivated serum for 30 min (RBCs) or 120 min (PBMCs) at 4°C. After washing with PBS, 10% goat serum was used to prevent non-specific binding. Next, the cells were incubated with FITC-conjugated goat anti-human IgM or IgG antibodies (Invitrogen) for 30 min at 4°C in the dark. After washing, the treated cells were analyzed by flow cytometry. The extent of IgM and IgG binding was evaluated by relative mean fluorescence intensity (MFI), using the following formula:

$$\text{Relative MFI} = (\text{actual MFI}) / (\text{MFI obtained with secondary antibodies only without serum})$$

Antibody-mediated complement-dependent cytotoxicity

The assay was conducted as previously described [30]. Briefly, PBMCs (2 × 10⁶) were incubated with various diluted serum samples in 200 µl for 30 min at 37°C. PBMCs were incubated with heat-inactivated serum as a negative control. After washing twice with

PBS, the cells were incubated with propidium iodide (PI, 1:1000; Invitrogen) for 15 min at 4°C. After washing, the treated cells were analyzed by flow cytometry. The PI-positive cells were used to assess cell death. The percentage of cytotoxicity was calculated by the following formula:

$\% \text{cytotoxicity} = (A-B)/(100-B) \times 100\%$, where A represents the percentage of PI-positive cells incubated with serum, and B represents the percentage of PI-positive cells incubated with heat-inactivated serum.

Statistical analysis

Data are presented as the mean \pm SEM. A two-tailed Student's *t* test was used for analysis of the differences between the groups. All *P* values < 0.05 were considered statistically significant.

Results

Generation of bi-allelic *GGTA1* and *CMAH* DKO cells

sgRNAs were designed to target exon 6 of pig *GGTA1* and exon 1 of pig *CMAH* (Fig. 1A). We selected at least three sgRNAs of each gene locus and tested their cleavage activities *in vitro* via PK15 cell transfection and T7E1 Surveyor assay. One sgRNA within each gene locus exhibited cleavage activity (Fig. 1B). Then, nucleofection was performed on Wuzhishan PFFs and G418-resistant cell colonies were selected. One candidate positive cell colony (#8) was found via Surveyor assay among the 11 cell colonies selected (Fig. 2A), and the PCR and sequencing results validated that the genotype of the #8 cell colony was a bi-allelic *GGTA1* and *CMAH* DKO cell colony (Fig. 2B–C). For the *GGTA1* gene locus, one allele was inserted with 1 base-pair, which led to a frameshift mutation exactly after the CRISPR cut site in exon 6, and the another allele was inserted with 421 base-pairs, in which there was a partial DNA sequence of the pcDNA3.1 vector (Fig. 2B–C). For the *CMAH* gene locus, there was a 103 base-pair deletion after the CRISPR cut site in exon 1, and the genotype was homozygous by validation of the electrophoresis assay and PCR product sequencing (Fig. 2B–C).

Production of *GGTA1/CMAH* DKO pigs via HMC

The #8 colony cells were used as nuclear donors of HMC, and the *GGTA1/CMAH* DKO pigs were generated (Fig. 3A–B). A total of 1,307 reconstructed embryos derived from *GGTA1/CMAH* DKO cells were created. The overall D6 blastocyst rate was 45%, which was statistically higher than that of the average rate in our laboratory over the past 4 years (45% vs. 36%), and also higher than that of previous knockout cells (45% vs. 34%) (Table 1) [23]. Then, 517 blastocysts were transferred into eight recipients, and three of them became pregnant, indicating a pregnancy rate of 38% (Table 1). All three surrogate mothers developed to term, which led to the birth of 13 piglets, of which two piglets were stillborn and 11 piglets were live born (Table 1). Total cloning efficiency was 2.5% (Table 1). Genomic DNA extracted from ear skin biopsies of the 11 cloned piglets was assessed for mutagenesis at the targeted sites by PCR-based assays and sequencing analysis to determine the genotype of the pigs. The results indicated that all 11 cloned piglets were derived from one nuclear donor and the genotypes of the *GGTA1* and *CMAH* loci were the same as that of the #8 cell donor (Fig. 3C–D).

Next, we checked Neu5Gc and Gal antigen expression in the DKO pigs we generated. Histological analysis of various tissues obtained from two *GGTA1/CMAH* DKO pigs indicated that neither the Neu5Gc nor the Gal antigen were detected on any of the tissues from the DKO pigs, whereas antigen expression was normal in wild-type pigs (Fig. 4A–B). Flow cytometry analysis of PAECs and PBMCs from the *GGTA1/CMAH* DKO pigs revealed similar results (Fig. 4C–D), and demonstrated that the DKO pigs lacked both Neu5Gc and Gal antigen expression.

Off-target analysis of the newborn piglets

A major concern of utilizing the CRISPR/Cas9 system for genome editing is the potential for off-target effects [31]. Several studies have shown that CRISPR/Cas9 cleaves genomic DNA with mismatches in the guide RNA strand [32–34]. To test whether there was an undesired off-target mutagenesis in the genome-modified cloned piglets, the predicted off-target sites (OTS) were screened and identified, as previously described [14, 35]. Genome DNA extracted from the ear tissues of one piglet was used to perform off-target analysis. Thirteen and 12 potential OTS of the *GGTA1* and *CMAH* locus, respectively, were PCR amplified and sequenced. Primers for amplification of the OTS are listed in Supplementary Table 1 (online only). Results showed that the *GGTA1*-OTS6, *CMAH*-OTS9, and *CMAH*-OTS11 presented undesired DNA mutations, which were 3 bp, 4 bp, and 2 bp deletions, respectively. However, the three deletions were in non-coding regions of the genome, which were deemed to have no negative effects on functional roles of any genes. Moreover, these 11 gene-modified pigs were healthy and some of them seemed even more active than some wild-type pigs.

Human antibody binding to cells from *GGTA1/CMAH* DKO pigs

Having generated these DKO pigs (without expression of Neu5Gc and Gal), we determined the level of xenoantigen expression by flow cytometry. After incubation with heat-inactivated serum from several healthy human volunteers, the mean relative MFI of IgM binding to RBCs from the wild-type, *GGTA1*-knockout (KO), and *GGTA1/CMAH* DKO pigs was 134.95, 3.09, and 1.31, respectively, whereas the mean relative MFI of IgG binding to RBCs from the above pigs was 17.28, 3.87, and 1.06, respectively (Fig. 5A). Human IgM and IgG binding to RBCs from *GGTA1*-KO pigs was much less than to RBCs from wild-type pigs, and there was further reduction of IgM and IgG binding to RBCs from the *GGTA1/CMAH* DKO pigs. We also obtained similar results with PBMCs (Fig. 5B). The data revealed that the majority of human IgM and IgG was anti-Gal antibody, and the majority of non-Gal IgM and IgG was anti-Neu5Gc antibody. Anti-Neu5Gc IgM and IgG accounted for >50% of non-Gal IgM and IgG.

Effect of *GGTA1/CMAH* DKO PBMCs on antibody-mediated complement-dependent cytotoxicity

Next, we assessed immune rejection using an antibody-mediated complement-dependent cytotoxicity assay by flow cytometry *in vitro*. Using 40%, 80%, and 100% serum concentrations, cytotoxicity of PBMCs from *GGTA1/CMAH* DKO pigs was 2.11%, 2.86%, and 3.44%, respectively, whereas cytotoxicity of PBMCs from

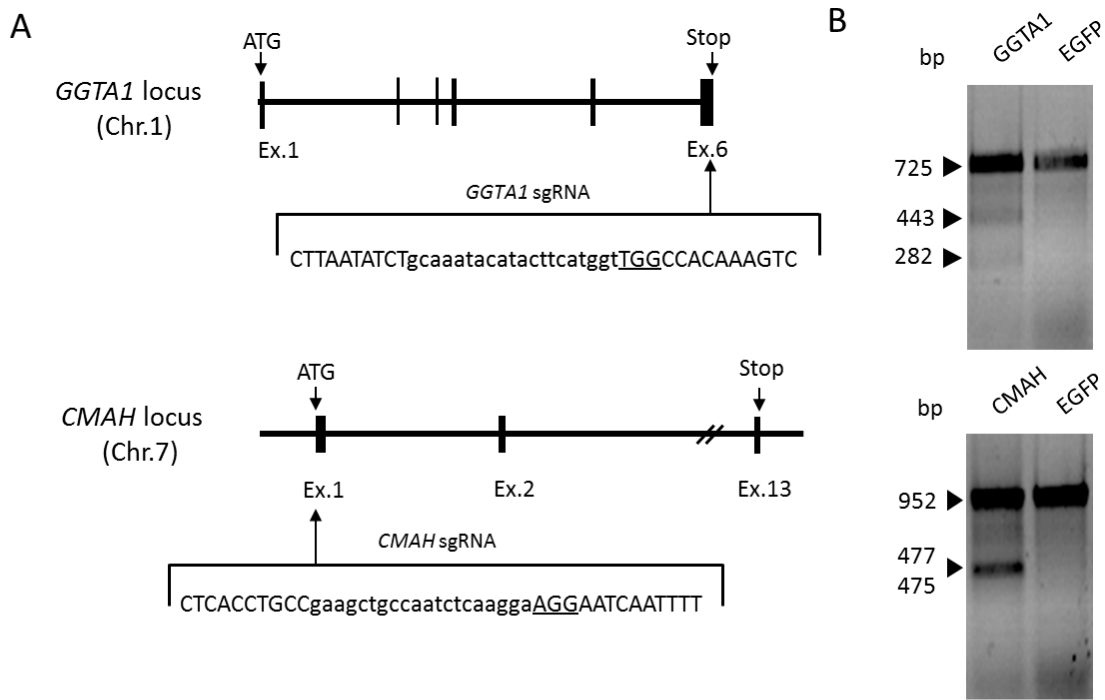


Fig. 1. CRISPR/Cas9 system targeting pig *GGTA1* and *CMAH* loci. (A) Schematic of sgRNAs targeting *GGTA1* and *CMAH* loci. The target sgRNAs were designed in the last exon of the *GGTA1* locus and the first exon of the *CMAH* locus. sgRNA-target sequences are labeled in lowercase and the protospacer adjacent motif (PAM) is underlined. (B) Surveyor assay (T7E1) for Cas9-mediated cleavage at *GGTA1* and *CMAH* loci in PK15 cells.

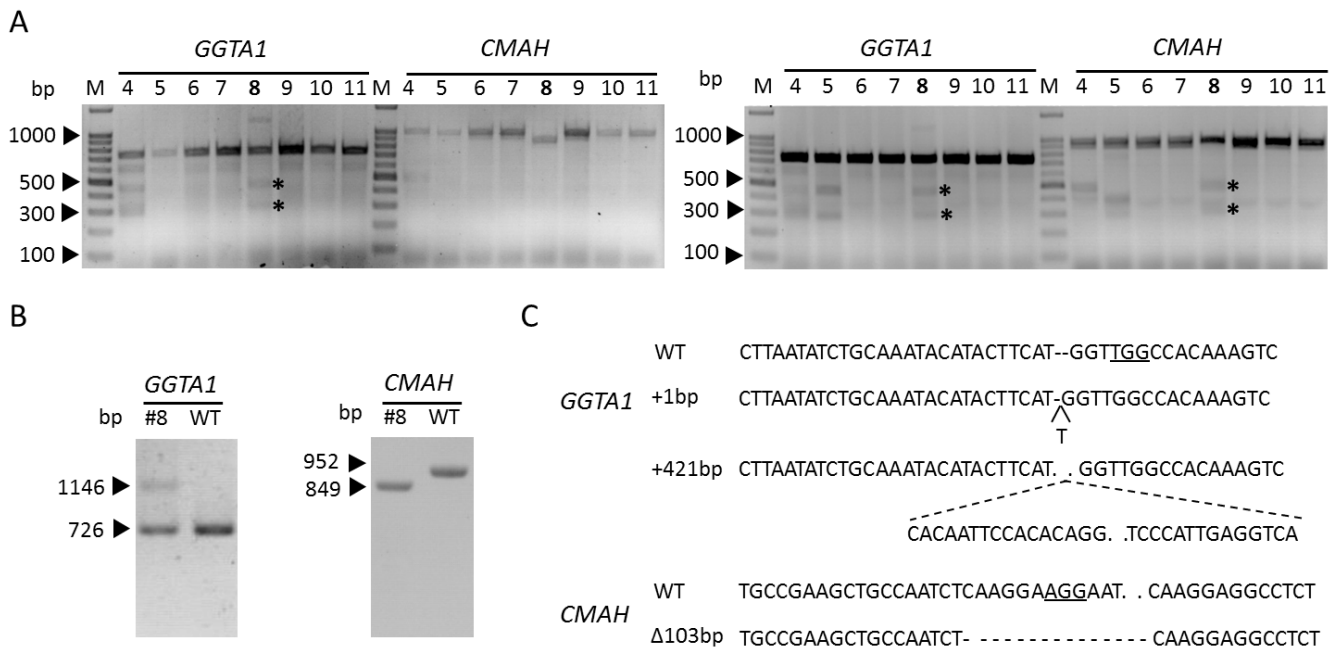


Fig. 2. Selection of *GGTA1* and *CMAH* gene bi-allelic DKO cell colonies. (A) Cell colonies were selected and subjected to two rounds of T7E1 Surveyor assays. The first round (left, PCR products of the candidate colonies were digested by T7E1) excluded heterozygotes, and the second round (right, PCR products of the candidate colonies and wild-type cells were mixed and then digested by T7E1) identified the candidate homozygotes. * indicates T7E1-digested fragments of the #8 cell colony at the *GGTA1* and *CMAH* loci. (B) PCR amplicons of *GGTA1* (left) and *CMAH* (right) loci of the #8 cell colony and wild-type cells. (C) Genotypes of the #8 cell colony at the *GGTA1* and *CMAH* loci. PAM sequence is underlined.

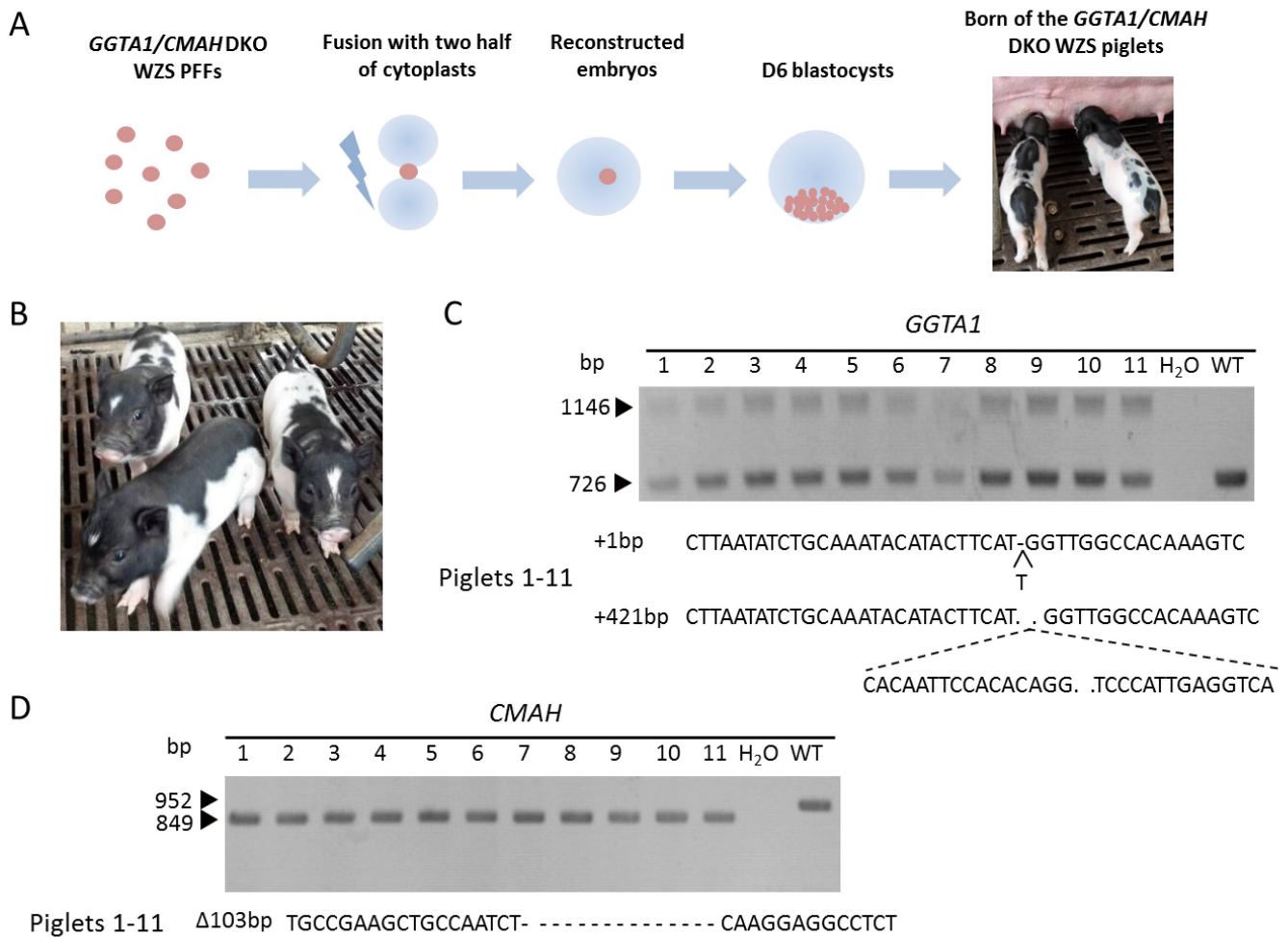


Fig. 3. Generation of *GGTA1* and *CMAH* gene DKO piglets by HMC. (A) A schematic description of *GGTA1/CMAH* Wuzhishan PFFs used as nuclear donors and subjected to HMC resulting in the birth of *GGTA1* and *CMAH* DKO piglets. (B) Photograph of three *GGTA1/CMAH* DKO piglets. (C) PCR products (above) and genotype analyzed by Sanger sequencing (below) of the 11 cloned piglets at the *GGTA1* loci. (D) PCR products (above) and genotype analyzed by Sanger sequencing (below) of the 11 cloned piglets at the *CMAH* loci.

Table 1. Handmade cloning for generating *GGTA1/CMAH* double-knockout pigs

Recipients	Reconstructed embryos	D6 blastocyst (%)	Blastocyst transferred	Pregnancy	Still born	Live born	Cloning efficiency %
01	219	79 (36)	63	No	–	–	–
02	243	128 (53)	103	No	–	–	–
03	239	96 (40)	81	Yes	0	3	3.7
04	117	58 (50)	55	Yes	2	4	10.9
05	105	52 (50)	49	No	–	–	–
06	105	47 (45)	46	Yes	0	4	8.7
07	104	45 (43)	45	No	–	–	–
08	175	77 (44)	75	No	–	–	–
Total	1307	582 (45)	517	3 (38%)	2	11	2.5

GGTA1-KO pigs was 6.14%, 11.58%, and 14.52%, respectively (Fig. 5C). Cytotoxicity of PBMCs from *GGTA1*-KO pigs was much higher than that of PBMCs from *GGTA1/CMAH* DKO pigs. These data indicated that the Neu5Gc antigen is a major non-Gal antigen, and suggests that it will be critical for clinical xenotransplantation.

Discussion

Organ/tissue transplantation is an important therapy for end-stage organ failure and some other human diseases, but the shortage of human organs/tissues is a major limitation [36]. Pigs are considered to be the most likely animal to solve the problem, and there are several reasons to choose pigs as xenograft ‘donors’, (e.g., physiological, anatomical, economic, and ethical) [37, 38]. However, immune rejection is a major hurdle for pig-to-human organ/tissue transplantation.

Gal is a major xenoantigen, and *GGTA1*-KO pigs were produced in 2002, thus reducing the humoral barrier to xenotransplantation [39]. Transplantation experiments employing hearts and kidneys from *GGTA1*-KO pigs into baboons proved an advance [40–42]. Several studies revealed that Neu5Gc, which is controlled by *CMAH*, is a major non-Gal antigen and would be problematic for clinical xenotransplantation [43–46]. Thus, generation of *GGTA1/CMAH* DKO pigs became a matter of urgency. Herein, we showed that the combination of CRISPR/Cas9 and HMC is a new strategy to generate gene DKO pigs.

We generated *GGTA1/CMAH* DKO pigs by targeting the *GGTA1* and *CMAH* genes together in PFFs with CRISPR/Cas9 and HMC. We knocked out these two genes simultaneously using wild-type PFFs in a single step using the CRISPR/Cas9 system. Additionally, we obtained bi-allelic *GGTA1/CMAH* DKO pigs with only one round of nucleofection, clone selection, and HMC. Previously, Miyagawa *et al.* (2015) generated *GGTA1/CMAH* DKO pigs using *GGTA1*-KO cells and a combination of ZFN and TALEN technology [6]. This group also obtained heterozygous pigs, and subsequently, homozygous pigs, by two rounds of SCNT. Lutz *et al.* also produced these pigs with two rounds of clone screening using ZFNs [5, 6]. In summary, the method we used is efficient and convenient in the production of gene-modified pigs.

Tector’s group generated the *GGTA1/CMAH* DKO pigs by CRISPR/Cas9 and SCNT [19, 20]. However, we used a different strategy. First, we obtained a single mutation (*GGTA1/CMAH* DKO) cell colony by G418 selection, Surveyor assay, and sequencing prior to HMC (instead of lectin selection prior to SCNT). We screened the cells dependent on a resistance gene and genome sequence. This method could be applied to any other gene selection to generate new genetic-modified pigs without the limitation of specific antibody availability for the target gene product. In addition, the 11 piglets we obtained were theoretically of identical genotype, whereas the 10 piglets obtained by Li *et al.* had different genotypes [20]. For the *CMAH* gene, three of the 10 piglets were wild-types, one was heterozygous, and six were homozygous; however, the genotypes of the homozygous piglets differed from one another [20].

We used HMC technology where there is no need for microinjection, which is associated with some technical difficulties and is not easy to master, although more oocytes were required for HMC because of the two oocytes essential for the reconstruction of a single embryo.

Moreover, HMC is less costly without the expensive micromanipulator; however, it is as efficient as SCNT [15]. Using HMC, approximately 100–140 constructed embryos can be generated by two skilled technicians in 2.5–3 h and the average blastocyst rate can be 30–50% [23, 47]. Although a skilled person using SCNT can produce 100–120 cloned embryos in 1 h, this method is dependent on an expensive micromanipulator. As such, HMC is suitable for an ‘industry-scale’ cloning platform. It benefits from the micromanipulator-free system and has the potential for automation, with which we could produce cloned pigs at a large scale. Additionally, we used the PFFs from Wuzhishan miniature pigs that have potential economic benefits and comparatively lower cost. Therefore, we used different cells from a different strain of pigs with a new method, which differed from those reported previously.

Our data indicated that both *GGTA1* and *CMAH* genes were completely disrupted by the CRISPR/Cas9 system, as Gal and Neu5Gc were not expressed in our DKO pigs. Our results also revealed that, whereas a large percentage of IgM and IgG from human serum is targeted for Gal, approximately half of non-anti-Gal IgM and IgG bound to Neu5Gc. The data suggest Neu5Gc is a major non-Gal antigen target. Antibody-mediated complement-dependent cytotoxicity using different human serum concentrations confirmed that anti-Neu5Gc antibodies are major antibodies directed toward non-Gal antigens and will be important in the success or failure of clinical xenotransplantation. As we expected, these results demonstrated that *GGTA1/CMAH* DKO cells are more profoundly protected from the humoral response than *GGTA1*-KO cells.

In summary, this study provided a new strategy, combining CRISPR/Cas9 with HMC, which can efficiently produce gene-modified pigs. All our observations support the conclusion that this technique provides a new, less costly strategy to produce pigs with multiple gene modifications. *GGTA1/CMAH* DKO pigs should be useful for xenotransplantation research and may be essential for clinical xenotransplantation.

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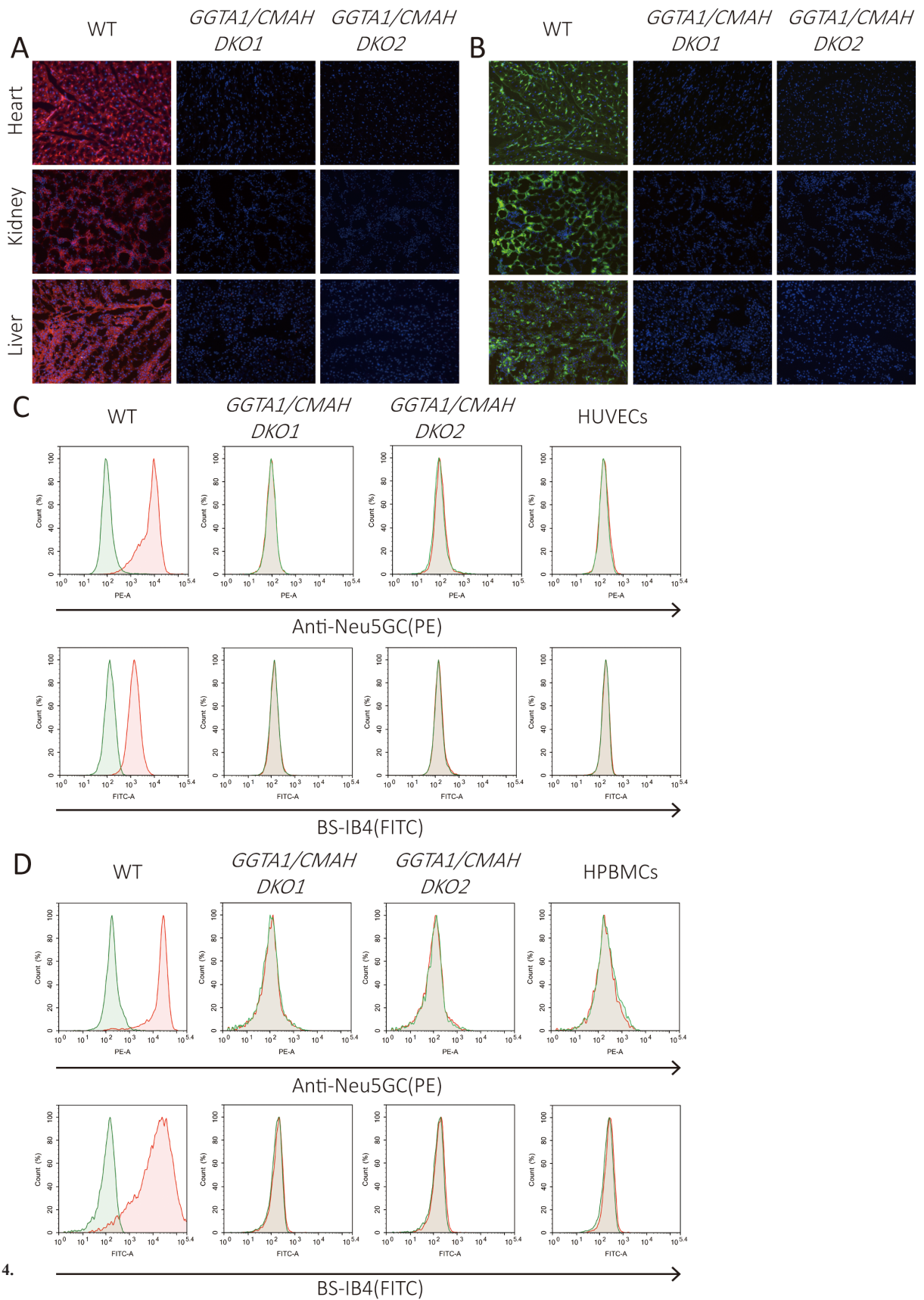


Fig. 4.

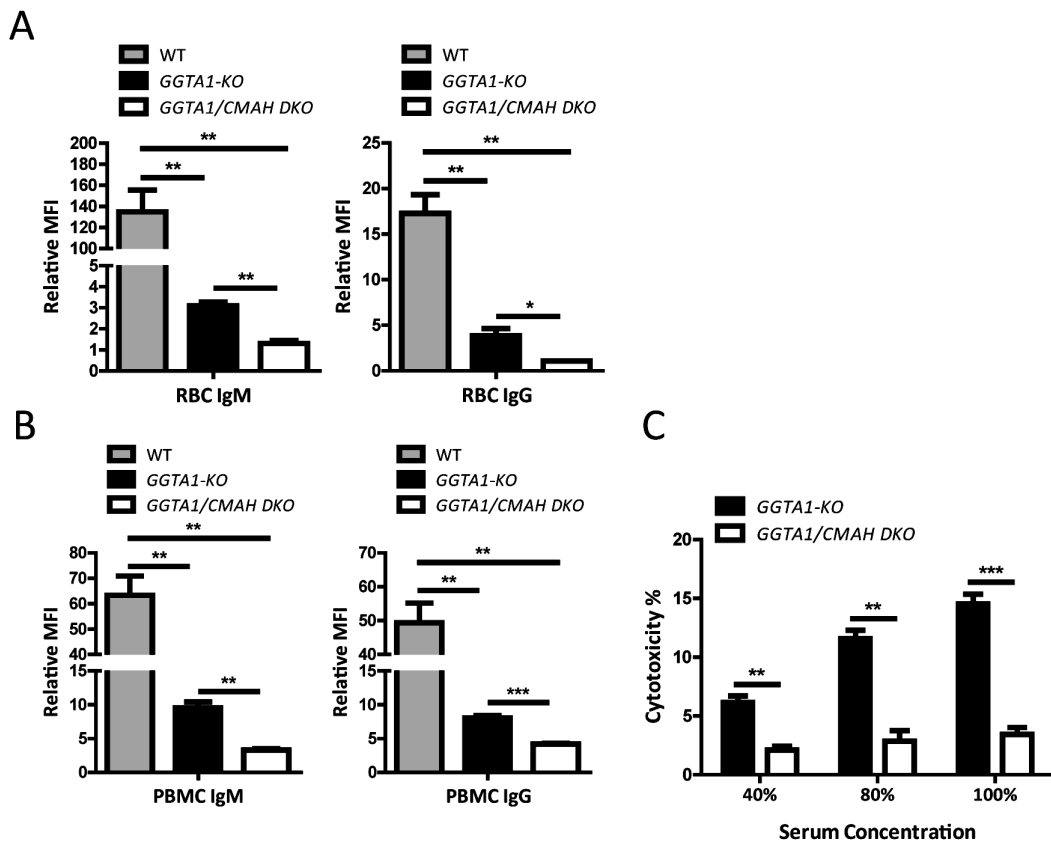


Fig. 5. Human antibody binding and antibody-mediated complement-dependent cytotoxicity of cells from *GGTA1/CMAH* DKO pigs. (A–B) Flow cytometry analysis of human IgM and IgG binding to RBCs (A) and PBMCs (B) from wild-type (n = 3), *GGTA1*-KO (n = 3), and *GGTA1/CMAH* DKO (n = 3) pigs. (C) Flow cytometry analysis of cytotoxicity of PBMCs from *GGTA1*-KO (n = 3) and *GGTA1/CMAH* DKO (n = 3) pigs incubated with different human serum concentrations. (*P < 0.05, ** P < 0.01, *** P < 0.001).

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Fig. 4. Immunofluorescence and flow cytometry analysis of Gal and Neu5Gc expression in *GGTA1/CMAH* DKO pigs. (A–B) Immunofluorescence of various tissues from wild-type or *GGTA1/CMAH* DKO pigs stained with anti-Neu5Gc antibody (A) and BS-IB4 lectin (B) (Magnification \times 200; Neu5Gc, red; Gal, green; Nuclei, blue). (C–D) Flow cytometry analysis of AECs (C) and PBMCs (D) from the pigs indicated in A–B stained with anti-Neu5Gc antibody and BS-IB4 lectin. Human umbilical vein endothelial cells (HUVECs) and human PBMCs (HPBMCs) were negative controls, respectively. The green lines represent negative controls; the red lines represent the experimental groups.

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