Reducing immunoreactivity of porcine bioprosthetic heart valves by genetically-deleting three major glycan antigens, GGTA1/β4GalNT2/CMAH

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Bioprosthetic heart valves (BHVs) originating from pigs are extensively used for heart valve replacement in clinics. However, recipient immune responses associated with chronic calcification lead to structural valve deterioration (SVD) of BHVs. Two well-characterized epitopes on porcine BHVs have been implicated in SVD, including galactoseα1,3-galactose (αGal) and N-glycolylneuraminic acid (Neu5Gc) whose synthesis are catalyzed by α(1,3) galactosyltransferase (encoded by the GGTA1 gene) and CMP-Neu5Ac hydroxylase (encoded by the CMAH gene), respectively. It has been reported that BHV from αGal-knockout pigs are associated with a significantly reduced immune response by human serum. Moreover, valves from αGal/Neu5Gc-deficient pigs could further reduce human IgM/IgG binding when compared to BHV from αGal-knockout pigs. Recently, another swine xenoantigen, Sd(a), produced by β-1,4-N-acetyl-galactosaminyl transferase 2 (β4GalNT2), has been identified. To explore whether tissue from GGTA1, CMAH, and β4GalNT2 triple gene-knockout (TKO) pigs would further minimize human antibody binding to porcine pericardium, TKO pigs were successfully produced by CRISPR/Cas9 mediated gene targeting. Our results showed that the expression of αGal, Neu5Gc and Sd(a) on TKO pigs was negative, and that human IgG/IgM binding to pericardium was minimal. Moreover, the analysis of collagen composition and physical characteristics of porcine pericardium from the TKO pigs indicated that elimination of the three xenoantigens had no significant impact on the physical proprieties of porcine pericardium. Our results demonstrated that TKO pigs would be an ideal source of BHVs.

STATEMENT OF SIGNIFICANCE

Surgical heart valve replacement is an established lifesaving treatment for diseased heart valve. Bioprosthetic heart valves (BHVs) made from glutaraldehyde-fixed porcine or bovine tissues are widely used in clinics but exhibit age-dependent structural valve degeneration (SVD) which is associated with the immune response against BHVs. Three major xenoantigens present on commercial BHVs, Galactosa α1,3 galactose (αGal), N-glycolyneuraminic acid (Neu5Gc) and glycan products of β-1,4-N-acetyl-galactosaminyl transferase 2 (β4GalNT2) are eliminated through CRISPR/Cas9 mediated gene targeting in the present study. The genetically modified porcine pericardium showed reduced immunoreactive binding to human serum when compared to unaltered porcine pericardium. These results demonstrate the potential of TKO pigs as an ideal source of BHVs.

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1. Introduction

More than 250,000 patients with valvular heart disease receive heart valve replacement worldwide each year [1]. Options for heart valve replacement include either mechanical heart valves or bio-prosthetic heart valves (BHV), both of which have been clinically applied for more than 50 years [2]. There has been an increasing trend towards the use of BHVs as they offer several advantages over mechanical prostheses [3]. A BHV has superior hemodynamic properties and no requirement for lifelong anticoagulant therapy when compared to a mechanical prosthesis, thus reducing the risk of stroke and bleeding [4]. BHVs, usually originated from bovine or porcine tissues, possess the potential to provide an unlimited number of valves of various sizes.

However, BHVs have limited durability as compared to mechanical prostheses. This is largely due to structural valve deterioration (SVD) caused by calcification [5,6]. A high incidence of calcification has been seen in BHVs implanted into younger patients. While 90% of older recipients (>65 years) did not develop SVD for 10 years' post-surgery [7,8], up to 100% of BHV failure has been observed within 5 years in aged patients younger than 35 years [4]. A considerable effort has been made to develop chemical anti-calcification methods based on glutaraldehyde fixation to reduce BHV calcification [9–12], but with only variable success.

Inflammatory responses are known to be involved in the BHV calcification process [4,13]. There is evidence that residual xenoantigens on glutaraldehyde-pretreated BHV can promote immune cell infiltration into the BHV matrix, which leads to calcification [14–17]. Prevention of calcification of xenograft valves could be achieved when an early immune response was inhibited by immunosuppressive therapy in an animal model [18], supporting the notion that calcification of BHV is at least partly a consequence of antibody-mediated immune injury.

xGal has long been known to be the major xenoantigen in porcine tissues that stimulates the human immune response [19] and, on this basis, has been suspected of being a major factor in the SVD that occurs in commercially-available BHVs [20,21]. Human anti-Gal antibody enhances calcification of fixed WT, but not GTKO, pig pericardium in the rat and rabbit subcutaneous implant models [22,23]. There is evidence that the immune response in non-human primate recipients is greatly reduced when the pericardium is derived from GGT1-knockout (GTKO) pigs [24]. Another carbohydrate antigen, N-glycoly neuraminic acid (Neu5Gc), has been detected in various porcine organs, including heart valves [25,26], which can be recognized by highly-specific circulating human anti-Neu5Gc antibodies [27,28]. Lee et al. [7] demonstrated that binding of human IgM/IgG to GGT1/CMAH-KO pig heart valves was further decreased as compared to GTKO pig valves, implying a role for Neu5Gc in BHV structural deterioration.

Recently, an additional xenoantigen, Sd(a), produced by β-1,4-N-acetyl-galactosaminytransferase 2 (β4GalNT2) has been identified [29,30]. Estrada et al. [31] reported that disruption of porcine β4GalNT2 concomitantly with GGT1 and CMAH further reduced human antibody binding to pig peripheral blood mononuclear cells (PBMCs) compared to GGT1/CMAH-deficient pigs.

However, human antibody binding to pericardium derived from GGT1/CMAH/β4GalNT2 triple gene-knockout (TKO) pigs was not evaluated in the previous study. Although McGregor et al. [3] showed the physical equivalency of pericardial tissues between GGT1-deficient and wild-type (WT) pigs, it has not yet been addressed whether additional gene modifications could affect the physical properties of pig pericardium. The aim of the present study was to generate GGT1/CMAH/β4GalNT2-KO pigs, to evaluate human immunoreactivity to them, and to determine the physical characteristics of TKO pericardial tissues.

2. Materials and methods

2.1. Animals

Wild-type mature Landrace gilts (average body weight approximately 105 kg) were purchased from Chia Tai (Jiangsu Huaiyin) Co., Ltd. and housed in a large animal facility affiliated with Nanjing Medical University, Nanjing, China. Standard pig husbandry procedures were applied to all animals. Embryo transplantation and euthanasia were conducted under isoflurane anesthesia to minimize animal suffering. All animal experiments were carried out in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University.

2.2. CRISPR/Cas9 plasmid constructs

To target the porcine GGT1, CMAH and β4GalNT2 genes, single guide RNAs (sgRNAs) were designed using online tools (http://crispr.mit.edu/). The DNA oligos of sgRNAs were purchased from Genscript (Nanjing, China) and are listed in Table 1. These complementary DNA oligos were annealed and ligated into BbsI digested pX330 (Addgene plasmid 423230) using thermal conditions 37 °C for 30 min, 95 °C for 5 min, and annealed by decreasing 5 °C/min to 25 °C to generate the Cas9-sgRNA targeting plasmids.

2.3. T7E1 cleavage assay

Porcine primary fetal fibroblast cells (PFFs) were isolated from Chinese Landrace fetuses at day 35 of gestation using 200 U/mL collagenase (Invitrogen, Carlsbad, USA) and 25 kU/mL DNase (Invitrogen) and cultured as previously described [32]. PFFs transfected with or without Cas9-sgRNA targeting plasmids (as described above) were cultured for 48 h. Genomic DNA was extracted using a DNA extraction kit (TianGen, Beijing, China) and subjected to agarose gel electrophoresis. The PCR products were amplified. PCR primers are listed in Table 2. The PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 40 s, and a final 72 °C for 7 min. A total of 250 ng of the purified PCR product was mixed with NEB Buffer 2, denatured, and annealed to allow formation of heteroduplex using the following conditions: 95 °C for 5 min, 95 °C to 85 °C ramping at –2 °C/s, 85 °C to 25 °C at –0.1 °C/s, and 4 °C hold. After reannealing, the products were digested with 1 μL of T7 endonuclease I (NEB, Beverly, USA) at 37 °C for 15 min and then subjected to agarose gel electrophoresis.
applied to the post-transfection cells 48 h later and maintained colonies. To obtain the transgenic cell Kit (Amaxa Biosystems/Lonza, Cologne, Germany), according to the manufacturer’s instructions. Early passage of PFFs using the Basic Fibroblast Nucleofection of the remaining cells were used for SCNT. The primers for birth. The perinatal period. All cloned piglets were delivered by natural estrus-synchronized recipient gilt. Pregnancy was examined 250 reconstructed oocytes were transplanted into the uterus of Tokyo, Japan) and 15–20 individual clones were picked and products were subcloned into a pMD18-T vector (Takara Clontech, 30 s, and 72°C for 30 min, 95°C for 10 s, 60°C for 30 s, and 72°C for 60 s, and a final 72°C for 5 min. The PCR products were subcloned into a pMD18-T vector (Takara Clontech, Tokyo, Japan) and 15–20 individual clones were picked and sequenced.

2.4. PFFs transfection and selection

To establish GCT/A/CMAH/β4GalNT2-knockout (TKO) cell lines, 2 μg of each targeting plasmid were co-transfected with 1 μg of the neomycin-expression plasmid (pCMV-ttdTomato) into 1 × 10^6 early passage of PFFs using the Basic Fibroblast Nucleofection Kit (Amaxa Biosystems/Lonza, Cologne, Germany), according to the manufacturer’s instructions. To obtain the transgenic cell colonies, 800 μg/mL of G418 (Gibco, Grand Island, USA) was applied to the post-transfection cells 48 h later and maintained for 7–10 days thereafter. The G418 resistant colonies were seeded in 24-well plates and then passed to 12-well plates. Approximately 1/5 of the cells of a single colony were lysed with NP-40 (55°C for 30 min, 95°C for 10 min) for PCR screening, and 4/5 of the remaining cells were used for SCNT. The primers for GCTA1-sgRNA1, β4GalNT2-sgRNA2, and CMAH-sgRNA1 listed in Table 2 were used for genotyping. The PCR conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 60 s, and a final 72°C for 5 min. The PCR products were subcloned into a pMD18-T vector (Takara Clontech, Tokyo, Japan) and 15–20 individual clones were picked and sequenced.

2.5. Somatic cell nuclear transfer (SCNT) and embryo transfer

The ovaries from six months-old gilts were purchased from a local slaughterhouse. The oocytes were collected and cultured for 42–44 h for maturation. The matured oocytes were removed to isolate cumulus cells and their nuclei. A single donor PFF was injected into the perivitelline space of the enucleated oocyte. After electrofused membranes between the donor cell and recipient cytoplasm, the reconstructed embryos were cultured in embryo-development medium (108 mM NaCl, 10 mM KCl, 0.35 mM KH2PO4, 0.4 mM MgSO4 7H2O, 25.07 mM NaHCO3, 0.2 mM Na-pyruvate, 2 mM CaCl2·5H2O, 1 mM L-glutamine, 5 mM Hypotaurine, 2% BME amino acid solution, 1% MEM non-essential amino acid solution, 0.05% gentamicin, 0.3% Fatty acid free BSA, pH 7.2–7.4, Osmolarity 288 ± 2 mOsm) at 38.5°C for 24 h. About 250 reconstructed oocytes were transplanted into the uterus of an estrus-synchronized recipient gilt. Pregnancy was examined by ultrasound 30 days after transplantation and monitored until the perinatal period. All cloned piglets were delivered by natural birth.

2.6. Genotyping

Genomic DNA was prepared from punched ear tissue using QIAamp DNA Mini Kit (QIAGEN, Duesseldorf, Germany). Genotyping was performed with standard PCR with primers for GCTA1-sgRNA1, β4GalNT2-sgRNA2, and CMAH-sgRNA1 (listed in Table 2).

2.7. Flow cytometry analysis

PBMCs were isolated from heparinized peripheral whole blood using Lysing Solution (BD Biosciences, San Jose, CA, USA). αGal staining was carried out using Alexa Fluor® 488 conjugated isoleucin GSI-B4 (Thermo Fisher Scientific, Waltham, MA, USA). β4GalNT2 phenotype was determined with fluorescein dolichos biflorus agglutinin (Vector Laboratories, FL-1031, Burlingame, CA, USA). Neu5Gc expression was assessed by chicken anti-Neu5Gc antibody (BioLegend, 146901, San Diego, CA, USA). Alexa Fluor® 488 goat anti-chicken (Thermo Fisher Scientific, A11039) was used as secondary antibody, and chicken IgY isotype used as negative control (BioLegend, 402101). Samples were analyzed on a flow cytometer (BD, Franklin Lakes, USA).

2.8. Histology and immunohistochemistry

The pericardia from 3 WT and 3 TKO pigs were used for the staining experiments. Fresh and 0.2% glutaraldehyde fixed pericardium was embedded in optimal cutting temperature (OCT) compound and cryosectioned into 4 μm thickness. All the sections were air dried, fixed with acetone for 10 min and stored at −80°C until used. Glutaraldehyde-fixed samples were used for Masson’s trichrome staining. Fresh samples were used for collagen and antigen staining. The slides were washed with PBS, then blocked with Endogenous Biotin-Blocking Kit (Thermo Fisher Scientific) for 15 min at room temperature. To detect collagen expression, sections were incubated with murine Anti-Collagen Type I (Abcam 6308, Cambridge, UK), Anti-Collagen Type III (Abcam 23445), and Anti-Collagen Type V (Abcam 112551). Mouse anti-collagen antibody binding was detected with biotin conjugated goat anti-mouse IgG (Sigma-Aldrich, St Louis, MO, USA) and HRP-conjugated streptavidin. Visualization was performed using diaminobenzidine (DAB) staining and haematoxylin counter staining. To detect the αGal antigen, tissues were stained with biotin-conjugated GSI-B4 (Enzo Life Science, ALX-650-001B, Raamsdonksveer, The Netherlands). Please cite this article in press as: R. Zhang et al., Reducing immunoreactivity of porcine bioprosthetic heart valves by genetically-deleting three major glycan antigens, GCTA1/β4GalNT2/CMAH, Acta Biomater. (2018), https://doi.org/10.1016/j.actbio.2018.03.055

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<td>Synthesized oligos for GCTA1, β4GalNT2, and CMAH sgRNAs.</td>
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<tr>
<td>GCTA1 oligos</td>
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<tr>
<td>5’-CACCGAAAATTAGAAATGCCCTCA-3’</td>
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<td>5’-AAACCCTCCTCTCATTTTCTC-3’</td>
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<td>Primers used for GCTA1, β4GalNT2, and CMAH genotyping.</td>
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<td>Forward primers (5’-3’)</td>
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<tr>
<td>GCTA1-sgRNA1</td>
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Netherlands). GSI-B4 binding was detected with VECTASTAIN ABC Kit (Vector Laboratories, PK6100 standard, Burlingame, CA) and visualized by DAB staining. Neu5Gc staining was carried out using a chicken-derived anti-Neu5Gc immunohistochemistry kit (BioLegend) following the manufacturer’s instructions, and Alexa Fluor 488 goat anti-chicken IgY (H + L) (Invitrogen, Waltham, MA, USA) was used as secondary antibody. For detection of β4GalNT2, dolichos biflorus agglutinin labeled with fluorescein (Vector Laboratories, FL-1031) was used. 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) was applied for nuclear staining. The slides were examined by fluorescent microscopy (Nikon, Tokyo, Japan).

2.9. Human immunoglobulin binding assay

IgM and IgG binding assays were carried out on PBMCs using commercial human serum (Sigma Aldrich) of AB blood type. 1 × 10^7 PBMCs were incubated with heat-inactivated human serum diluted in RPMI Medium Modified (HyClone, South Logan, UT, USA) for 120 min at 4 °C, washed with PBS containing 0.1% FBS and blocked with 10% goat serum (Sigma-Aldrich) for 30 min at room temperature. FITC-conjugated goat-derived anti-human IgM (Invitrogen A18830, Carlsbad, CA, USA) or IgG (Invitrogen A18842) was applied for 30 min at 4 °C to detect IgM or IgG binding. Sample analysis was completed on a flow cytometer (BD, Franklin Lakes, USA). IgM and IgG binding assays were also performed on pericardial tissue sections. Goat anti-human IgM (μ chain) antibody (Invitrogen A18845) and goat anti-human IgG Fc antibody (Invitrogen A18833) were used. VECTASTAIN ABC Kit (Vector Laboratories, PK1600 standard) was applied for detection. Visualization was performed using diaminobenzidine (DAB)/imidazole staining.

2.10. Hydroxyproline content estimation

Hydroxyproline is a major component of collagen and a useful estimate of collagen content. Pericardial samples of WT and TKO pigs were hydrolyzed in 6 N HCl at 105 °C for 22–24 h. Hydrolyzed samples were mixed with 1 ml Chloramine T and 1 ml deionized water for 20 min at room temperature followed by 15 min at 60 °C with dimethylamino-benzaldehyde reagent (Sigma Aldrich). The absorbance at 550 nm was measured. The hydroxyproline content of pericardium was determined by reference to a standard curve.

2.11. Uniaxial mechanical test

Pericardium was harvested from each of the WT and TKO pigs and washed with phosphate buffered saline (PBS). After removal of adipose tissue, the pericardium was fixed with glutaraldehyde. Three days later, the fixed pericardium was prepared into 12 dog-bone-shaped samples (6 for WT and 6 for TKO) according to the American Society for Testing and Materials (ASTM) standard [33]. Thickness was measured by a vernier caliper (with the smallest scale being 20 μm, or 0.02 mm). All the samples were uniaxially tensioned at room temperature by a single column tabletop testing machine equipped with a 10 N load cell (Instron 5943, Norwood, MA, USA). A thermostatic bath was not used. The velocity of the cross-head was the loading rate, which was set at 10 mm/min. The stress-strain curve, maximum stress, and strain at the maximum stress were recorded. The stress and strain were calculated by σ=P/A and ε=Δl/l, respectively, where P is the applied load, A is the cross-sectional area of the sample, Δl is the displacement of the cross-head, and l is the initial length of the sample.

2.12. Statistical methods

Data were analyzed with Microsoft Excel and presented as the mean ± standard error. P values were determined by using student’s t tests and ANOVA for all quantifications. P values < 0.05 were considered as statistically significant.

3. Results

3.1. Simultaneous disruption of GGTA1, β4GalNT2, and CMAH genes in pigs

To disrupt the GGTA1, β4GalNT2, and CMAH genes in Landrace pigs, two candidate sgRNAs were designed for each target gene. The precise target sites of these sgRNAs are shown in Fig. 1A. The cleavage efficiency of the 6 sgRNAs was first tested by T7EN1 digestion assay. The results indicated that all 6 sgRNAs could induce indels in their targeting regions, with cleavage efficiency ranging from 2.5% to 21.8% (Fig. 1B). sgRNAs specific for GGTA1, β4GalNT2, and CMAH with higher cleavage efficiency were selected for subsequent gene targeting. To establish GGTA1/CMAH- /β4GalNT2-knockout (TKO) cell lines, a TD-tomato plasmid carrying a neoymycin resistance (Neo) gene and three plasmids encoding the Cas9 endonuclease and sgRNA for each target gene were simultaneously transfected into the early passage of primary PFFs. A total of 27 resistant colonies were obtained after G418 selection for 10 days. Genotyping of these cell colonies was performed using TA-cloning and Sanger sequencing. The results indicated that 15 colonies had biallelic modifications in the GGTA1 targeting region, 11 had biallelic modifications in the β4GalNT2 targeting region, and 17 had biallelic modifications in the CMAH targeting region, respectively (Fig. 1C). Among them, one colony (#50) was identified with biallelic frame-shift mutations in the target regions of all three genes (Fig. 1D) and used as donor cells for SCNT to produce GGTA1/β4GalNT2/CMAH TKO pigs. Eight live-born male piglets were delivered from 3 recipients (Fig. 1E). The genotype of each cloned piglet was determined by TA-cloning and sequencing using DNA isolated from ear tissues. The results showed that all cloned piglets bore a 1 base insertion in the GGTA1 and CMAH target regions, and a 10 base deletion in the β4GalNT2 target region, which is identical to the genotype of #50 donor cells.

3.2. Assessment of αGal, β4GalNT2, and Neu5Gc antigen expression

We expected that the biallelic frameshift modifications of GGTA1, β4GalNT2, and CMAH genes would eliminate the αGal, Sd(a) and Neu5Gc carbohydrates on the tissues and cells of these cloned piglets. To confirm the deficiency of αGal, Sd(a) and Neu5Gc antigens in these TKO piglets, flow cytometry analysis was performed on PBMCs collected from both cloned pigs (n = 5) and WT pigs (n = 5). The results showed that WT PBMC were 84.3% αGal, 72.9% Neu5Gc, and 92.6% β4GalNT2 positive. In contrast, TKO piglet PBMC were all negative for these three antigens (Fig. 2).

Of the 5 examined TKO piglets, 3 were euthanized to examine the αGal, Sd(a), or Neu5Gc antigens on their pericardium, one of the main materials for producing commercial BHVs. Immunohistochemistry analysis was performed with antibodies specific for αGal, Sd(a), or Neu5Gc glycans. Results showed that αGal, Sd(a) and Neu5Gc were strongly positive on the pericardium of WT piglets, while these antigens were barely detected in pericardial samples from TKO pigs (Fig. 3), which was consistent with the flow cytometry results.
3.3. Human IgM and IgG antibody binding assay

Most humans have variable levels of antibodies against αGal, Sd(a), and Neu5Gc epitopes [31]. To determine whether disruption of αGal, Sd(a) and Neu5Gc expression reduced their immunoreactivity, immunohistochemistry analysis was conducted on pericardial tissue sections from WT and TKO piglets by staining for human IgM and IgG binding. Pericardial samples were fixed with 0.2% glutaraldehyde for 48 h prior to the test. The TKO group showed a significantly lower level of IgM and IgG binding to pericardial tissue. The WT group showed strong positive binding even when incubated with a weak (5%) concentration of human serum. This indicated that TKO porcine pericardium expressed significantly reduced xenoantigens (Fig. 4A). To further evaluate binding of human serum antibody quantitatively, WT and TKO pig PBMCs were isolated and analyzed by flow cytometry using human PBMCs as a negative control. GTKO, DKO (GGTA1/CMAH-KO) pigs (provided by Dr. Pan) were used as positive controls. The mean fluorescence intensity of IgM and IgG was determined after incubation with heat-inactivated human serum. Compared to WT PBMCs, human IgM and IgG binding to GTKO and DKO pigs was significantly diminished. As we expected, disrupting GGTA1, β4GalNT2, and CMAH further minimized IgM and IgG binding to GTKO and DKO pigs to a level comparable to human serum (Fig. 4B).

3.4. Collagen composition of porcine WT and TKO pericardium

Gross morphology of pericardium from the TKO piglets was examined, demonstrating that it was similar to WT control pericardium in terms of texture (data not shown). Trichrome Masson staining showed that both the TKO and WT pericardium were composed of a large matrix of extracellular protein with massive collagen (Fig. 5A). No obvious structural difference between the two groups was observed. Immunohistochemistry analysis was carried out on pericardial sections with antibodies against either collagen I, III or V. All three types of collagen were similarly expressed in both TKO and WT tissues. Collagen I was more abundant in tissue from both groups than collagen III and V (Fig. 5B). The total collagen content was also determined by the hydroxyproline estimation method, which indicated that there was no significant difference between the WT and TKO pericardium (Fig. 5C).

3.5. Mechanical test of WT and TKO pericardium

Biomechanical properties of the WT and TKO pericardial samples were compared by uniaxial stress testing. The thicknesses for WT and TKO groups were 0.22 ± 0.06 mm and 0.20 ± 0.01 mm (mean ± standard deviation), respectively. The lengths for WT and TKO groups were 15.25 ± 1.72 mm and 14.67 ± 1.03 mm while...
Fig. 2. Flow cytometry detection of xenoantigen expression of WT and TKO pig PBMCs. A. Representative flow cytometric images of PBMCs with the numbers in quadrants indicating the percentage of respective xenoantigen positive cells. IB4 lectin specific for αGal carbohydrates, chicken antibodies specific for the Neu5Gc carbohydrate and DBA lectin specific for carbohydrates produced by the β4GalNT2 gene were used to detect the antigen expression. B. Quantification of xenoantigen positive cell percentages (n = 5/group). All values represent the mean ± SEM. (**P < 0.01, WT group to TKO group, Student's t test).

Fig. 3. Representative images of immunohistochemistry analysis of αGal, Sd(a), and Neu5Gc expression in WT and TKO pericardial tissue sections (n = 3/group). Negative controls have been included. A scale bar of 100 μm is shown. All images have the same magnification.

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the widths for WT and TKO groups were 2.48 ± 0.47 mm and 2.15 ± 0.23 mm, respectively. There is no significant difference in the geometry of WT and TKO samples. The stress-strain curve, maximum stress, and strain at the maximum stress of the tested WT and TKO samples are plotted in Fig. 6. All tested samples showed similar nonlinear stress curves, while the mechanical response of the TKO samples is slightly superior to the WT as shown by smaller individual variations (Fig. 6A). Furthermore, the maximum stress was not significantly different, measuring 10.56 ± 4.39 MPa and 13.54 ± 2.61 MPa for the WT and TKO samples, respectively (Fig. 6B). In terms of tensile strength, the values for the WT and TKO groups were not significantly different (74.62 ± 12.43% vs. 67.81 ± 6.44%, respectively, p > 0.05) (Fig. 6C). From the above analysis, the mechanical properties of pericardium were similar in all aspects between the WT and TKO groups.

4. Discussion

GBHVs, derived from porcine or bovine tissues, have been used as a replacement for failed human heart valves worldwide for many years. However, clinical application of GBHV implants has been limited by calcification that frequently occurs over time, mainly caused by the recipients’ immune responses [1,4,34,35]. αGal and Neu5Gc, two xenogeneic antigen targets for human antibodies, have been detected in native porcine tissues as well as in commercially-available GBHVs [21,28]. Besides the well-characterized αGal and Neu5Gc antigens, the Sd(a) antigen, which is associated with the β4GalNT2 glycosyltransferase, has been recently shown to be recognized by human IgG/IgM [29,30].

In the present study, we simultaneously disrupted porcine GGTA1, β4GalNT2, and CMAH genes, and investigated antigen expression and immunoreactivity with human antibodies to TKO porcine pericardium (which has similar antigen expression and immunoreactivity to porcine valve leaflets [7]). We demonstrated that αGal, Neu5Gc, and Sd(a) antigens were undetectable in TKO pigs and that human IgG/IgM antibody binding to TKO pig pericardial tissues was significantly reduced to a level comparable to binding to human cells. Our results substantiate that minimizing the immunoreactivity of porcine BHV through multiple gene modifications could prolong the life-span of a BHV in a patient.

With the advantage of high gene targeting efficiency, CRISPR/Cas9 technology has been extensively employed for gene modification in various species [32,36]. In the present study, the CRISPR/Cas9 system achieved high cleavage efficiency as 15, 17 and 11 of 27 colonies harbored biallelic modifications for the respective
GGTA1, CMAH and β4GalNT2 loci. However, only 1 of 27 colonies showed biallelic modifications in all 3 genes, indicating the efficiency of CRISPR/Cas9 was significantly decreased when multiple genes were needed to be simultaneously targeted. Therefore, dual or multiple sgRNA instead of a single sgRNA could be used for multiple gene targeting, as suggested by previous studies [37–39]. In addition, GGTA1, CMAH, and β4GalNT2 genes resulted in homozygous biallelic modifications (GGTA1: +1; CMAH: +1; β4GalNT2: −10), which induced frameshift mutations. This is contrary to the genotypes of TKO pigs described by Estrada et al. [31]. The pigs

Fig. 5. Morphology and collagen composition of WT and TKO pericardium. A. Representative images of trichrome stained WT and TKO pericardial tissues. Negative controls have been included. A scale bar of 200 μm is shown. All images have the same magnification. B. Immunohistochemistry of collagen expression. Pericardium was stained with antibodies specific for collagen I, III, and V. A scale bar of 200 μm is shown. All images have the same magnification. C. Hydroxyproline content of WT and TKO pericardial samples (n = 3/group). Data bars are shown as mean ± SEM.

Fig. 6. Uniaxial stress test of WT and TKO pig pericardium (n = 3/group). A. Stress–strain curves. Pericardium was harvested from the WT and TKO pigs and 12 dog-bone-shaped pericardia (6 for WT and 6 for TKO) were tested. B. Box plot of the maximum stress (10.56 ± 4.39 MPa and 13.54 ± 2.61 MPa for the WT and TKO, respectively). C. Box plot of the strain at the maximum stress (WT was 74.62 ± 12.43%, TKO was 67.81 ± 6.44%). NS: not significant.
in the previous study by Estrada et al. harbored heterozygous modifications with in-frame deletions in CMAH and \(\beta\)GalNT2 genes (CMAH: –12/–3/+5; \(\beta\)GalNT2: –12/+1/–5). This approach may give rise to truncated proteins with partially-retained function. We have observed that a 39 bp deletion could not totally disrupt the function of ApoE (unpublished data). Thus, to completely eliminate xenografts, in-frame mutations should be avoided.

Consistent with our results, \(\beta\)Gal and Neu5Gc antigens have been shown to be highly expressed on pericardial collagen fibers, and are associated with calcification of GBVHs [3,7,28]. Elimination of \(\beta\)Gal from pericardium by genetic modification or alphagalactosidase treatment has been proven to have no negative impact on the physical characteristics of porcine pericardium [3,40]. Our results showed that the collagen composition and tensile stress properties of TKO pericardium did not significantly change, suggesting that the additional absence of Neu5Gc and Sd (a) antigens would not substantially affect pericardial physical properties and tissue integrity. The immunoreactivity of pericardium from the GGTa1/\(\beta\)GalNT2/CMAH TKO pigs was significantly decreased, suggesting that this tissue could serve as a promising new source of BHVs. Although the results presented here are encouraging, our study has some limitations. The number of TKO pericardia used for uniaxial testing was limited, and, based on the data presented in this study, potential epigenetic effects on cloned pig pericardial tissues cannot be excluded. The establishment of female TKO pigs is in process in our lab, more TKO piglets will be produced via natural breeding and these issues will be studied once we have enough F1 TKO pigs. Moreover, whether the rate of calcification of TKO BHVs is reduced needs to be investigated in vivo in a non-human primate transplantation model.

The availability of TKO pigs has important practical implications for clinical valve replacement using BHVs. A BHV has significant advantages over a mechanical prosthesis, as chronic anticoagulant therapy is not required. However, in young patients (e.g., <35 years of age), and particularly in teenagers, SVD of a BHV is rapid, sometimes occurring within one or two years. This is almost certainly related to both the more vigorous immune response and higher metabolic activity in this age group. The likely mechanism is binding of anti-pig antibody, activating and damaging the graft endothelial cells, with rapid calcification taking place. The greatly reduced (or no) binding of antibody to TKO cells should result in a much slower rate of injury to the cells.

The use of BHVs from TKO pigs would initially result in a significantly higher cost of each BHV, as herds of these pigs would need to be developed. However, pigs being used as sources of kidneys, islets, or corneas in patients could possibly also be used as sources of heart valves (as long as the heart was not being transplanted). These pigs will be bred and housed under biosecure isolation conditions and the costs will be relatively high. However, once a herd has been established, if they will only be sources of BHVs, it is unlikely that they will need to be housed under any different conditions from those of WT pigs housed today as sources of BHVs. The costs will therefore be comparable to those associated today with BHVs from WT pigs. The major impact for the companies producing BHVs commercially will be that re-transplantation will hopefully not be required so early or frequently as it is now.

Will it be ethical to carry out a clinical trial of TKO BHVs without prior animal experimentation? Only the regulatory authorities will be able to answer that question. However, nonhuman primates, e.g., baboons, have a similar (though not identical) immune response to pig tissues as humans [41], and so they could provide a suitable animal model for BHV transplantation studies. The aortic valves of even quite large baboons are small compared with those of human adolescents or adults, and so perhaps a BHV leaflet could be transplanted into the descending aorta of the baboon, though this would not be ideal. Alternatively, the BHV could be more readily transplanted to replace the baboon’s mitral valve, where a larger BHV could be accommodated [42].

Is a TKO pig the ultimate source of organs and BHVs for humans, or are there additional xenografts that need to be deleted? Although approximately one-third of patients on the wait-list for a kidney transplant in the USA express no antibodies against TKO pig cells [43], there are undoubtedly other antigen targets against which some patients with a BHV (who will not be receiving immunosuppressive therapy) could make antibodies, e.g., swine leukocyte antigens. There are also possibly other carbohydrate antigens that we have yet to identify. Therefore, this question cannot be answered yet. Further studies are needed to identify additional pig antigens that could be recognized by human natural antibodies and to minimize the immunoreactivity of pig tissues through genetic engineering. However, the evidence indicates that BHVs from TKO pigs are likely to function significantly longer in human recipients than the current WT BHVs implanted today.

5. Disclosures
The authors do not have any conflict of interest to disclose.

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References