Genetically engineered sheep: A new paradigm for future preclinical testing of biological heart valves

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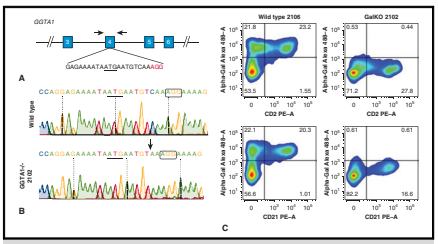
ABSTRACT

Background: Heart valve implantation in juvenile sheep to demonstrate biocompatibility and physiologic performance is the accepted model for regulatory approval of new biological heart valves (BHVs). However, this standard model does not detect the immunologic incompatibility between the major xenogeneic antigen, galactose-α-1,3-galactose (Gal), which is present in all current commercial BHVs, and patients who universally produce anti-Gal antibody. This clinical discordance leads to induced anti-Gal antibody in BHV recipients, promoting tissue calcification and premature structural valve degeneration, especially in young patients. The objective of the present study was to develop genetically engineered sheep that, like humans, produce anti-Gal antibody and mirror current clinical immune discordance.

Methods: Guide RNA for CRISPR Cas9 nuclease was transfected into sheep fetal fibroblasts, creating a biallelic frame shift mutation in exon 4 of the ovine α-galactosyltransferase gene (*GGTA1*). Somatic cell nuclear transfer was performed, and cloned embryos were transferred to synchronized recipients. Cloned offspring were analyzed for expression of Gal antigen and spontaneous production of anti-Gal antibody.

Results: Two of 4 surviving sheep survived long-term. One of the 2 was devoid of the Gal antigen (GalKO) and expressed cytotoxic anti-Gal antibody by age 2 to 3 months, which increased to clinically relevant levels by 6 months.

Conclusions: GalKO sheep represent a new, clinically relevant advanced standard for preclinical testing of BHVs (surgical or transcatheter) by accounting for the first time for human immune responses to residual Gal antigen that persists after current BHV tissue processing. This will identify the consequences of immune disparity preclinically and avoid unexpected past clinical sequelae. (J Thorac Cardiovasc Surg 2023;■:1-10)



CRISPR Cas9 engineering of GGTA1 creates a new GalKO sheep immune model for BHV testing.

CENTRAL MESSAGE

For the first time, genetically engineered GalKO sheep producing cytotoxic anti-galactose-α-1,3-galactose antibody as humans do have been created, representing a new, clinically relevant immune model for future more optimal testing of biological heart valves.

PERSPECTIVE

Clinical discordance between current biological heart valves (BHVs; surgical or transcatheter), all of which contain the xenogeneic galactose-α-1,3-galactose (Gal) antigen, and patients who universally produce anti-Gal antibody is linked to age-dependent BHV tissue calcification and structural valve degeneration (SVD). The genetically engineered sheep from this study can be used to directly test the impact of this immune discordance, define the role of immune injury in SVD, and create a new model for future BHV testing.

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BS

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Abbreviations and Acronyms

BHV = biological heart valve
ELISA = enzyme-linked immunosorbent assay
Gal = galactose-α-1, 3-galactose
GalKO = α-galactosyltransferase-deficient mutation
GGTA1 = α-galactosyltransferase gene
GLUT = glutaraldehyde
GSIB-4 = *Griffonia simplicifolia*-IB4 lectin
HEK = human embryonic kidney
PCR = polymerase chain reaction
PBMC = peripheral blood mononuclear cell
SCNT = somatic cell nuclear transfer
SVD = structural valve degeneration

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BS

Valve implantation into sheep is the primary experimental and regulatory testing model used for the development and approval of new mechanical and biological heart valves (BHVs). For mechanical heart valves, sheep implantation is used to test physiological compatibility and hemodynamic function with special focus on the degree of valve-dependent thrombogenesis. For BHVs, both surgical and percutaneous, the sheep model tests physiological compatibility and hemodynamic function but is also used to test additional biological responses. Juvenile sheep are preferentially used to assess tissue fixation methods and their effectiveness in preventing tissue calcification, a hallmark of structural valve degeneration (SVD). Adolescent and adult sheep are used for testing valve function, durability, and tissue inflammation and also for testing novel regenerative valve designs that require recellularization of the implanted valve. The standard valve implantation sheep model has been important for developing and refining the effective BHVs currently in use; however, the standard sheep model is not an effective immune model for detecting clinical immune responses to the dominant xenogeneic antigen galactose-α-1, 3-galactose (Gal).

All current commercial BHVs are made of porcine valve tissue, porcine pericardium, or bovine pericardium. These tissues all contain the dominant xenogeneic antigen Gal, and humans universally produce abundant amounts of anti-Gal antibody.[1,2] This Gal-specific clinical immune discordance results in induction of anti-Gal antibody in BHV recipients but not in recipients of mechanical heart valves or after coronary artery bypass procedures.[3,4] The immune response to BHVs is especially strong in children[5] and has been replicated in nonhuman primates,[6] confirming that the Gal antigen on BHVs remains immunogenic despite modern tissue fixation techniques. Both preformed and induced anti-Gal antibody bind to BHV tissue, and clinical and experimental studies indicate that bound antibody enhances the rate of tissue calcification, likely contributing to SVD.[7-10]

SVD is a complex, strongly age-related, multifactorial process associated with tissue calcification, leaflet tearing, and perforation, leading to hemodynamic dysfunction.[11] A host of long-recognized passive physiochemical processes contribute to tissue calcification and SVD in all patient populations, including calcium binding to unreacted glutaraldehyde (GLUT) and carboxylic acid, organic phosphate, acidic phospholipids, and calcium-binding proteins exposed in the devitalized cellular cytosol, membranes, and organelles. Direct calcification of extracellular matrix also occurs and is exacerbated by mechanical and oxidative injury and ineffective elastin fixation.[11] These physiochemical processes occur in all patients, and manufacturers have adopted fixation processes aimed at preventing passive tissue calcification by blocking unreacted GLUT with primary amines, minimizing GLUT oxidation, and using detergents or alcohols to extract charged acidic membrane phospholipids that serve as calcium nucleation sites.[12-15] These commercial treatments reduce tissue calcification in the standard juvenile sheep mitral valve model by approximately 10-fold[12,13] but have not prevented BHV calcification in patients, especially younger patients.[16,17] Indeed, current practice guidelines predict a 15-year risk of requiring reoperation from SVD of 50% for patients age 20 years, 30% for patients age 40 years, and 22% for patients age 50 years.[18] In children and teenagers, the use of BHVs has largely been abandoned owing to the high rates of SVD.

The effectiveness of commercial anticalcification treatments is clear in animal models, but their inability to mitigate age-dependent SVD in patients suggests that for modern commercial BHVs, passive physiochemical calcification is no longer the key remaining mechanism of clinical SVD.[7,11] This suggests that an immune-mediated process, created by the clinical Gal-specific discordance between Gal-containing BHVs and patients producing anti-Gal antibody, may contribute substantially to age-dependent clinical tissue calcification and SVD. Using standard sheep, we and others have shown in vitro[19-21] and in vivo[22] that porcine GalKO tissues can be used to make effective and durable BHVs. This hypothesis has not been fully testable to date, because standard sheep synthesize the Gal antigen and thus do not produce anti-Gal antibody. Here we describe the production of the first viable GalKO

sheep, which lack expression of the Gal antigen and begin producing cytotoxic anti-Gal IgG at clinically relevant levels by age 2 to 3 months. GalKO sheep represent the first large animal BHV testing model that accounts for human immune responses to the major xenogeneic antigen and will enable direct tests of the role of antibody in age-dependent SVD. This model will be critical for further refinement of BHVs to resist immune-mediated tissue calcification and SVD and as an immune model for regenerative heart valve development.

METHODS

All animal studies were approved and monitored by Utah State University's Institutional Animal Care and Use Committee (protocol 11908) and conformed to National Institutes of Health guidelines.

Sheep Fetal Fibroblast Transfection and Screening

Polymerase chain reaction (PCR) primers were designed according to the sheep *GGTA1* genome sequences (GenBank, NC_040254.1) and used to amplify exon 4 with partial flanking intron. Three guide RNAs (gRNAs) were designed targeting exon 4 (Table 1) and synthesized with chemical modifications (Synthego). Male sheep fetal fibroblasts (SFFs) at early passages were cultured in Dulbecco's Modified Eagle's medium (DMEM, high-glucose; Gibco) supplemented with 15% fetal bovine serum (Hyclone) and 100 U/mL penicillin/streptomycin (Life Technologies) at 38.5 °C in an atmosphere of 5% CO_2 in air.[23]

Cells were transfected with complexes of gRNA and Cas9 protein by electroporation, and the gene mutation efficiency was determined 3 days after transfection by Sanger sequencing of exon 4 PCR products cloned into a T-vector (Promega). Single-cell–derived colonies were isolated by limiting dilution and screened by PCR and Sanger sequencing. Four GGTA1$^{-/-}$ male colonies were used as donor cells for somatic cell nuclear transfer (SCNT).

SCNT

Domestic sheep (*Ovis aries*) used as embryo recipients in this study were 2 to 5 years old. Sheep SCNT was performed with slight modification as described by Yang and colleagues[24] for goats. SFFs were grown to 80% to 90% confluence and used as nuclear donor cells for SCNT after 24 hours of serum starvation (0.5% fetal bovine serum). The cloned embryos were cultured in synthetic oviduct fluid medium for 10 to 12 hours and then transferred into estrus synchronized recipients as described previously.[23] A total of 130 cloned embryos were transferred into 10 estrus-synchronized recipients. Exon 4 of GGTA1 was amplified from genomic DNA (forward primer: 5′-TCCAGCTCTTTGCAACGCTA-3′; reverse primer: 5′-TAGGGCTCAGGGAAACAGGA-3′), cloned, and then analyzed by Sanger sequencing.

Detection of Gal Antigen

Sheep peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep centrifugation (Stemcell Technologies) and washed in Minimal Essential Medium for Suspension Culture (S-MEM; Gibco). Cells were resuspended in fluorescence buffer (phosphate-buffered saline [PBS] containing 0.1% bovine serum albumin and 0.01% sodium azide) at 10^6/mL, washed, and stained with a combination of affinity-purified human anti-Gal IgG[8] and antibody to sheep CD2 (1.5 μg of MUC2A; Novus Biologicals) or CD21 (1.5 μg of GB25A; Novus Biologicals). Human antibody binding was detected with a 1:100 dilution of Alexa Fluor 488–conjugated goat anti-human IgG secondary monoclonal antibody (Southern Biotech; catalog no. 2040-30). Murine anti-CD2 or anti-CD21 binding was detected using a 1:100 dilution of phycoerythrin-conjugated F(ab')2 fragment goat anti-mouse IgG (H&L) (Jackson ImmunoResearch; catalog no. 115-116-146). Before analysis, cells were stained for gating of viable cells using Live/Dead Blue Fixable Cell Stain (Invitrogen; catalog no. L34961) and fixed in fluorescence fixative (1% formaldehyde in PBS). Flow cytometry analysis was done using a BD FACSAria II flow cytometer running FACSDiva v6.1.3 software (BD Biosciences).

Anti-Gal Detection of Sheep Anti-Gal Antibody

Enzyme-linked immunosorbent assay (ELISA) plates were coated with 50 μL of 10 μg/mL human serum albumin (HSA) conjugated to galactose-α-1,3-galactose β-1,4 N-acetylglucosamine (HSA-Gal; Dextra Labs) or HSA in carbonate buffer (pH 9.6) overnight at 4 °C. Plates were blocked with 100 μL of ELISA buffer (PBS containing 2% HSA and 0.1% Tween 20) for 30 minutes at room temperature. A dilution series of sheep serum (1:20-1:1024) in ELISA buffer was added (50 μL) to HSA-Gal (duplicate) and has-coated wells and incubated for 90 minutes at 4 °C. Plates were washed 3 times (150 μL each) with wash buffer (PBS containing 0.1% Tween 20) and then incubated for 60 minutes at room temperature with horseradish peroxidase–conjugated rabbit anti-sheep IgG or IgM (Bethyl Labs). Antibody binding was detected with 100 μL of 1-Step Turbo TMB (Thermo Fisher Scientific), and the color reaction was stopped by adding 100 μL of 3 M sulfuric acid. The optical density at 450 nm (OD_{450}) was measured with a FLUOstar Omega plate reader (BMG Labtech). Anti-Gal–specific antibody was calculated as:

$$\text{Anti-Gal} = (\text{OD}_{\text{HSA-Gal}} - \text{blank}) - (\text{OD}_{\text{HSA}} - \text{blank})$$

Detection of Gal-Specific Staining Using Human Embryonic Kidney Cells

Human embryonic kidney 293 (HEK) cells and stably transfected HEK cells expressing the porcine *GGTA1* gene (HEK-Gal) were grown in DMEM media supplemented with 10% fetal calf serum, 1% glutamine, 1× nonessential amino acids, and 110 mg/L sodium pyruvate to 80% confluence. Cells were collected by trypsin digestion, washed, and resuspended in FACS buffer (PBS with 2% human serum albumin) at a concentration of 4.6×10^6 cells/mL. For antibody staining, 44 μL of cells (~200,000 cells) were combined with 6.25 μL of heat-inactivated neat sheep serum (1:8 dilution) and then incubated at 4 °C for 45 minutes. HEK and HEK-Gal cells were also stained with fluorescein isothiocyanate –conjugated Gal-specific lectin *Griffonia simplicifolia*–IB4 (GSIB-4; Vector Laboratories, catalog no. FL-1201) to detect the Gal antigen. The cells were washed with FACS buffer and stained with Dylight 488–conjugated donkey anti-sheep IgG (Bio-Rad) diluted 1:200 in PBS with 5% donkey serum at 4 °C for 35 minutes to detect IgG binding.

TABLE 1. Guide RNAs and mutation frequencies for sheep GGTA1 exon 3

gRNA	Sequence (5′–3′)	PAM	Mutation efficiency, n/N (%)*
gRNA1	GAGAAAATAATGAATGTCAA	AGG	5/9 (55.6)
gRNA2	AAAAGTGATTCTGTCAATGC	TGG	1/8 (12.5)
gRNA3	TGTTTTGGGAATATATCCAC	AGG	9/9 (100)

gRNA, Guide RNA; *PAM*, protospacer-adjacent motif. *The mutation efficiencies were determined by polymerase chain reaction/T-vector cloning assays.

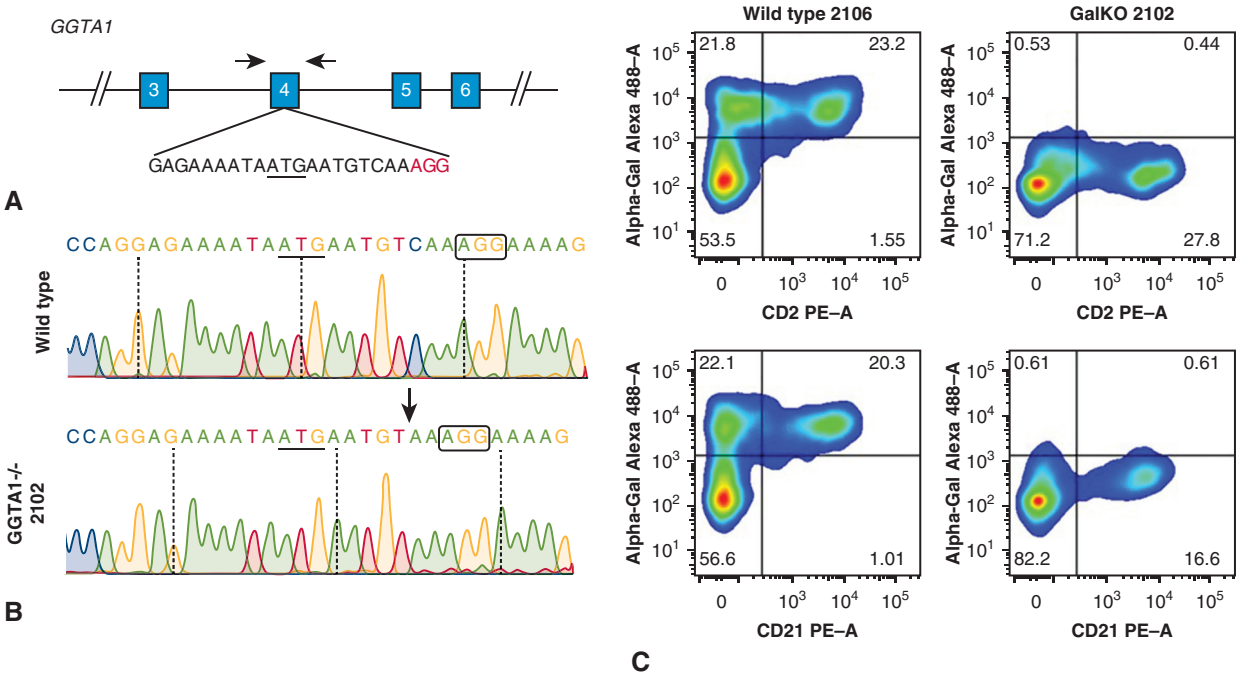

FIGURE 1. Analysis of cloned galactose-α-1,3-galactose (*Gal*) knockout (*GalKO*) sheep. A, Schematic diagram of the ovine α-galactosyltransferase 1 gene (*GGTA1*) showing the position of the guide RNA (*gRNA*) sequence in exon 4. The translation start codon, ATG, for *GGTA1* is in the gRNA and is underscored. Letters in red indicate the protospacer-adjacent motif (*PAM*). *Arrows* indicate locations of polymerase chain reaction primers. B, Representative sequence analysis for the *GGAT1*$^{-/-}$ FO lamb. The sequences for a wild-type *GGTA1* lamb and the 2102 *GGTA1*$^{-/-}$ FO lamb are shown. The *arrow* indicates the mutation site. C, FACS analysis of peripheral blood mononuclear cells (*PBMCs*) from wild-type sheep 2106 and healthy *GGTA1*$^{-/-}$ sheep (GalKO 2102). Approximately 41% of the PBMCs in the wild-type sheep were Gal^{+} with CD2^{+} α/β T cells and CD21^{+} B cells. The *GGTA1* KO sheep did not express Gal but showed normal T cell and B cell profiles.

Cell staining was analyzed by flow cytometry using a BD FACSCanto II and FACSDiva software.

Detection of Gal-Specific Cytotoxicity on HEK Cells

An equal volume of HEK or HEK-Gal cells (2 × 106 cells/mL) were mixed with 50 μL of dilute heat-inactivated sheep serum (final serum concentration of 1:8-1:32) and incubated at 4 °C for 30 minutes. HEK and HEK-Gal cells were also incubated with a serial dilution of affinity- purified human anti-Gal IgG (2 μg/mL-15.5 ng/mL) to create a standard cytotoxicity curve.[8] After antibody incubation, cells were washed once with 2 mL of FACS buffer, resuspended in 100 μL of 10% baby rabbit complement (Cedarlane; catalog no. CL3441) and incubated at 37 °C for 90 minutes. Propidium iodide (PI; 200 μL of 10 μg/mL) was added to each tube, and the cells were analyzed by flow cytometry. Specific lysis was calculated as the ratio of:

$$(\%\text{PI} + \text{cells treated with serum and complement}) - (\%\text{PI} + \text{cells treated with complement only}) / (100 - \%\text{PI} + \text{cells treated with complement only}).$$

transfected with gRNA and Cas9 protein using an electroporation-based method. The mutation efficiencies of 3 targeting compounds ranged from 12.5% to 100% (Table 1). Single-cell–derived mutated fibroblast colonies were isolated by limiting the dilution of cells transfected with the gRNA1 (Figure 1, *A*). Targeted biallelic disruption at *GGTA1* was achieved in colonies at a screening efficiency of 38.5% (10 of 26). Four *GGTA1*$^{-/-}$ colonies were used as donor cells for SCNT. A total of 130 embryos were transferred to 10 ewes, and 5 pregnancies were established (Table E1). Four of the 10 pregnancies developed to term (40%) and gave birth to 4 cloned lambs, of which 1 was a stillbirth and 1 died within 48 hours of hypoxia resulting from pulmonary atelectasis and large offspring syndrome, commonly observed in cloned sheep. One of the 2 remaining healthy lambs showed evidence of continued Gal expression (data not shown) and was not analyzed

RESULTS

Generation of *GGTA1*$^{-/-}$ Cloned Male Sheep by CRISPR/Cas9 and SCNT

Three gRNAs targeting exon 4 of *GGTA1* were designed and synthesized. Male SFFs at early passages were

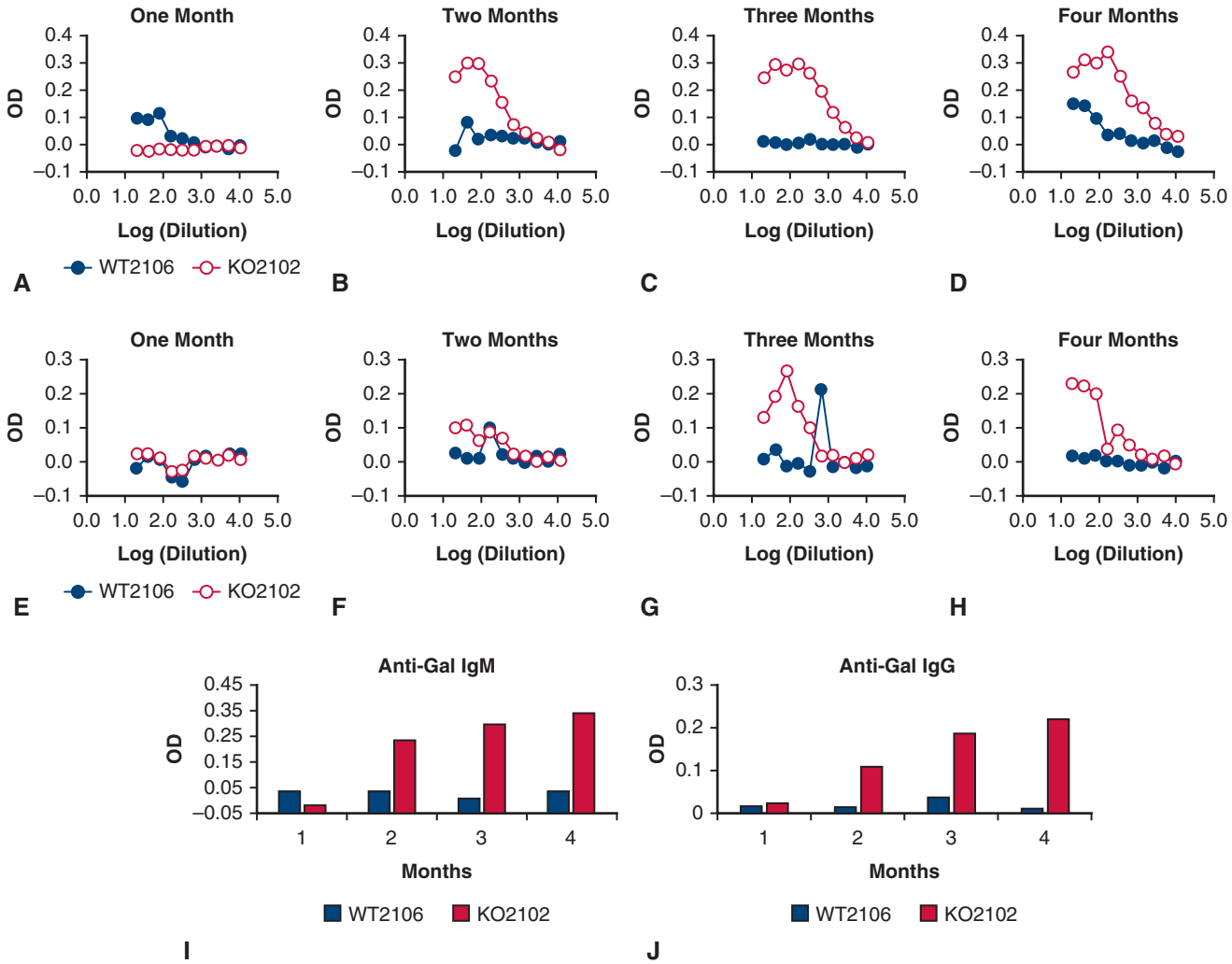

FIGURE 2. Enzyme-linked immunosorbent assay of anti–galactose-α-1,3-galactose (*Gal*) IgM and IgG in wild-type sheep 2106 (*WT2106*) and GalKO sheep 2102 (KO2102). A-D, Anti-Gal IgM dilution profiles at age 1 to 4 months. E-H, Anti-Gal IgG dilution profiles at age 1 to 4 months. Serum dilutions ranged from 1:20 to 1:10,240. I, Anti-Gal IgM at a 1:160 dilution for age 1 to 4 months. J, Anti-Gal IgG at a 1:40 dilution for age 1 to 4 months. *OD*, Optical density.

further, and the other (KO2102) is presently alive at 1.5 years of age. Sequencing results indicated that the cloned lambs carried the same mutations as those of the donor cells from which they originated. The genotyping results indicate a single cytosine base deletion at the gRNA target site of cloned lamb KO2102 (Figure 1, *B*).

Expression of Gal Antigen in GalKO Sheep

PBMCs from wild-type WT2106 and GalKO KO2102 were analyzed for Gal antigen expression on $CD2^+$ and $CD21^+$ lymphocytes (Figure 1, *C*). Approximately 40% of ovine lymphocytes express the Gal antigen. In wild-type sheep, 2106 Gal expression is evident on $CD2^+$ α/β T cells and $CD21^+$ B lymphocytes. The proportion of $CD2^+$ α/β T cells and $CD21^+$ B lymphocytes in GalKO sheep is similar to that seen in wild-type sheep; however, Gal antigen is not present.

Spontaneous Expression of Anti-Gal Antibody in GalKO Sheep

Anti-Gal ELISA. Genetically engineered pigs, mice, and rabbits with mutations in *GGTA1* show a loss of tolerance to the Gal antigen and spontaneously produce serum anti-Gal antibody.[25-27] We analyzed consecutive monthly serum samples from KO2102 for the presence of anti-Gal IgM and IgG using a Gal-specific ELISA (Figure 2). At age 1 month, there was no evidence of anti-Gal IgM or IgG reactivity in either KO2102 or the wild-type sheep WT2106 (Figure 2, *A* and *E*). In months 2 to 4, however, HSA-Gal–specific serum IgM binding from KO2102 was consistently higher across a wide range of serum dilutions, indicating the expression of anti-Gal IgM (Figure 2, *B-D*). Consistent HSA-Gal–specific IgG reactivity in KO2102 was first clearly apparent at age 3 months (Figure 2, *F* and *G*). At serum dilutions of 1:160 for IgM

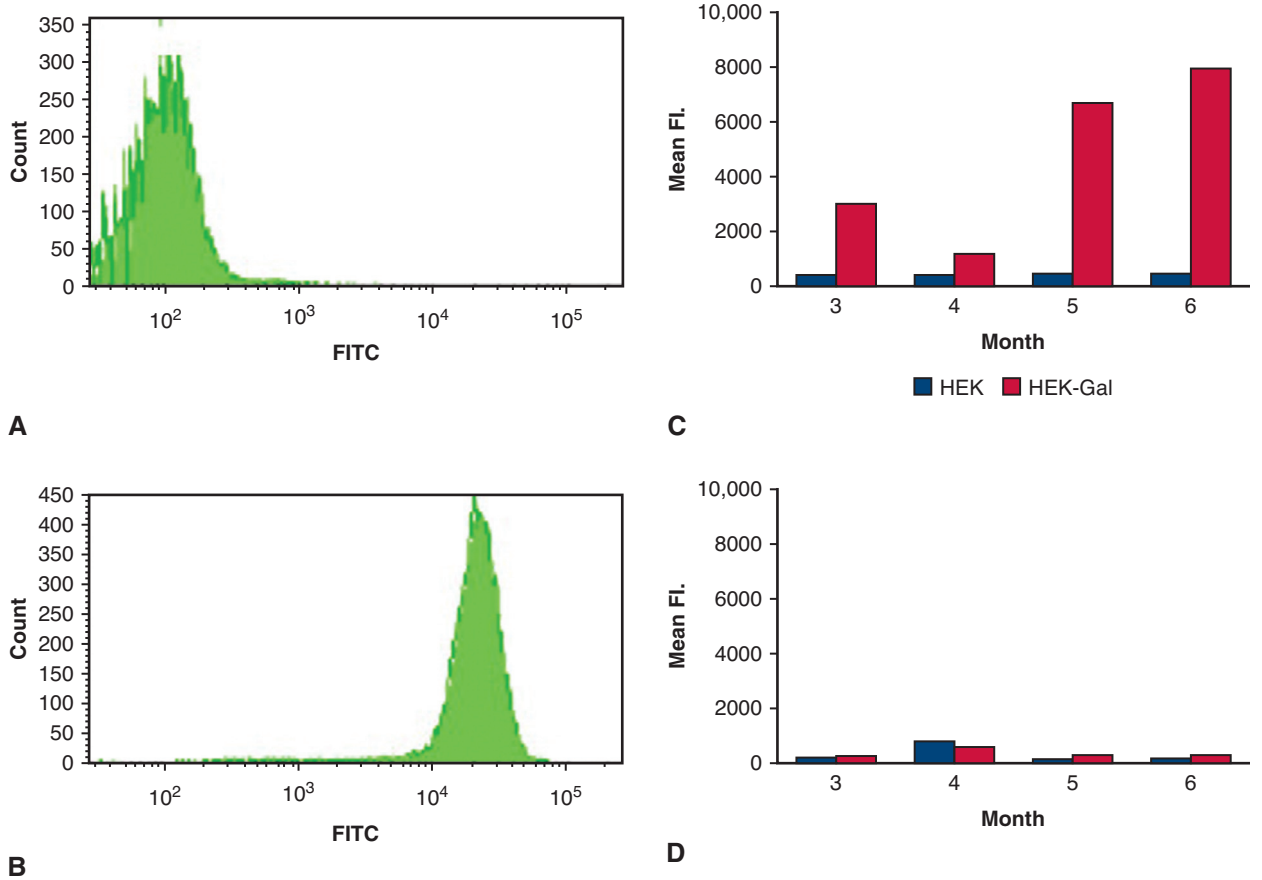

FIGURE 3. Analysis of sheep anti–galactose-α-1,3-galactose (*Gal*) antibody using human embryonic kidney 293 (*HEK*) and HEK-Gal cells. HEK cells and HEK cells expressing the porcine *GGTA1* gene were stained with Gal-specific Griffonia simplicifolia-IB4 lectin (*GSIB-4*). A, GSIB-4 staining of HEK cells. B, GSIB-4 staining of HEK-Gal cells. C and D, Sheep IgG binding to HEK (*black bars*) and HEK-Gal cells (*white bars*) at age 3 to 6 months for IgG reactivity of GalKO sheep 2102 (C) and IgG reactivity of wild-type sheep 2106 (D). *FITC*, Fluorescein isothiocyanate; *FI*, fluorescence.

and 1:40 for IgG, there was a progressive increase in HSA-Gal reactivity in KO2102 and little reactivity in WT2106 (Figure 2, *I* and *J*).

Antibody reactivity and cytotoxicity to HEK and HEK-Gal cells. To confirm the expression of anti-Gal IgG in KO2102 serum, we compared serum IgG reactivity to HEK and HEK-Gal cells. HEK cells are an O blood group human cell line that does not express the Gal antigen and does not bind the Gal-specific lectin GSIB-4 (Figure 3, *A*). HEK-Gal cells are a stable HEK cell line expressing the porcine *GGTA1* gene that produce abundant Gal antigen on the cell surface (Figure 3, *B*). Serum collected at age 3 to 6 months from KO2102 showed high IgG reactivity to HEK-Gal cells with minimal binding to HEK cells (Figure 3, *C*). Serum from WT2106 bound at very low levels to both HEK and HEK-Gal cells (Figure 3, *D*). Antibody in KO2102 serum was highly cytotoxic to HEK-Gal cells but minimally cytotoxic to HEK cells, consistent with the low level of anti-Gal IgG reactivity to HEK cells (Figure 4, *A*). We compared the cytotoxicity of KO2102 serum at serum dilutions of 1:8, 1:16, and 1:32 to the cytotoxicity of known amounts of affinity-purified human anti-Gal IgG (Figure 4, *B*). KO2102 serum showed Gal-specific cytotoxicity of HEK-Gal cells equivalent to the cytotoxicity induced by 1.07, 0.87 and 0.26 μg/mL of human anti-Gal IgG, respectively (Figure 4, *B*). When the serum dilutions are accounted for, KO2102 sheep serum cytotoxicity at 1:8, 1:16, and 1:32 dilutions was comparable to 8.5, 14.0, and 8.4 μg/mL of human anti-Gal antibody, giving an overall mean serum cytotoxicity equal to 10.3 ± 3.2 μg/mL of human anti-Gal IgG.

DISCUSSION

The adaptation of CRISPR Cas9 nuclease for gene modification in mammals has greatly facilitated the development of new large animal models. Here we report the successful engineering of a sheep containing a biallelic frameshift-inactivating mutation in the *GGTA1* α-galactosyltransferase gene. Analysis of PBMCs confirmed the absence of the major xenogeneic glycan Gal. Furthermore, the spontaneous production of cytotoxic

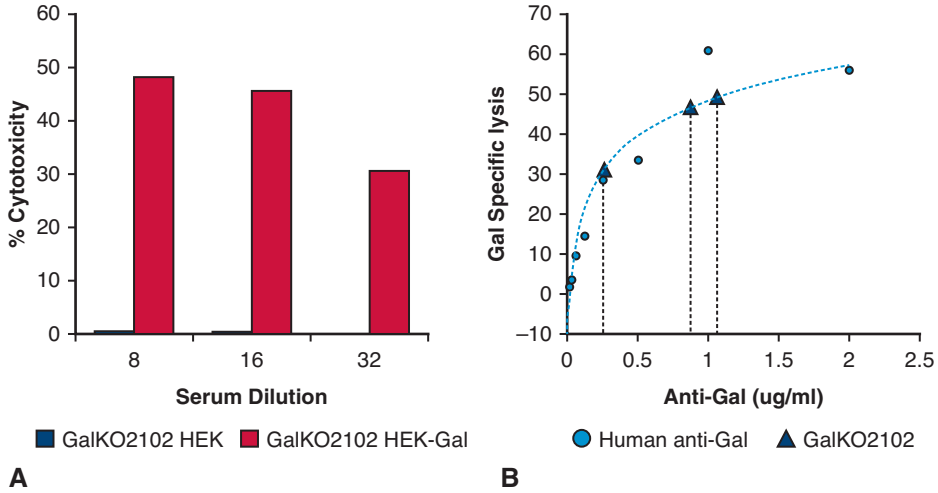

FIGURE 4. Galactose-α-1,3-galactose (*Gal*)-specific complement dependent cytotoxicity. A, Gal knockout (*GalKO*) sheep KO2102 cytotoxicity against human embryonic kidney 293 (*HEK*) and HEK-Gal cells. B, HEK-Gal cytotoxicity of purified human anti-Gal antibody (*blue circle*). Purified human anti-Gal antibody was used to create a standard curve for Gal-specific cytotoxicity (*dotted blue line*). The cytotoxicity of sheep KO2102 (*blue triangle*) was mapped to this curve to derive an approximate estimate of anti-Gal antibody equivalence cytotoxic activity. The coefficient of correlation (R^2) for the fitted logarithmic curve is 0.92. GalKO KO2102 cytotoxicity at 1:8, 1:16, and 1:32 dilutions is equivalent to 1.07, 0.87, and 0.26 μg/mL, respectively, of human anti-Gal antibody (*vertical dashed lines*).

anti-Gal antibody beginning at age 2 to 3 months in GalKO sheep but not in standard sheep confirms the loss of tolerance to this glycan. This is consistent with previous analysis of engineered GalKO mice, rabbits, and pigs.[25-27] An earlier attempt to generate GalKO sheep embryos using targeted homologous recombination did not produce live animals.[28] Given our present results, this was most likely due to the low targeting frequencies and prolonged culture times required for isolating cells targeted by homologous recombination, as suggested by Denning and colleagues.[28]

Sheep have provided an essential model for testing the physiologic compatibility and function of new BHV designs. As such, they have been instrumental in improving BHV durability and minimizing passive tissue calcification and, more recently, as a model for regeneration of tissue engineered heart valves. Standard sheep have major immunologic limitations, however. The Synergraft homograft is an effective decellularized homograft valve reported to have greater durability and lower immunogenicity than standard allografts for pulmonary valve replacement.[29] When this same technology was adapted to xenogeneic pig tissue, the cryopreserved heart valve worked well in preclinical standard adolescent sheep studies, showing good hemodynamic function and little tissue calcification.[30] However, early clinical application was followed by the premature death of 3 of the 4 children owing to severe valve degeneration from an aggressive inflammatory response and significant calcific deposits.[31] Subsequent analysis indicated that the decellularization procedure had not been successful, and that cellular debris and Gal antigen remained on the devices.[32] The potential roles of Gal antigen and antibody in this rapid inflammatory process are supported by the premature valve degeneration reported in patients developing Gal allergies after heart valve replacement.[10]

Human and Zilla,[7] using experimentally generated immune serum, demonstrated that BHV-specific antibody bound to GLUT-fixed tissue would enhance tissue calcification in a subcutaneous implant model. They described a model in which BHV-specific antibody binding initiates opsonization and complement activation, creating a self-sustaining inflammatory process that releases anaphylatoxins (C3a and C5a), recruits and activates monocytes (macrophages and neutrophils), and promotes BHV matrix degradation via release of protease and superoxide radicals. This immune injury enhances tissue calcification and SVD by weakening the BHV matrix, allowing further antibody binding, cellular infiltration, and passive tissue calcification. We showed that preexisting human anti-Gal antibody bound to standard GLUT-fixed pig pericardium increased tissue calcification after subcutaneous implantation in rats or rabbits, thereby identifying a clinically plausible source of anti-BHV antibody and obviating the need for an induced BHV-specific immune response.[8] Furthermore, we demonstrated that this antibody-induced calcification occurred even when tissue was treated with anticalcification processing,[9] showing that passive and immune induced tissue calcification are distinct processes.

Animal tissues (including standard sheep) express 2 additional xenogeneic glycans that bind human antibody:

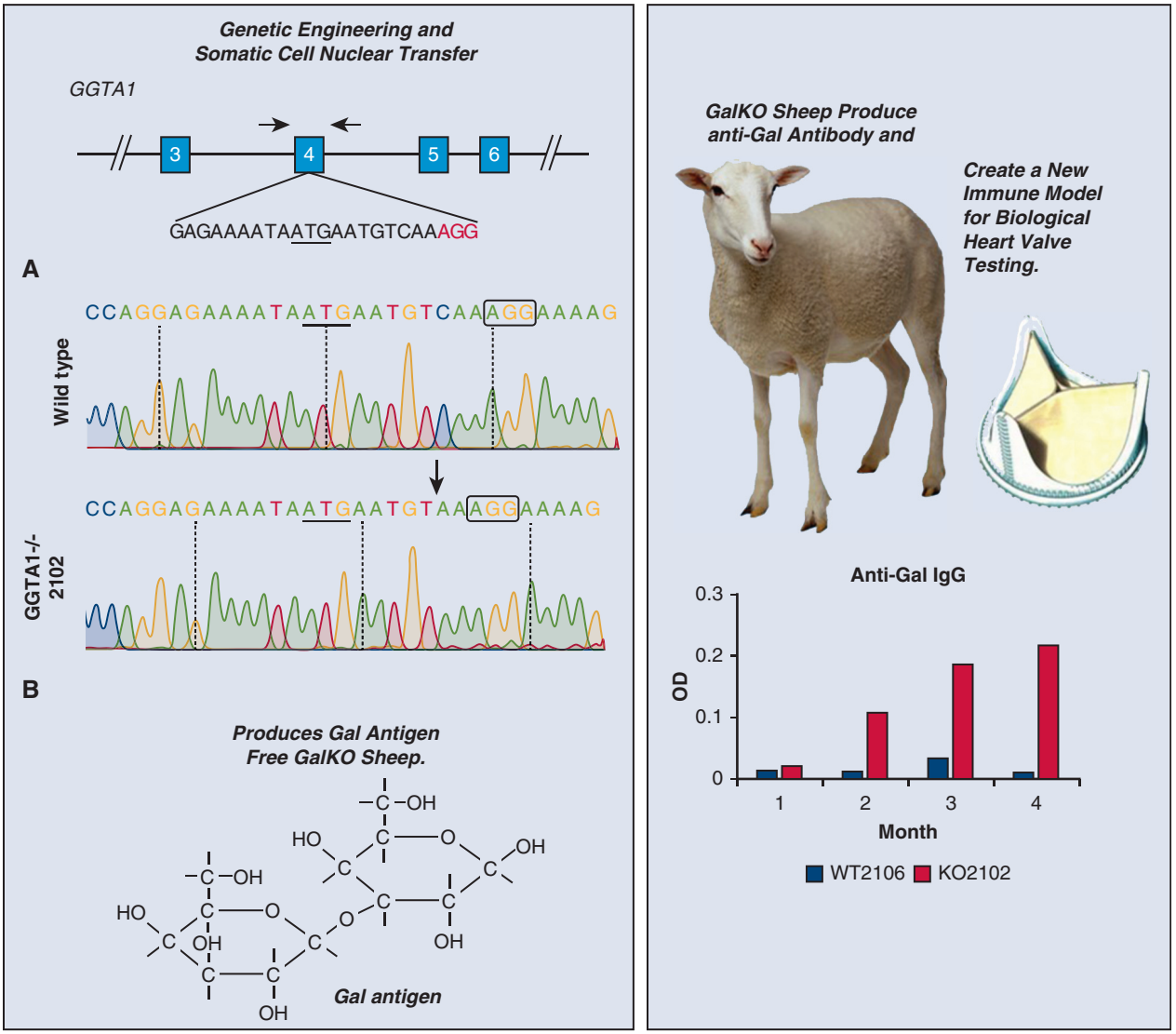

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FIGURE 5. Graphical abstract showing the use of CRISPR Cas9 nuclease to create an inactivating frame shift mutation in exon 4 of the ovine α-galactosyltransferase (*GGTA-1*) locus. The GGTA-1 locus is essential for producing the common xenogeneic carbohydrate galactose-α-1,3-galactose (*Gal*), and as a consequence of this CRISPR-induced mutation, Gal knockout (*GalKO*) sheep lack the Gal carbohydrate and spontaneously produce anti-Gal antibody. These sheep represent a new immune model for biological heart valve testing.

N-glycolylneuraminic acid modified glycans (Neu5Gc) and the SDa blood group antigen (encoded by the *B4GALNT2* locus). Gene modifications eliminating each of these xenogeneic glycans, Gal (*GGTA1*$^{-/-}$), Neu5Gc (*CMAH*$^{-/-}$), and SDa (*B4GALNT2*$^{-/-}$), have been produced in pigs and when combined, greatly reduce the level of human antibody binding to pig cells compared with standard pig tissue.[33] High expression levels of all 3 glycans are known to be present on pig endothelial cells, porcine pericardium, and commercial porcine BHVs.[21] Elimination of these other xenogeneic glycans has no apparent detrimental effect on the biophysical properties of porcine pericardium.[21] Exogenous addition of purified anti-Neu5Gc antibody has been shown to increase calcification of fixed tissue in a subcutaneous implant model,[4] and elimination of Neu5Gc-modified glycans from BHV tissue would be expected to further reduce human antibody binding. In sheep, the *B4GALNT2* gene shows high ovarian expression, and coding and noncoding polymorphisms in ovine *B4GALNT2* are associated with the *FecL* mutation, which promotes

high fecundity in Lacaune sheep and increased litter size in Small Tail Han sheep.[34] It appears that overexpression of *B4GALNT2* results in altered glycosylation of follicular proteins, leading to increased ovulation and prolificacy. The effects of eliminating *B4GALNT2* expression on sheep fertility are unknown. In any case, the Gal antigen has by far the greatest impact on human antibody reactivity to porcine cells and tissues, suggesting that elimination of anti-Gal antibody reactivity to BHV tissue may have a disproportionate impact on BHV immune injury.

The GalKO sheep presented in this report represent a new model for the development and testing of biomedical devices, particularly new BHVs (Figure 5). The spontaneous production of cytotoxic anti-Gal antibody in GalKO sheep allows these animals to model the current immune discordance between patients and current commercial clinical BHVs. The industry standard orthotopic BHV implantation model in juvenile/adolescent sheep is well characterized, reproducible, and accepted by regulatory bodies with excellent preclinical outcomes. This long history of BHV testing in sheep confers advantages to the GalKO sheep over other engineered GalKO species (mice, rabbits, and pigs), which also produce anti-Gal antibody, This will, for the first time, allow a detailed examination of the role of immune injury in BHV tissue calcification and the progression to SVD.

CONCLUSIONS

Consistent with other mammals that have been engineered for mutations in the *GGTA1* gene, this GalKO sheep lacks expression of the Gal antigen and produces cytotoxic anti-Gal antibody by age 2 to 3 months. GalKO sheep represent a new standard for preclinical testing of BHVs (surgical or transcatheter) and innovative emerging regenerative technologies because they are the first time to account for human immune responses to the major xenogeneic antigen.

Conflict of Interest Statement

C.G.A.M. is founder of a xenotransplantation startup company, FIOS Therapeutics, LLC. All other authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

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Key Words: biological heart valve, immune injury, structural valve degeneration, sheep

BS

TABLE E1. Development rates following somatic cell nuclear transfer using *GGTA1*$^{-/-}$ fibroblast colonies

Colony ID/sex	Cell line	No. of embryos, transferred/recipients	Pregnancy rate, n/N (%)	Term rate, n/N (%)	No. of lambs alive at 1 mo
13/male	SFF4	30/2	2/2 (100)	2/2 (100)	2
16/male	SFF4	34/3	2/3 (66.7)	2/3 (66.7)	0
20/male	SFF4	43/3	0/3	0/3	0
23/male	SFF4	23/2	1/2 (50)	0/2	0
Total		130/10	5/10 (50)	4/10 (40)	2

The frequencies of pregnancies from somatic cell nuclear transfer (*SCNT*), pregnancies going to term, and the number of live born offspring in this study are similar to the results of larger SCNT cloning efforts to create cystic fibrosis *CFTR*$^{-/-}$ sheep[23] and for the broader experience in the field in other sheep and goat cloning models (see Polejaeva IA. Generation of genetically engineered livestock using somatic cell nuclear transfer. *Reproduction*. 2021;162:F11-F22. doi: 10.1530/REP-21-0072.2021).