

Original Article

Cloning and expression of porcine β 1,4 N-acetylgalactosaminyl transferase encoding a new xenoreactive antigen

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Abstract: Background: Xenograft rejection of pigs organs with an engineered mutation in the GGTA-1 gene (GTKO) remains a predominantly antibody mediated process which is directed to a variety of non-Gal protein and carbohydrate antigens. We previously used an expression library screening strategy to identify six porcine endothelial cell cDNAs which encode pig antigens that bind to IgG induced after pig-to-primate cardiac xenotransplantation. One of these gene products was a glycosyltransferase with homology to the bovine β 1,4 N-acetylgalactosaminyltransferase (B4GALNT2). We now characterize the porcine B4GALNT2 gene sequence, genomic organization, expression, and functional significance.

Methods: The porcine B4GALNT2 cDNA was recovered from the original library isolate, subcloned, sequenced, and used to identify a bacterial artificial chromosome (BAC) containing the entire B4GALNT2 locus from the Children's Hospital Oakland Research Institute BAC-PAC Resource Centre (#AC173453). PCR primers were designed to map the intron/exon genomic organization in the BAC clone. A stable human embryonic kidney (HEK) cell line expressing porcine B4GALNT2 (HEK-B4T) was produced. Expression of porcine B4GALNT2 in HEK-B4T cells was characterized by immune staining and siRNA transfection. The effects of B4GALNT2 expression in HEK-B4T cells was measured by flow cytometry and complement mediated lysis. Antibody binding to HEK and HEK-B4T cells was used to detect an induced antibody response to the B4GALNT2 produced glycan and the results were compared to GTKO PAEC specific non-Gal antibody induction. Expression of porcine B4GALNT2 in pig cells and tissues was measured by qualitative and quantitative real time reverse transcriptase PCR and by *Dolichos biflorus* agglutinin (DBA) tissue staining. **Results:** The porcine B4GALNT2 gene shares a conserved genomic organization and encodes an open reading frame with 76 and 70% amino acid identity to the human and murine B4GALNT2 genes, respectively. The B4GALNT2 gene is expressed in porcine endothelial cells and shows a broadly distributed expression pattern. Expression of porcine B4GALNT2 in human HEK cells (HEK-B4T) results in increased binding of antibody to the B4GALNT2 enzyme, and increased reactivity with anti-Sd^a and DBA. HEK-B4T cells show increased sensitivity to complement mediated lysis when challenged with serum from primates after pig to primate cardiac xenotransplantation. In GTKO and GTKO:CD55 cardiac xenotransplantation recipients there is a significant correlation between the induction of a non-Gal antibody, measured using GTKO PAECs, and the induction of antibodies which preferentially bind to HEK-B4T cells.

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Abbreviations: B4GALNT2, β 1,4 N-acetylgalactosaminyltransferase; BLAST, Basic local alignment search tool; DBA, *Dolichos biflorus* agglutinin; Gal, galactose α 1,3, galactose; GalNAc, N-acetylgalactosamine; GTKO, pigs with a GGTA-1 α -galactosyltransferase mutation; HEK, human embryonic kidney cells; HEK-B4T, HEK cells expressing the porcine B4GALNT2 gene; HUVEC, Human umbilical vein endothelial cells; Mvwf-1, modifier of von Willebrand factor-1 mutation; Neu5GC, N-acetylneuraminic acid; PAEC, porcine aortic endothelial cell; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcriptase dependent polymerase chain reaction; Sd^a, The SID blood group expressing a GalNAc β 1-4[Neu5Ac α 2-3]Gal β 1-4GlcNAc β 1-3Gal terminal glycan.

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Conclusion: The functional isolation of the porcine B4GALNT2 gene from a PAEC expression library, the pattern of B4GALNT2 gene expression and its sensitization of HEK-B4T cells to antibody binding and complement mediated lysis indicates that the enzymatic activity of porcine B4GALNT2 produces a new immunogenic non-Gal glycan which contributes in part to the non-Gal immune response detected after pig-to-baboon cardiac xenotransplantation.

Introduction

Carbohydrate modifications on glycoproteins and glycolipids are involved in a wide array of biological processes including protein stability, development, and cell growth. Variations in carbohydrate modification between individuals and between species define a form of immune self-recognition. Differences in expression of ABH blood group antigens between individuals has long been recognized as a prohibitive boundary across which blood transfusion and organ transplantation is generally not performed. Individuals which do not express a particular ABH antigen are stimulated by intestinal microflora to produce antibody to that glycan. This preformed antibody can induce a hemolytic reaction or promote hyperacute or accelerated allograft rejection of ABH incompatible grafts. The same process occurs across species. Humans and Old World primates do not produce oligosaccharides with terminal galactose α 1,3, galactose (α Gal), whereas all other mammals synthesize terminal α Gal glycans [1]. As a consequence, humans make high levels of anti-Gal antibody. Anti-Gal antibody is a major immune barrier to xenotransplantation [2]. This antibody has also been implicated in other clinical pathologies, notably the accelerated calcification of glutaraldehyde-fixed porcine bioprosthetic material and degeneration of bioprosthetic replacement heart valves [3,4].

Targeted genetic modification has been used to mutate the α -galactosyltransferase (GGTA-1) gene of the pig (GTKO) [5–7]. This mutation eliminates the enzyme function and, when homozygous, blocks the synthesis of terminal α Gal moieties on glycoproteins [8–11]. Porcine GTKO tissue does not bind anti-Gal antibody and transplantation of GTKO cells, tissue and organs does not induce a specific anti-Gal antibody response. Elimination of this antigen has not eliminated GTKO xenograft rejection which remains a predominantly antibody-mediated process now directed to non-Gal antigens [12,13]. We have used proteomic analysis of porcine aortic endothelial cell (PAEC) membranes and expression library screening of PAEC

cDNA libraries to identify immunogenic non-Gal target antigens that may contribute to xenograft rejection [14,15]. Using sera, obtained after pig-to-baboon cardiac xenotransplantation, these studies identified 43 potential non-Gal target antigens including six cDNAs, isolated from PAEC expression libraries. When expressed on human embryonic kidney 293 cells (HEK) these cDNAs each produce a porcine antigen that binds to baboon non-Gal IgG. A BLAST search identified one of these recovered cDNAs as a porcine glycosyltransferase with homology to a predicted *Bos Taurus* β 1,4 N-acetylgalactosaminyltransferase (B4GALNT2) sequence [14]. In humans and mice the B4GALNT2 gene catalyzes the terminal addition of N-acetylgalactosamine to a sialic acid modified lactosamine to produce GalNAc β 1-4[Neu5Ac α 2-3]Gal β 1-4GlcNAc β 1-3Gal, the Sd^a (Sid blood group, also known as CAD or CT) blood group antigen [16,17]. In this study, we further characterize the porcine B4GALNT2 coding sequence, genomic organization, expression, and functional significance.

Materials and methods

Cells, culture conditions, and transfection methods

Porcine aortic endothelial cells (PAECs) were isolated from GTKO A- and O-type blood group pig aortas as previously described [18] and cultured in EGM media (Lonza Inc., Allendale, NJ, USA). Porcine peripheral blood mononuclear cells (PBMCs) were isolated from GTKO pig blood by density gradient centrifugation using Ficoll-Hypaque. Human embryonic kidney 293 cells (HEK) and HEK cells expressing the porcine B4GALNT2 cDNA were grown in DMEM media with 10% FBS at 37 °C in a 5% CO₂ incubator. The full length pig B4GALNT2 open reading frame was amplified from the original library isolated clone using a primers set containing a Kozak consensus sequence and in-frame translation stop signals, respectively (Forward: 5'- ACCATGACTTCGTA CAGCCCTAG-3', Reverse: 5'- CAGATACCTTA GGTGGCACATTGGAG-3'). The PCR product

was inserted into pcDNA3.1/V5-His TOPO expression vector (Life Technologies, Paisley, UK) and transfected into HEK cells using Lipofectamine-2000 (Life Technologies). A stable G418 resistant HEK clone expressing the porcine B4GALNT2 genes (HEK-B4T) was established and used for further real-time RT-PCR, immunohistochemical staining, complement dependent cytotoxicity, and flow cytometry analysis.

siRNA isolation and transfection

B4GALNT2 specific siRNA and control siRNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were generated from the coding region of each gene using a BLOCK-iT Dicer RNAi Kit (Life Technologies) according to the manufacturer's instruction. RNAi primers for B4GALNT2 were: Forward: 5'-ATGACTTCGTACAGCCCTAG-3' and Reverse: 5'-CAGATACCTTAGGTGGCACATTGGAG-3'. RNAi primers for GAPDH were Forward: 5'-AGGTGAAGGTCGGAGTCAAC-3' and Reverse: 5'-AGTGGTTCGTTGAGGCAATG-3'. The diced siRNA was purified through a RNA Spin Cartridge (Life Technologies). B4GALNT2 and GAPDH diced siRNAs were transfected into HEK-B4T cells using Lipofectamine-2000 (Life Technologies). After 48 h, total RNA was extracted from cells using RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA, USA). B4GALNT2 expression was monitored by real-time RT-PCR and immunohistochemical staining.

Real-time and qualitative RT-PCR analysis of B4GALNT2 expression

Total RNA was extracted from cultured cells using RNeasy Plus Mini kit (Qiagen Inc.). Porcine tissue RNA was extracted using 1 g of frozen pig tissue homogenized in 10 ml cold STAT-60 RNA (Tel-Test Inc., Friendswood, TX, USA) and processed as recommended. For both qualitative and quantitative reverse transcriptase PCR (RT-PCR) RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

To survey the expression of porcine B4GALNT2 a gene specific one-step reverse transcriptase PCR (RT-PCR) assay using 0.5 μ g of total tissue RNA and primers for pig B4GALNT2 (Forward: TACAGCCCTAGATGTCTGTC in exon 1, Reverse: CTCTCCTCTGAAAGTGTTCGAG in exon 3) was used to amplify a 334 bp product. Primers for beta-actin (Forward: CAAGATCATCGCGCCTC CA exon 6, Reverse: ACTCCTGCTTGCTGAT CCACATCT, exon 6) produce a 108 bp product

which was used as a loading control. The authenticity of each amplified PCR product was confirmed by sequencing. Gene specific amplification was performed in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) using one-step RT-PCR reaction (USB-Affymetrix, Santa Clara, CA, USA) with reverse transcription performed at 50 °C for 30 min. Amplification conditions consisted of 95 °C for 10 min, and 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Amplification products were run in a 1.5% agarose gel.

For quantitative real-time analysis (Figs 3C and 4B) QuantiTect SYBR Green one-step RT-PCR was used (Qiagen Inc.) according to the manufacturer's instruction. The cycling conditions were as follows: 50 °C for 30 min for reverse transcriptase, 95 °C for 15 min, and 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The B4GALNT2 primers for quantitative real time PCR (Fig. 3C) were: rt-Forward: 5'-GCGACTCCAAA-GAATTGGCTTC-3' (exon 10) and rt-Reverse 5'-TGGTGACCTATGATCACGTGTG-3'(exon 11) which produces a 120 bp RT-PCR product. A normalized dCT was calculated using β -actin expression and the relative change in B4GALNT2 expression, ddCT, was calculated based on B4GALNT2 expression in the absence of siRNA [19]. Pig and PAEC AO blood group determination was performed as described [20].

Cell staining and flow cytometry

Fluorescein isothiocyanate conjugated *Dolichos biflorus* agglutinin (FITC-DBA; Vector Labs, Burlingame, CA, USA) and an anti-Sd^a antibody KM694 (kindly provided by Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) were used to detect the glycan products of porcine B4GALNT2 expression in cultured human cells. Cells (2.5×10^5) in FACS buffer (phosphate buffered saline (PBS) with 1% bovine serum albumin) were stained with 1 to 5 μ g/ml KM694 or 10 μ g/ml of FITC-DBA for 45 min at 4 °C. Cells stained with KM694 were washed in FACS buffer and subsequently stained with a FITC conjugated goat anti-mouse IgM (2 μ g/ml Southern Biotech, Birmingham, AL, USA). All cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA, USA) cytometer and CellQuest software.

Immunohistochemical staining of HEK and HEK-B4T cells with rabbit anti-human B4GALNT2 (Sigma-Aldrich, Saint Louis, MO, USA) was used to assess B4GALNT2 protein expression after siRNA transfection. Cells were fixed in cold methanol, washed with PBS containing 0.1% Triton

X-100 and blocked with 10% non-immune serum. Cells were incubated with rabbit anti-human B4GALNT2 in FACS buffer (1 : 75 dilution) for 2 h and washed with PBS. Anti-B4GALNT2 binding was detected with an anti-rabbit diaminobenzidine kit (ZymedLife Technologies, Paisley, UK) as recommended by the manufacturer.

Induced antibody directed to the B4GALNT2 produced porcine glycan after pig-to-baboon heterotopic GTKO or GTKO:CD55 cardiac xenotransplantation was measured by comparing differential antibody binding to HEK and HEK-B4T cells. Cells (2.5×10^5) were stained with pre-transplant, and post-explant serial serum dilutions (1 : 5 to 1 : 40) at 4 °C for 45 min in FACS buffer. After washing, bound antibody was detected using a phycoerythrin conjugated goat anti-human IgM or IgG secondary. Induction of antibody specific for the B4GALNT2 produced glycans was estimated as the ratio of induced antibody binding to HEK-B4T cells compared to HEK cells (anti-B4GALNT2 glycan = MFI post explant (HEK-B4T)/pretransplant (HEK-B4T): MFI post-explant (HEK)/pretransplant (HEK)). Post-explant induction of non-Gal antibody was measured using GTKO PAECs as previously described using serum samples from earlier GTKO or GTKO:CD55 heterotopic cardiac transplants [11] or transplant recipients subject to similar immune suppression. The induced antibody binding data were used to calculate a Spearman rank correlation coefficient for IgM and IgG specific reactivity to HEK-B4T cells and GTKO PAECs. A two tailed non-directed P-value < 0.05 was considered significant. In Table 2, the induced HEK-B4T antibody response is represented in a semi-quantitative scale with ratios <2 scored as negative, 2.0 to 2.5 +, 2.5 to 5.0 ++, 5.0 to 10.0 +++, and greater than 10 ++++.

Complement-mediated lysis

Human embryonic kidney and HEK-B4T cells (2×10^5) were incubated with a dilution of heat inactivated baboon serum at 4 °C for 45 min, washed with PBS, and resuspended in 20% fresh rabbit complement (Sigma-Aldrich) at 37 °C for 1 h. Cells were stained with propidium iodide (3 µg/ml) placed on ice and analyzed on a FACS Calibur flow cytometer. Samples were analyzed in triplicate and the average proportion of propidium iodide stained cells is reported. Baboon serum came from previously described pig to primate cardiac xenograft recipients [14,21]. Serum heat inactivation was performed at 56 °C for 30 min using a temperature controlled water bath.

DBA lectin staining of pig tissues

Fresh pig tissues were embedded and snap frozen in optimal cutting temperature material (OCT, Miles Laboratories, Elkhart, IN, USA). Sections (8 µm) were cut, air dried, and acetone fixed prior to staining. Sections were cleared of OCT by washing in distilled water and PBS, blocked with FACS buffer and stained with FITC-DBA (4 µg/ml) in FACS buffer at 4 °C overnight, washed with PBS and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Stained sections were photographed using a Leica DMI4000B fluorescent microscope (Leica Microsystems, Buffalo Grove, IL, USA).

Results

Sequence analysis and genomic organization

Expression screening of a porcine aortic endothelial cell (PAEC) library using IgG induced after pig-to-baboon cardiac xenotransplantation isolated a porcine cDNA with 54 base pairs of 5' untranslated sequence, a 1509 base pair open reading frame and 459 bp of 3' untranslated sequence including a putative polyadenylation sequence (Fig. 1A, GenBank accession: KF501048). A BLAST search identified this cDNA as a porcine glycosyltransferase with homology to known or predicted β 1,4 N-acetylgalactosaminyltransferase (B4GALNT2) products from several mammalian species including the human and mouse B4GALNT2 proteins. A comparison of these protein sequences shows the porcine open reading frame to have 76 and 70% amino acid identity to human and murine B4GALNT2 proteins, respectively (Fig. 1B). In contrast amino acid identity to the related human and murine B4GALNT1 protein is <50% (data not shown). Analysis of the porcine protein sequence with the NCBI Conserved Domain Database [22] identified a putative GT-A type fold (underlined). This region includes a conserved structural motif (SQVTTKYVLWVDDDF), present in both human and murine B4GALNT2, which contains an acidic divalent cation binding site (DXD) commonly found in glycosyltransferases which use a UDP-sugar as the donor substrate [23].

To map the genomic organization, the porcine B4GALNT2 cDNA was used to identify a porcine BAC clone obtained from the Children's Hospital Oakland Research Institute BACPAC Resource Centre (#AC173453). Based on the intron/exon structure of human and mouse B4GALNT2 and the porcine genomic sequences derived from the *Sus scrofa* working draft of AC173453.2, clone

Porcine B4GALNT2 encodes a new xenoreactive antigen

A

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aatacgcggagtggtgaccaccagccagaaatggtccgagggcggcgggattaag
ATGACTTCGTACAGCCCTAGATGTCTGTGATCCTCAAGAATATGATGGTCTTTTGGTCTGAGCGTTGACTCTTTAT134
GTTCCAAAGCGTGTTCCTCGATACAGACTCAGTCTCCTCACTCACCCATCCCCTCCACCCCTGGATCGCAGACGC160
TAAAGCTTCTACCTGAGAAAACCCGATTTCTACGGTGAAAAACGGGCTGTTCGAAAAAACAGTGCCCAATGTGACGCTTC240
GGGCATCAGGAAAGCTATACTTGGAGGATGCCTACGACCAGCAAGACCTCCCCGCAGTAACTGAGGAGACAGGCTGA320
GCTCGAACACCTTTCAGAGGAGAGAAGGGCTCCCTCGCCCAACGGCCCTGCTGGCTCAGCCCAACTCCCCCTTTGGGTACC400
CGGTCACCGGGTGAAGTATGCCTCTACACACATCCCATCCCAGGCTCCCGGTTGAAAGACCTGATGCTCCCATC480
TATGAGGTCAACCTGACAGCTTCTCTGGGGACACTGAAACGGCTGTGACGCTCCAGACAATGTGGTGAAGGCGAGAG560
CCAGAAGCAGCTGAACATTTGACCAGTAGCCGGGAGCTTTGAATTTCACTCCAGCATGTGACATACACGAGCACAG640
AGTACCACCTCCACAGAGTGATGTGGTGAATGTGGAGTCAAGTCTCTCATGTGGCCAAGTTCCAGTGACATCCCGCTAT720
CCTGTCTATGCCAAGTTATGACCTTGGACAGAGAGGAGTCCGAGAGCTGGTGGTACCATTGCCACCAAACTTCTCT800
CCGTCCCGCAAGTCAATGACATGCTCCGAGTGTCTGCTGACTACTACAGACCTGACGGTGATCGTGGGATGACA880
GCAAGGAGCCCTGAAAATCCTGACAGCCACGTGGAGTATACACCATGCCATTTGGGAAGGGCTGGTTGCTGGCAGG960
AACCTGGCCAATCTCAGGTCAACACAAAATATGTGCTCTGGGTGGACGATGACTTCATCTAACACGACAGACAGGAT1040
CGAGGCGCTGCGGACGCTCTAGAAAAACGAACTGGAGTGGTAGGTGCAGCGTGATGAAAACACATTCAGTTCA1120
AGCTGTCTGAGGACGAGGAAAGATGGGCTCCACTGAGTGAACAGCAAGATTTTCCGGCCCTGGGATGCTTCCCC1200
GACTGCGTGGTACCAGTGGTGTGTCTGCTTCTCTGCTCACACAGAGGACTCCAAAGAATTGGCTCGACCCCG1280
GCTGACGAGTGGCTCACTCAGAGTCTTATGATGGCTCGGGAGCTGCTCGTGGGTCCTGCCCAACAGTGATCA1360
TAGGTACCAACCCCATTTAAGTGTGGTACCCAGAGTGGCCACCCTGAGGGGAACACACAGTTATCGGGCCA1440
ACCGAAGCCAGATCAATGCAAGTGGCTCTCCACTACTTCTCAATGCCACCTAGGCCACCTAAGtatctgggc1520
attgaaaagcgctgagctgctggttgaagtatttaagtcagcggatcggtggctggataccaatattgaactcc1600
tcataaaataagcattgtaaagcccaggagcagggttaggcaggtggcttgactccgtactggaagtaacaataaaag1680
tacagggtcattaaaatgaccagtcactgaggtgggacatggagactcattcataacgattacggcgaggtttccat1760
ccgtgctcagagtagcaagccagactgctatcccaagagatcggagttgatccctggccttgctcagtggtcaaga1840
tctggcattgctgtgctgtggcataggctggcagctgctctgtatgccccctagctgggaactccagatgcta1920
agtgtggggccacaaaaaaaataaaaaaaaataaaaaaaaataaatgt1968

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B

Pig	MTS---YSPRCLSILKILMVLVLSVGLFMFQSVFLDITDFSLNLSPIPSPTLDAQTLKLLPEK--PDFY	64
Human	MTS---GSRFLWLLKILVILVLGIVGFMFGSMFLQAVFSSPKPELPSAPGVQKLLKLLPEERLRNLF	66
Mouse	MTSSVSFASFRFPWLLKTFVLMVGLATVAFMVRKVSITDFSTFKPKFPEPARVDPVLKLLPEEHLRKL	70
	*** * . ** . . . * ** . * . ** * * . ***** .	
Pig	GENG--LFSKNQCQDAFGHQESYNLEDAYDPQDLPAVNLRRQAELEHFQRREGLPRPPLLAQPNLFPF	132
Human	SYDGIWLFPNKQCKEANKEQGGYNFQDAYGQSDLPVAVKARRQAEFEHFQRREGLPRPLPLLVQPNLFPF	136
Mouse	TYSDIWLFPKNQCDNCSGKLRMKYKFQDAYNQKDLPAVNARRQAEFEHFQRREGLPRPPLLAPPNLPF	140
	** **** * . * . **** ***** ***** ***** ***** *****	
Pig	YPVHGVEVMPHLTIPIPLRFEGDPAPIYEVTLTASLGLTALADVPDNNVVRGRGQKLNILTSRELLN	202
Human	YPVHGVEVMPHLTVPIPLQFEGDPAPVYEVTLTASLGLTALADVPDVSVVQGRGQQLIISTSDRKLK	206
Mouse	YPVHGVEVMPHLTILIPGLQYEGDPAPVYEVILKASLGLTALADVPDDEVQGRGQRLTISTRHRKVLN	210
	***** . ***** . ***** . ***** . ***** . ***** . ***** . *****	
Pig	FILQHVTYTSLEYHLHRVDVVSLESKSSVAKFPVTIRYPVMPKLYDPGPERKLRDLVTIATKTFLRPHKL	272
Human	FILQHVTYTSSTGYHQKVDIVSLESRSSVAKFPVTIRHPVLPKLYDPGPERKLRNLVTIATKTFLRPHKL	276
Mouse	FILQHVTYTSLEYLHKVDVSMYEYSSVAKFPVTIKQQTVPKLYDPGPERKLRNLVTIATKTFLRPHKL	280
	***** * . ** ** * ***** . ***** . ***** . ***** . *****	
Pig	MTMLRSVREYYPDLTVIVADDSKEPLKIDTSHVEYYTMPFGKGFAGRNLAISQVTTKYVLVWDDDFL	342
Human	MIMLRSIREYYPDLTVIVADDSQKPLEIKDNHVEYYTMPFGKGFAGRNLAISQVTTKYVLVWDDDFL	346
Mouse	KILLQSIKRYYPDLTVIVADDSKEPLEINDDYVEYYTMPFGKGFAGRNLAISQVTTKYVLVWDDDFL	350
	* . * . * **** . ***** . * * * * ***** . ***** . ***** . ***** . *	
Pig	SKTRIEALADVLEKTELDVVGGSVIENTFQFKLLEQKNGDCLHQQPGFFRVPDGFPCVVTSGVVSFF	412
Human	EETKIEVLVDVLEKTELDVVGGSVLGNVQFKLLEQSENGACLHKRMGFQPLDGFPCVVTSGVNVFF	416
Mouse	DKTKIEVLVDVLEKTELDVVGGSVQGNFYQFRLLYEQTNGSCLHQRWGFSQALDGFPCVVTSGVNVFF	420
	* . * * ***** . * . * . *	
Pig	LAHTERLQRIGFDRPRLQVAHSEFFIDGLGSLLVGSCPHVIIGHQPHLPVMDPELATLEGNYTSYRANTE	482
Human	LAHTERLQRRVGFDRPRLQVAHSEFFIDGLGTLVGVSCPEVIIGHQSRSPVVDSELALEKTYNTYRSNTL	486
Mouse	LAHTEQLRRVGFDPILQVAHGEFFIDGLGRLLVGVSCPGVIVINHQRVTPPKDPKLALEKTYDKYRANTN	490
	***** . * . * . *	
Pig	AQIKFKLALHYFKNYLQCATN	503
Human	TRVQFKLALHYFKNHLQCAANRC	509
Mouse	SVIQFKVALQYFKNHLYCSTN	511
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Fig. 1. Sequence and homology comparison of porcine B4GALNT2. (A) Sequence of the porcine B4GALNT2 cDNA (GenBank: KF501048). Numbering begins with the start codon. Uppercase letters are the protein coding sequences, lower case letter are untranslated sequences. (B) Amino acid homology comparison between porcine, human and murine B4GALNT2 protein sequences. An asterisk indicates a shared amino acid identity, a line indicates a conserved amino acid substitution and a space indicates a non-conserved amino acid residue. The underlined region represents a conserved GT-A fold [22] and the boxed region highlights the conserved divalent cation binding domain [23].

RP44-32E24, we generated a series of primers (Table 1) to confirm the intron exon structure of the porcine B4GALNT2 gene (Fig. 2A). These primers amplified exons 1 to 11, including variable lengths of neighboring intron sequence, and amplified introns 5 to 8 and 10. All PCR products were sequenced and compared to the original B4GALNT2 cDNA to identify intron-exon junction sequences (Fig. 2B). The cDNA coding region is composed of 11 exons spread across approximately 40 000 base pairs. Exon 1 contains translation initiation start site and 5' untranslated sequences. We have not directly mapped the transcriptional start site of the gene so there may be additional 5' untranslated sequence or an upstream exon(s) not depicted in this figure. During the course of this study, a sequence for porcine B4GALNT2 99% identical to the clone we reported was added to the database (NM_001244330).

Expression of porcine B4GALNT2 in human HEK cells

A stable G418 resistant HEK cell line expressing the porcine B4GALNT2 cDNA (HEK-B4T) was produced. This cell line bound higher levels of rabbit anti-human B4GALNT2 antibody (Fig. 3A)

Table 1. Primers for mapping the porcine B4GALNT2 genomic structure

No.	ID	Sequence	BP to exon	Site
1	Exon1-F	AAGGCAGGACCTAGAAGGTG	90	flank exon 1
2	Exon1-R	AACGAGCAGGAACCTCTCAAC	185	flank exon 1
3	Exon2-F	GCTGTGCAAAAGCTGTGTCAGTTTGC	186	flank exon 2
4	Exon2-R	CACAGTAAAGCCACAGGAGGAG	41	flank exon 2
5	Exon3-F	CCCAATCTGTGATCTTTGAC	24	flank exon 3
6	Exon3-R	GGAATGAGTAGAGAGCTTCC	41	flank exon 3
7	Exon4-F	ATCTGGCATGGTCAGTGCATTG	145	flank exon 4
8	Exon4-R	CAGTGGTGGAAACAGTGAG	150	flank exon 4
9	Exon5-F	AACTCTGGCATCCTCTC	51	flank exon 5
10	Exon5-R	TGAGACCAGCCACCATCTCA	73	flank exon 5
11	Exon7-F	AGTGACCCAGATACCGTG	29	flank exon 6
12	Exon7-R	CTAAGGATCCAGTGTGTGCAC	224	flank exon 6
13	Exon8-F	TCCACAACACTCGAGACAG	84	flank exon 7
14	Exon8-R	TGGGTAGTACTCAGAACACTC	n/a	in exon 8
15	Exon9-F	AGAGAGTCCCTCTCCTTCTTC	152	flank exon 9
16	Exon9-R	TCAGATGGCAATGGGGTAGAAC	22	flank exon 9
17	Exon10-F	ACAGGCTCCTTGGATATGGAG	36	flank exon 10
18	Exon10-R	TTGACAACACTGGTCACCAC	n/a	in exon 10
19	Intron5-F	GTTTGAAAGACCTGATGCTC	n/a	in exon 5
20	Intron5-R	TGTTCACTGTCCCCAGAGAAG	n/a	in exon 6
21	Intron6-F	ACACGAGCACAGAGTACCACC	n/a	in exon 6
22	Intron6-R	AACTTGGCCACTGAGGACTTG	n/a	in exon 7
23	Intron8-F	GACAGCCACGTGGAGTATTAC	n/a	in exon 8
24	Intron8-R	TGAGATATGGCCAGGTTTCTG	n/a	in exon 9
25	Intron10-F	GCGACTCAAAGAATTGGCTTC	n/a	in exon 10
26	Intron10-R	TGGTGACCTATGATCACGTGTG	n/a	in exon 11

The table list the names, sequences, and location of primers used to confirm the genomic organization of the porcine B4GALNT2 gene. Primer numbers (No.) correspond to values in Fig. 2A. Not applicable, n/a.

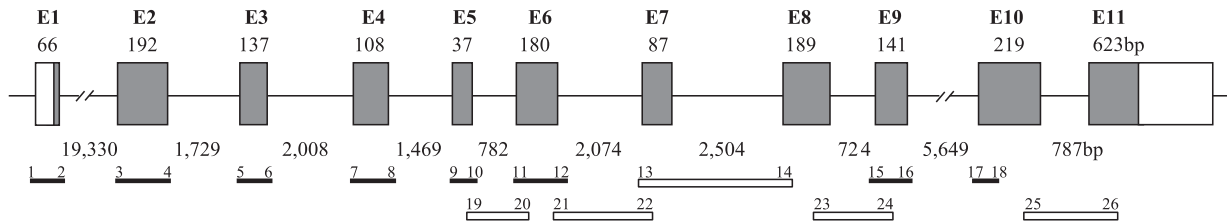
compared with control HEK cells. The increased antibody binding was specific for expression of porcine B4GALNT2 mRNA as HEK-B4T cells transfected with siRNA to porcine B4GALNT2 exhibit an 80% decrease in porcine B4GALNT2 mRNA expression and a proportionate reduction in anti-human B4GALNT2 antibody binding (Fig. 3B,C). No change in anti-B4GALNT2 staining or expression of B4GALNT2 mRNA was evident when cells were transfected with siRNA for GAPDH.

The human and mouse B4GALNT2 gene produces GalNAc β 1-4[Neu5Ac α 2-3]Gal β 1-4GlcNAc β 1-3Gal, the Sd^a blood group glycan, by the addition of a β 1,4 N-acetyl galactosamine to a sialic acid modified lactosamine acceptor. HEK cells, but not HUVECs, bind a low level of anti-Sd^a hybridoma KM694 (Fig. 3D). The HEK-B4T cell line shows increased binding of KM694 consistent with increased synthesis of the Sd^a antigen as a result of expression of porcine B4GALNT2. The *Dolichos biflorus* agglutinin (DBA) binds alpha linked terminal GalNAc structures [24], but also binds beta GalNAc residues as presented in the Sd^a glycan. The DBA lectin has been used to isolate the Sd^a pentasaccharide from murine small intestine [25], shows differential binding to humans Sd^{a+} and Sd^{a-} glycoproteins [26], and is commonly used to detect the Sd^a antigen on cells and in tissue sections [17,27,28]. HEK-B4T cells bind high levels of DBA and are strongly agglutinated by this lectin (Fig. 3E). Expression of porcine B4GALNT2 in HEK-B4T cells increases cell sensitivity to complement mediated cytotoxicity (Fig. 3F). HEK-B4T cells show a 20-fold enhancement of antibody-dependent complement-mediated lysis compared to HEK cells when challenged with pig-to-baboon cardiac xenotransplantation sensitized recipient serum.

Immunogenicity of B4GALNT2 produced antigens

Heterotopic GTKO or GTKO:CD55 cardiac xenograft recipients show variable induction of IgG and IgM binding to GTKO PAECs (Table 2). Comparing pretransplant and post explant serum (obtained from immune suppressed recipients 1 to 3 weeks after organ recovery) three recipients (survival 28, 27, and 22 days) showed a clear 2- to 8-fold increase in non-Gal antibody and four recipients (survival 71, 31, 21, and 18 days) showed a minimal non-Gal antibody response. We used differential antibody binding to HEK-B4T and HEK cells to determine if this induced non-Gal antibody response included antibody reactivity specific to the glycans expressed on the HEK-B4T cell

A



B

Exon	Splice donor	Intron	Splice acceptor	Exon
E1	ATGACTTCGTAC	aggtgagtggtcc...ttgtgtcccaac	AGCCCTAGATGT	E2
E2	GGTGAAAACGGG	tgagatggcaag...tcttatttttagg	CTGTTCCCGAAA	E3
E3	CTTTCAGAGGAG	gtattgcagatg...gtttccctgcag	AGAAGGGCTCCC	E4
E4	CCCCATCCCAGG	taggtgcacatctc...gtgacctccagg	CCTCCGGTTTGA	E5
E5	CCCATCTATGAG	gcaagagtcccc...cctttatcctag	GTCACCCTGACA	E6
E6	AGAGTGGATGTG	ggtgagtcgcgc...tttctccctcca	GTGAGTCTGGAG	E7
E7	GACCCTGGACCA	ggtaagaccctc...ctttcttgccca	GAGAGGAAGCTC	E8
E8	CCATTTGGGAAG	gtatatctcctcc...ctgcctcgcag	GGCTGGTTTGCT	E9
E9	GAAGTGGACGTG	gtaagtgagctg...cccgtggtctag	GTAGGTGGCAGC	E10
E10	GTGGCTCACTCA	cgtggggaggct...cctctcttgcca	GAGTTCTTTATT	E11

Fig. 2. Schematic representation of the intron-exon organization (A) and splice junction sequences (B) of the porcine B4GALNT2 gene. (A) Shaded boxes represent exon coding sequences numbered E1–E11. Open boxes correspond to untranslated sequences present in the cDNA. The size of each exon is indicated in base pairs above each box and the intron distances are indicated below between each exon. The primer pairs (1–26, see Table 1) and amplified products used to analyze the genomic structure from a BAC clone are illustrated below the genomic structure as solid bars for products covering exon/intron boundaries and open bars for products spanning entire introns. The primer numbering corresponds to the primers listed in Table 1. (B) The proximal splice donor and distal splice acceptor nucleotide sequences (upper case) for each exon are shown. Adjacent intervening intron sequences (lower case) are shown. Exon numbering corresponds to (A).

surface. The induction of specific post transplant IgM ($r = 0.8929$) and IgG ($r = 0.8571$) reactivity to HEK-B4T cells was significantly correlated with the induced non-Gal antibody response to GTKO PAECs (Table 2). Recipients with a clear induced antibody response to GTKO PAECs also showed increased antibody reactivity for HEK-B4T cells. The antibody (IgG) reactivity to HEK-B4T cells in sensitized baboon serum can be blocked by immune absorption with GTKO PAECs (Fig. 4A) but is unaffected by immune absorption using human umbilical vein endothelial cells (HUVECs) (Fig. 4B). This suggests that the induced xenoreactive antibody response in baboons includes antibody directed to glycan antigens present on both HEK-B4T and GTKO PAECs, but not present on HUVECs which are negative for B4GALNT2 expression and the Sd^a antigen (Fig. 3D).

Expression of B4GALNT2 in porcine tissue

A panel of pig tissues was assessed for B4GALNT2 expression by qualitative RT-PCR. Expression

of B4GALNT2 RNA was evident in cultured PAECs, peripheral blood mononuclear cells and at variable levels in most GT⁺ porcine tissues (Fig. 5A). Expression of B4GALNT2 RNA was not strongly affected by the GGTA-1 Gal genotype as heterozygous (GGTA-1^{+/-}) and GTKO pig tissues exhibited a similar pattern of B4GALNT2 expression (data not shown). Quantitative real time RT-PCR analysis of B4GALNT2 gene expression in cultured GT⁺ and GTKO PAECs of the A- and O-type blood groups showed only moderate variation in B4GALNT2 expression (Fig. 5B). Expression of porcine B4GALNT2 in HEK-B4T cells resulted in increased DBA and KM694 antibody binding in HEK-B4T cells (Fig. 3D,E), however, PAECs show only strong DBA agglutination (Fig. 5C) and do not stain with the anti-Sd^a antibody KM694 (Fig. 5D). DBA staining of GTKO pig tissues (O-type blood group) was observed in vascular endothelial cells of the heart, in glomeruli and larger blood vessels in the kidney and by the reticuloendothelial cells of the liver (Fig. 5E–G).

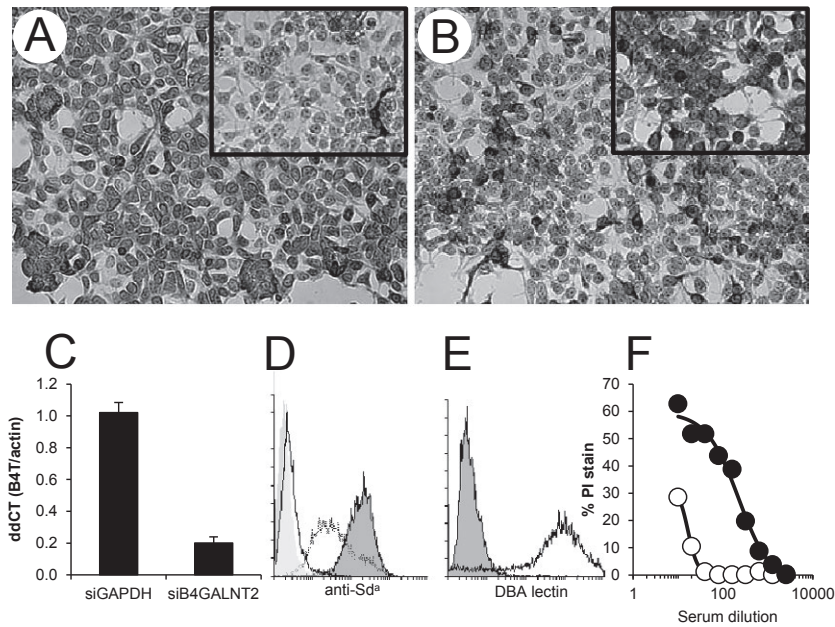


Fig. 3. Expression of B4GALNT2 protein and synthesis of the Sd^a antigen on HEK-B4T cells. (A) HEK-B4T cells show increased anti-human B4GALNT2 staining compared to HEK cells (insert). (B) Staining is dependent on porcine B4GALNT2 mRNA expression as HEK-B4T cells transfected with porcine B4GALNT2 siRNA show decreased staining compared to cell transfected with control GAPDH siRNA (insert in B). (C) Quantitative real-time RT-PCR analysis of B4GALNT2 RNA in HEK-B4T cells after transfection with control (siGAPDH) or porcine specific (siB4GALNT2) siRNA. Gene expression was normalized to pig beta-actin (dCT) and expressed as a ratio to B4GALNT2 expression in HEK-B4T cells without siRNA transfection (ddCT). Error bars are standard error of the mean. (D) Flow cytometry detection of anti-Sd^a antibody (KM694) binding to HEK (dotted line) and HEK-B4T cells (black line filled). Background is secondary antibody only binding to HEK cells (filled no line). KM694 binding to HUVECs (solid line no fill) is negative. (E) FITC conjugated *Dolichos biflorus* agglutinin (DBA) staining of HEK-B4T cells lines (black line) and control HEK cells (filled). (F) Complement-dependent cytotoxicity of HEK (open) and HEK-B4T (filled) cells challenged with primate serum after pig-to-primate xenotransplantation.

Table 2. Differential immune response to HEK-B4T cells after cardiac xenotransplantation

Donor	Survival (days)	HEK-B4T IgM	HEK-B4T IgG	GTKO PAEC IgM	GTKO PAEC IgG
GTKO:CD55	71	–	+	0.5	0.6
GTKO:CD55	31	–	–	0.5	0.4
GTKO:CD55	28	+++	+++	4.8	2.5
GTKO:CD55	27	+++	+	2.2	8.5
GTKO	22	+++	++++	1.9	7.3
GTKO	21	–	–	1.3	0.9
GTKO:CD55	18	–	–	0.4	0.4

Table shows the specific induced antibody response in pig-to-baboon heterotopic cardiac xenograft recipients directed to the B4GALNT2 (HEK-B4T) glycan or to GTKO PAECs. Antibody reactivity to HEK-B4T cells was estimated as described in the Materials and Methods. There was a positive and significant ($P < 0.05$) Spearman rank correlation coefficient between the anti-PAEC and anti-HEK-B4T IgM ($r = 0.8929$) and IgG ($r = 0.8571$) immune response.

Discussion

The porcine B4GALNT2 cDNA was originally isolated during a flow cytometry based library screen to identify human HEK cells expressing porcine cDNAs which encoded surface membrane antigens that bound to sensitized primate IgG after GT⁺ or GTKO pig cardiac xenotransplantation

[14]. The porcine cDNA and its encoded protein show the highest homology to the B4GALNT2 gene with 76 and 70% amino acid identity with the human and mouse gene, respectively (Fig. 1). We show that the porcine gene also shares with human and mouse a conserved intron/exon genomic organization (Fig. 2). Consistent with a conserved protein sequence a stable HEK cell line expressing the porcine cDNA (HEK-B4T) binds increased levels of rabbit anti-human B4GALNT2 antisera and, using siRNA inhibition, we show this binding is dependent on expression of the porcine cDNA (Fig. 3B,C). Expression of the porcine cDNA in HEK-B4T cells also results in increased binding of anti-Sd^a antibody and DBA lectin. On this structural and functional data, we conclude that the isolated porcine cDNA encodes the porcine B4GALNT2 gene.

Porcine B4GALNT2 enzymatic activity has been reported from swine large intestine mucosal cells [29] and immunostaining of the Sd^a antigen has been detected in porcine primordial germ cells [30]. To our knowledge, this is the first molecular analysis of porcine B4GALNT2 gene expression in pig tissues. In the pig, we find B4GALNT2 mRNA

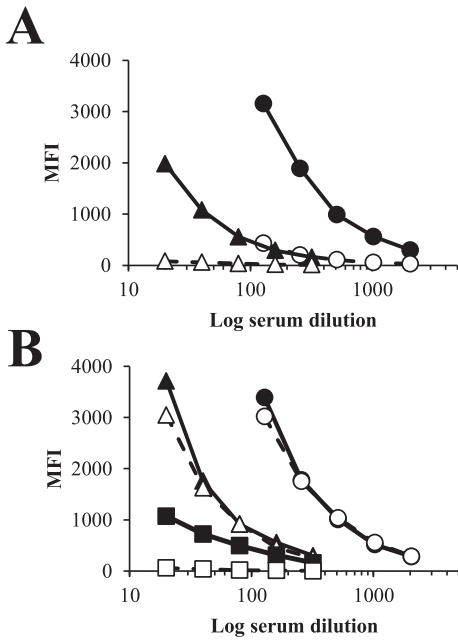


Fig. 4. Serum absorption of pig-to-baboon induced antibody to HEK-B4T (B4T) cells. Sensitized baboon serum was absorbed by incubating baboon serum (1 : 10 dilution) with 1×10^7 GTKO PAECs (A) or HUVECs (B) for 1 h at 4 °C. Absorption was performed 3 times for each cell type. IgG binding was analyzed by flow cytometry in dilutions from 1 : 20 to 20 : 48. (A) Closed symbols IgG binding from whole serum to GTKO PAECs (circle) and HEK-B4T cells (triangle). Open symbols are IgG binding after absorption with GTKO PAECs. Over the dilution range, absorption reduced IgG binding by an average of 89% for GTKO PAECs and 94% for HEK-B4T cells. (B) Closed symbols IgG binding from whole serum to GTKO PAECs (circle), HEK-B4T cells (triangle) and HUVEC (square). Open symbols are IgG binding after absorption with HUVECs. Absorption reduced HUVEC reactivity on average by 96%, had a minimal effect on IgG binding to GTKO PAECs (0.05%) and only a minor reduction of IgG binding to HEK-B4T (12%) cells.

expression in PAECs, peripheral blood mononuclear cells, and across a wide array of tissues (Fig. 5A). This porcine B4GALNT2 gene expression pattern is in distinct contrast to humans and most strains of mice where B4GALNT2 expression and the Sd^a antigen, generally detected by lectin staining, is largely restricted to gastrointestinal, skin and renal tissues and is notably absent from arteries, heart and skeletal muscle [23,27,31,32]. Vascular expression of B4GALNT2 is not unprecedented however as mice which carry the modifier of von Willebrand factor-1 (Mvwf1) mutation exhibit a shift from gastrointestinal epithelial cell expression to vascular endothelial cell expression of B4GALNT2 [27].

The B4GALNT2 cDNA was originally isolated by screening an expression library with post transplant IgG from pig-to-baboon cardiac heterotopic xenotransplantation recipients transplanted with-

out T-cell immune suppression [14,21]. In these recipients, the non-Gal immune response was especially strong and five of five transplant recipients showed an induced immune response with preferential binding to HEK-B4T cells [14]. In this study, we extend our previous results and show in recipients subject to substantial immune suppression that there is a correlation between an induced non-Gal antibody response and the induction of antibody with preferential binding to HEK-B4T cells (Table 2). We further show that expression of the porcine B4GALNT2 gene in HEK-B4T cells produced a 20-fold enhancement to complement mediated lysis (Fig. 3F). These results and the pattern of B4GALNT2 gene expression strongly indicate that the porcine B4GALNT2 enzyme produces an immunogenic non-Gal glycan on endothelial cells which contributes in part to the non-Gal immune response after pig-to-baboon cardiac xenotransplantation.

The precise structure of the glycan(s) produced on pig endothelial cells by the B4GALNT2 enzyme remains under investigation. Classically in humans and mice expression of B4GALNT2 produces the Sd^a antigen. Consistent with this we detect increased expression of the Sd^a antigen, based on KM694 antibody and DBA lectin binding, in HEK-B4T cells but surprisingly do not observe anti-Sd^a KM694 antibody binding to PAECs. It is unclear why this is the case. Ongoing glycan profiling of HEK and HEK-B4T cells suggests that the porcine B4GALNT2 enzyme may produce a wider variety of GalNAc glycans in HEK-B4T cells than normally attributed to the human or mouse enzyme (data not shown). Alternatively KM694 binding may be affected by the presentation of the Sd^a epitope on pig cells, possibly due to inclusion of N-glycolylneuraminic acid (Neu5GC) in the structure. In any case, our absorption experiments (Fig. 4) show that GTKO PAECs and HEK-B4T cells share B4GALNT2 dependent antigens which are not present on HUVECs. Interestingly, an induced antibody response in baboons to trace acidic cardiac glycolipids has been reported [8]. Whether this antibody response is related to the glycan produced by B4GALNT2 remains to be determined.

There are a limited set of potential antibody and glycan antigen combinations which may be involved in xenogeneic antibody dependent inflammatory processes. Antibody to the Gal antigen is the dominant xenoreactivity in both humans and Old World non-human primates [1]. Anti-Gal antibodies are known to be sufficient to induce organ rejection of Gal-positive vascular grafts [33–35] and to accelerate calcification of

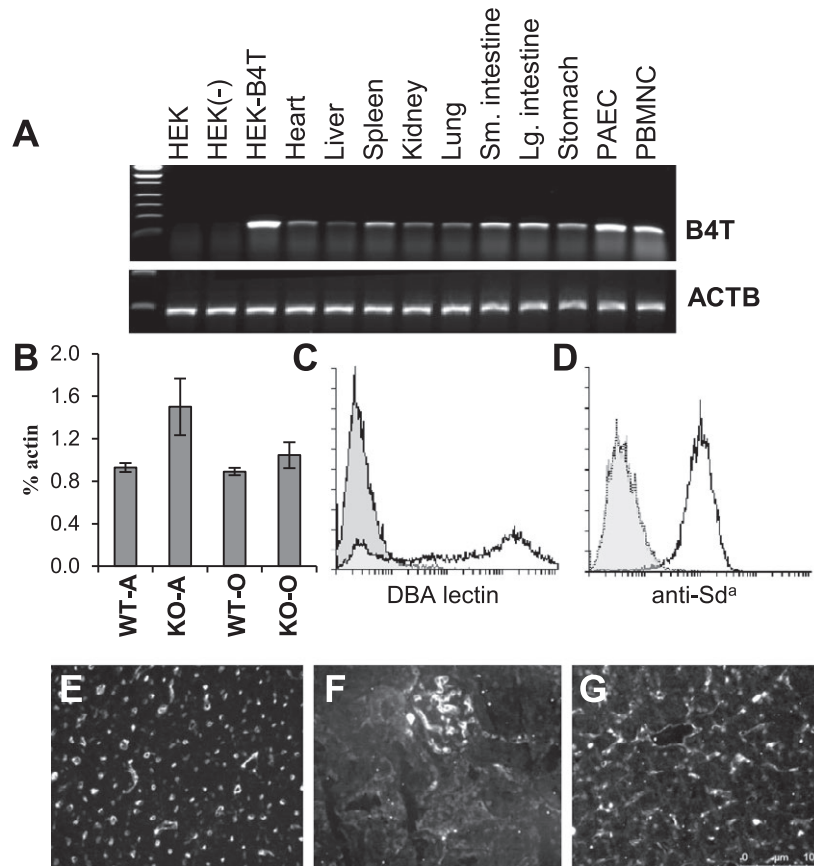


Fig. 5. Porcine B4GALNT2 gene expression. (A) Qualitative RT-PCR analysis of porcine B4GALNT2 RNA expression in cell lines and pig tissues. The top panel represents specific expression of B4GALNT2 (B4T) and the bottom panel shows expression of porcine actin (ACTB). Samples; Human embryonic kidney cells (HEK), HEK cells transformed with an unrelated porcine cDNA (HEK-), HEK cells expressing porcine B4GALNT2 (HEK-B4T), porcine tissue samples (heart, liver, spleen, kidney, lung, stomach, and small [Sm] and large [Lg] intestine), porcine aortic endothelial cells (PAEC) and porcine peripheral blood mononuclear cells (PBMC). (B) Quantitative real time RT-PCR analysis of porcine B4GALNT2 gene expression in Gal-positive wild type (WT) and Gal-negative (KO) endothelial cells. On the x-axis the A- and O-type blood group is indicated for each Gal genotype. B4GALNT2 expression is plotted as the average percentage of β actin expression. Error bars are the standard deviation. (C) Positive FITC-DBA staining (bold line) of O-type PAECs. Background (filled) staining with FITC conjugated *Solanum tuberosum* agglutinin which does not bind to PAECs. (D) Negative staining of O-type PAECs with KM694 (dotted line). Background (filled) is FITC conjugated anti-mouse IgM only staining of PAECs. Positive stain (solid line) is HEK-B4T stained with KM694. (E-G) FITC conjugated DBA lectin staining of O-type GTKO pig heart (E), kidney (F) and liver (G).

fixed bioprosthetic animal tissue [3,36]. Humans, but not nonhuman primates, also make a complex array of antibody to a common mammalian sialic acid modification Neu5GC [37]. This antibody reactivity is widely expected to contribute to xenograft rejection in humans [38–41] but its significance remains uncertain due to the absence of anti-Neu5GC antibody in experimental non-human primate models. Screening strategies based on panels of defined oligosaccharides have failed to detect xenogeneic sensitization to other common oligosaccharides (Forssman antigen and α - and β -lactosamine) [42,43]. The results presented in this study indicate that porcine B4GALNT2 produces an immunogenic non-Gal glycan which contributes to the induced non-Gal antibody response in the pig-to-baboon xenotrans-

plant model. It remains to be determined if antibody to this glycan contributes to xenograft rejection. Additionally, while most humans express low levels of anti-Sd^a antibody which agglutinates rare human red blood cells with high levels of Sd^a antigen (CAD or super Sd^a) [17], whether an anti-Sd^a immune response will occur in humans exposed to porcine tissues and the relationship between human anti-Sd^a antibody and the immune response in observed baboons remains to be determined.

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Author contribution

Guerard W. Byrne: Dr. Byrne contributed to the concept and design of the research, data acquisition, analysis and insight in interpreting the results. He was responsible for drafting and critical revision of the manuscript, contributed to securing funding, and gave final approval for the article.

Zeji Du: Dr. Du was responsible for data acquisition, analysis, and interpretation of the results. He contributed to the drafting of the manuscript and gave final approval.

Paul Stalboeger: Mr. Stalboeger was responsible for data acquisition, analysis, and interpretation of the results. He contributed to the drafting of the manuscript and gave final approval.

Heide Kogelberg: Dr. Kogelberg was responsible for data acquisition, analysis, and interpretation of the results. She contributed to the drafting of the manuscript and gave final approval.

Christopher G. A. McGregor: Dr. McGregor contributed to the concept and design of the research, was primarily responsible for securing funding to support this work, provided critical review and final approval of the article.

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