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Complete absence of the αGal xenoantigen and isoglobotrihexosylceramide in α1,3galactosyltransferase knock-out pigs

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Abstract

Background—Anti-Galα1,3Galβ-R natural antibodies are responsible for hyperacute rejection in pig-to-primate xenotransplantation. Although the generation of pigs lacking the α1,3galactosyltransferase (GalT) has overcome hyperacute rejection, antibody-mediated rejection is still a problem. It is possible that other enzymes synthesize antigens similar to Galα1,3Gal epitopes that are recognized by xenoreactive antibodies. The glycosphingolipid isoglobotrihexosylceramide (iGb_3) represents such a candidate expressing an alternative Galα1,3Gal epitope. The present work determined whether the terminal Galα1,3Gal disaccharide is completely absent in Immerge pigs lacking the GalT using several different highly sensitive methods.

Methods—The expression of Galα1,3Gal was evaluated using a panel of antibodies and lectins by flow cytometry and fluorescent microscopy; GalT activity was detected by an enzymatic assay; and ion trap mass spectroscopy of neutral cellular membranes extracted from aortic endothelial was used for the detection of sugar structures. Finally, the presence of iGb_3 synthase mRNA was tested by RT-PCR in pig thymus, spleen, lymph node, kidney, lung, and liver tissue samples.

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Authors' contributions

GPY, JDS contributed to the concept/design of the article; GPY, YL, LB, ALM, MBK, DZ performed data analysis/interpretation; GPY drafted the article; and DZ, LB, ALM, JDS were involved in the critical revision of the article.

Conflict of interest

DZ is a consultant for BioTex, Houston, TX, and an inventor involved in patents related to technologies mentioned in this article, issued or in application.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Specificity of 4F10 monoclonal antibody determined by the Glycan-array assay.

Figure S2. Tandem Mass Spectrometry detects iGb3 from isomeric mixtures after multiple rounds of fragmentation that leads to characteristic ions.

Figure S3. Precursor of fucosylated lactosylceramide in pig aortic endothelial cell membranes

Table S1. Structures recognized by the different antibodies and lectins used in the study.

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Results—Aortic endothelial cells derived from GalT knockout pigs expressed neither Galα1,3Gal nor iGb_3 on their surface, and GalT enzymatic activity was also absent. Lectin staining showed an increase in the blood group H-type sugar structures present in GalT knockout cells as compared to wild-type pig aortic endothelial cells (PAEC). Mass spectroscopic analysis did not reveal Galα1,3Gal in membranes of GalT knockout PAEC; iGb_3 was also totally absent, whereas a fucosylated form of iGb_3 was detected at low levels in both pig aortic endothelial cell extracts. Isoglobotrihexosylceramide 3 synthase mRNA was expressed in all pig tissues tested whether derived from wild-type or GalT knockout animals.

Conclusions—These results confirm unequivocally the absence of terminal Galα1,3Gal disaccharides in GalT knockout endothelial cells. Future work will have to focus on other mechanisms responsible for xenograft rejection, in particular non-Galα1,3Gal antibodies and cellular responses.

Keywords

α1; 3galactosyltransferase; isoglobotrihexosylceramide; knockout pigs; transplantation; xenoantigen

Introduction

Natural antibodies (Ab) are responsible for hyperacute rejection in pig-to-primate xenotransplantation [1,2]. The main target of these natural Ab is the disaccharide Galα1,3Galβ-R (αGal), which is mainly synthesized by α1,3galactosyltransferase (GalT) [3], an enzyme found in all mammals, except in higher primates and humans [4]. The generation of pigs with disrupted GalT by knocking out the GalT gene (GalT KO) has overcome the hurdle of hyperacute rejection [5,6]. However, after the generation of GalT KO animals, there was a controversy about the complete elimination of αGal epitopes coming from positive staining for αGal epitope in GalT KO and the discovery of a new enzyme able to add αGal to lipids [7]. A possible explanation for the reported residual expression of αGal on GalT KO pigs is provided by the findings in GalT KO mice and rats of a second enzyme able to synthesize αGal, the isoglobotrihexosylceramide synthase (iGb_3S) [8,9]. The latter belongs to the family of ABO-blood group glycosyltransferases and initiates the synthesis of isoglobo-series of glycosphingolipids [10]. In contrast to GalT, iGb_3S uses the common precursor lactosylceramide (LacCer) as substrate [9,10], suggesting the possibility of alternative pathways for the synthesis of αGal epitopes and, therefore, their expression in GalT KO pigs. The aim of the present work was to confirm the absence of the terminal Galα1,3Gal disaccharide in GalT KO pigs in comprehensive methodological approach using simultaneously different reagents, including a nearly complete panel of available monoclonal anti-Gal antibodies, and highly sensitive techniques such as ion trap mass spectroscopy (MS) to evaluate αGal expression, a novel enzymatic assay for GalT, and RT-PCR for iGb_3 synthase.

Material and methods

Cell lines and tissues

Porcine aortic endothelial cells isolated from wildtype (PAEC WT) and PAEC derived from GalT knockout pigs (PAEC GalT KO) [11] were kindly provided by R.J. Hawley (former Immerge Biotherapeutics, Cambridge, MA, USA). Thymus, spleen, lymph node, kidney, lung, and liver tissues from both WT and GalT KO miniature swine were kindly provided by D. Sachs and J. Hanekamp (Massachusetts General Hospital, Boston, MA, USA) [11]. Two different samples from each organ were obtained from a WT animal (#16517, female, AA haplotype, 2.5 yr old) and a GalT KO animal (#16183, female, DD haplotype, 3.5 yr old),

respectively. Tissue samples were snap frozen in a RNA stabilization solution and stored at −80 °C until RNA extraction and mRNA analysis. The human embryonic kidney cell line E293 transfected with rat GalT was a kind gift from M.S. Sandrin (University of Melbourne Department of Surgery, Austin Health, Heidelberg, Victoria, Australia). All cell lines were cultured as previously reported [12,13].

Anti-αGal and H-type structures reagents

For the detection of αGal epitopes on the cell surface, the following antibodies and lectins were used: (i) human polyclonal anti-αGal antibodies previously generated by affinity purification and characterized in our laboratory [14]; (ii) mouse monoclonal Ab (mAb) anti-αGal IgM M86 (Alexis Corporation, Lausen, Switzerland); (iii) FITC-conjugated isolectin B4 from *Bandeiraea simplicifolia* (BS-IB4; Sigma, Buchs, Switzerland); (iv) four different murine mAb: 15.101, 25.2, 24.7, and 22.121, which were a kind gift from M.S. Sandrin [13,15] and thoroughly described previously [15]; (v) two different clones of murine mAb clones GT4-31 and GT6-27, which were a kind gift from A.S. Chong (University of Chicago, Chicago, IL, USA) [16]; and the murine mAb 4F10 that was a gift of A. Bendelac (Howard Hughes Medical Institute and University of Chicago, Chicago, IL, USA). Additionally, the FITC-conjugated *Ulex europaeus* (UEA-I) lectin (Sigma) was used for flow cytometry.

Flow cytometry

Surface expression of the αGal epitope and blood group H-type structures on PAEC WT and GalT KO cells was analyzed using a FACSCanto (Becton Dickinson, Basel, Switzerland) by direct and indirect immunofluorescence using the above-mentioned reagents. Cells were incubated for 30 min at 4 °C with saturating amounts of these reagents. Goat anti-mouse IgM/IgG FITC-conjugated (BD-Pharmingen, Basel, Switzerland) was used as a secondary Ab and the following isotype-matched mAb as controls: mouse IgG1 (Sigma); mouse IgG3; and mouse IgM (BD-Pharmingen). For lectin staining, cells were incubated for 15 min at 4 °C. Propidium iodide staining was used to exclude dead cells. Data were analyzed with FlowJo® (TreeStar Inc, Ashland, OR, USA). To compare the levels of surface expression, the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity of each sample with the mean fluorescence intensity of the isotype control mAb or staining buffer-only in the case of lectins.

Fluorescent microscopy

Both WT and GalT KO PAEC were grown in 96-well plates (Milian, Geneva, Switzerland) coated with bovine fibronectin (Sigma-Aldrich, Buchs, Switzerland). After 24 h, the monolayers were fixed with a cold acetone–ethanol solution (1: 2) for 10 min or with 80% acetone for 5 min at room temperature, respectively. Then, the monolayers were incubated with 50 μl of a buffer containing the different anti-αGal for 30 min at 37 °C; washed three times with PBS; and incubated at 37 °C for 30 min with 50 μl of buffer containing the respective secondary antibody. Finally, nuclei were counterstained with DAPI. After extensive rinsing, coverslips were mounted on glass slides using Fluokeep (Argene, Varilhes, France) and analyzed with a fluorescent IX71 microscope (Olympus, Hamburg, Germany).

Detection of iGb_3 by multistep ion trap mass spectroscopy

A total of 10^8 cells were used to isolate neutral glycolipid membrane fractions of WT and GalT KO PAEC, which were permethylated as described by Li et al. [17]. The molecular ion profiles were obtained by linear ion trap mass spectrometry using the electrospray ionization mass spectroscopic method (LTQ-ESI-MS) as described [18]. Briefly, the iGb_3 in isobaric

mixture of Gb_3 (Galα1,4Lac-Cer) and iGb_3 standards was identified by comparing the different patterns of MS^4 product ions from the sodiated molecular ions *via* the glycan fragment *m/z* 667 and the terminal disaccharide 1-ene ion *m/z* 445 (i.e., X → 667 → 445 →) of pure permethylated iGb_3 standards *via* ESI-LIT-MS with those of permethylated Gb_3. Characteristic fragment ions at MS^4, 211, and 371 were used as evidence for the presence of iGb_3.

α1,3galactosyltransferase assay

The GalT enzymatic assay was performed as described elsewhere [19–22] with slight modifications. Briefly, fresh cell extracts containing 1% Triton X-100 were prepared from PAEC WT, GalT KO, and E293 cells; and protein concentrations were determined with a BCA protein assay reagent (Pierce Chemical Co, Rockford, IL, USA). A typical reaction mixture contained 100 mM Tris (pH 7.0), 20 mM $MnCl_2$, 101 μM UDP-Gal ([sim] 5000 cpm/nmol), mixture of UDP-[U-^{14}C]Gal (Amersham Biosciences, Arlington Heights, IL, USA) and UDP-Gal (Calbiochem, Darmstadt, Germany), 500 mg asialofetuin, and 150 μg of protein stemming from the cell lysates. Controls without acceptor were assayed in parallel under the same conditions. After different incubation times at 37 °C, the enzymatic reaction was stopped by adding ice-cold 5% phosphotungstic acid. The precipitates were collected by suction filtration over GFA Whatman glass-microfiber filters (Millipore, Zug, Switzerland), washed with absolute alcohol, and dried. Finally, ^{14}C-radioactivity was quantified in a Tri-Carb 2900TR liquid scintillation counter (Packard, Pangbourne, UK).

RT-PCR

Total RNA was isolated using Trizol (Invitrogen, Basel, Switzerland) and treated with DNase (Qiagen, Hombrechtikon, Switzerland) during RNA extraction to avoid possible genomic DNA contaminations. Before use, RNA quality was controlled on a 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Reverse transcription was achieved using ImProm II Reverse Transcriptase (Promega, Wallisellen, Switzerland) following the manufacturers protocol. Different sets of primers for pig iGb_3S were tested, three primers already published: 5′ GGCGCTGGCAGGAC 3′, 5′ CGGCCAGCGGTAGTG 3′, 5′ CAGTGCGCCGTCAG 3′, named K2, K3, and K4, respectively [23], and the primers 5′ TCTTAGGGCTGCTCCTGTTTGG 3′ and 5′ AATGGTGAGGTTCTGCTGGGTAG 3′, named 67 and 68 correspondently, which were designed based on the iGb_3S sequence reported in the published patents WO 2002081688 and WO 2005047469, respectively. Pig β2 microglobulin was used as housekeeping gene. All PCR products were visualized by agarose gel electrophoresis and cloned into a pGEM Easy vector (Promega) for further sequence analysis by using the flanking cloning site primers T7 and SP6. The sequence analysis was performed by Microsynth according to standards operating procedure (Microsynth, Balgach, Switzerland).

Results

Evaluation of αGal surface expression on pig endothelial cells

We first tested whether αGal was detectable on the cell surface of GalT KO PAEC using two different lectins and a panel of mAb directed against αGal. In addition, polyclonal human anti-αGal Abs were used as well as two mAb able to recognize iGb_3 and the shared Galα1,3Gal disaccharide xenoantigen. The mAb 4F10, which recognizes both αGal and iGb_3 (Fig. S1), unquestionably bound to WT but not to GalT KO PAEC, with a MFIR of 47.5 and 0.9, respectively (Fig. 1A). Similar results were obtained with the mAb 15.101, which has been reported to bind iGb_3 and globotriaosylceramide (Gb_3) [24], with a MFIR of 9.8 and 1.0 for WT and GalT KO PAEC, respectively (Fig. 1B). In addition, staining with M86; 25.2; 24.7; 22.121; GT4-31; and GT6-27 mAb, as well as polyclonal anti-αGal Ab

isolated from human serum and the BSI-B4 lectin, confirmed the lack of αGal expression on GalT KO PAEC (Fig. 1C–I).

Staining of PAEC with UEA-I lectin that has a high affinity for L-fucose structures showed that GalT KO PAEC express higher levels of fucose structures in comparison with WT PAEC (MFIR, 16.8 vs. 3.0, respectively, Fig. 1J). To further determine the cellular distribution of the αGal epitope, fluorescent microscopic analysis was performed using the 4F10 and M86 mAb on PAEC cultured in 96-flat well plates. Figure 2 shows that there were no traces of αGal in GalT KO PAEC, whereas a clear and uniform labeling of cell PAEC WT staining was detected, thus confirming our flow cytometry results. In summary, the αGal epitope was not detected by antibody or lectin binding in GalT KO PAEC, neither by flow cytometry nor by fluorescent microscopy using a panel of seven different mAb, polyclonal anti-αGal Ab, and two lectins.

Mass spectroscopy analysis of αGal or iGb_3 in cellular membranes of GalT KO PAEC

Next, we applied a highly sensitive MS technique to search for the presence of αGal carbohydrate structures in cellular membranes derived from PAEC. The mass spectroscopy 1 (MS^1) analysis of neutral glycolipid fractions stemming from WT PAEC showed the presence of molecular ions located at 1664; 1748; and 1774 *m/z*, which correspond to fragments belonging to the terminal Galα1,3Gal disaccharide (specifically, Galα1,3Galβ1,4GlcβNAcβ1,3Galβ1,4Glc-Cer) (Fig. 3A). On the contrary, these ions were totally absent in GalT KO PAEC (Fig. 3B). In addition, the molecular ions of Gb_4 (1460; 1486; 1514; 1544; and 1570 *m/z*); the Gb_3 (1215; 1299, and 1325 *m/z*); and the precursor of iGb_3, LacCer (1012 and 1120 *m/z*) were present in WT and GalT KO PAEC (Fig. 3). Because differences between Gb_3 and iGb_3 cannot be detected by MS^1, sequential "breakdown" of the MS^1 ions was performed by ion trap MS^4 analysis (Fig. S2) [18] confirming that only Gb_3 is present in both WT and GalT KO cells (data not shown). Additionally, we found the presence of a fucosylated-iGb_3 structure in both WT and GalT KO PAEC, using the 841 *m/z* fragment by precursor ion mapping method (Fig. 4). However, the exact position of fucose in the tetrasaccharideceramide structure remains to be determined. Candidate structures are Galα1, 3(Fucα1,2)Galβ1,4Glc-Cer, or Fucα1,2Galα1, 3Galβ1, 4Glc-Cer. In addition, the precursor of the fucosylated- iGb_3 (fucosylated LacCer) was found in WT PAEC (Fig. S3). Taken together, only WT PAEC cellular membranes express αGal bound to lipids, whereas Gb_3 and its precursor LacCer are present in both WT and GalT KO PAEC, iGb_3 is absent. A fucosylated-iGb_3-type of structure was found in WT and GalT KO PAEC, but the position of fucose remains undetermined due its low abundance.

α1,3galactosyltransferase activity in pig endothelial cell lysates

Hitherto, analysis of the GalT enzymatic activity in cells derived from GalT KO pigs has not been reported. Theoretically, other enzymes originally unable to compete against GalT for UDP-Gal in WT cells could be active in GalT KO cells. Thus, we analyzed the enzymatic activity in freshly prepared cell lysates from WT and GalT KO PAEC by measuring the transfer of ^{14}C-labeled UDP-Gal residues into the acceptor molecule asialofetuin. As shown in Table 1, a minimum (0.0018 mU/g), if not null, enzymatic activity was found in GalT KO PAEC lysates after 1 h, and this activity stayed the same during the 3 h of observation. On the contrary, WT PAEC clearly produced a labeled adduct at 1 h (0.0263 mU/g), which increased over time (0.0364 mU/g). Used as positive control, human E293 cells transfected with rat GalT, also showed an increase in αGal-asialofetuin formation over time, although the enzymatic activity detected was lower (0.0031 and 0.0061 mU/g at 1 and 3 h, respectively) than in WT PAEC lysates. Finally, 0.7 μg of commercially available purified human βGal1,4GalT with an enzymatic activity of 250 pmol/min/μg was tested under the

same conditions to analyze whether βGal1,4GalT enzymatic activity could account for the results obtained in the above-mentioned assays. We detected 1.03 nmol of UDP-Gal/1 h under saturating conditions, while the expected activity of β1,4GalT was nearly 10 times higher (10.5 nmol/h/0.7 μg). In conclusion, GalT enzymatic activity was detected in WT PAEC and not in GalT KO PAEC.

Levels of isoglobotrihexosylceramide synthase mRNA in WT and GalT KO pigs

As we could not find iGb_3 in WT and GalT KO PAEC, we wondered whether the enzyme that catalyzes the production of iGb_3 is actually expressed in pig cells. To this purpose, the mRNA levels of iGb_3S were analyzed not only in PAEC but also in different tissues derived from WT and GalT KO animals. We used three different sets of primers to detect iGb_3S: K2/K3, K2/K4, and 67/68, respectively (Fig. 5A). The products were inserted into the cloning site of the pGEM-T expression vector and sequenced. Whereas the K2/K4 and 67/68 amplified products presented homology to a predicted pig alpha-centractin and other DNA sequences present in pig chromosomes 4, 7, 17, and 18, respectively, only the K2/K3 amplified product matched the known sequence of *Sus scrofa* iGb_3S (patent WO 2005/047469). Due to the fact that the K2/K3 primer set spans exclusively exon 5 of the iGb_3S gene, negative controls without reverse transcriptase were always included to rule out genomic DNA contaminations. Both WT and GalT KO PAEC expressed iGb_3S mRNA. In accordance, all pig tissues tested expressed considerable amounts of mRNA for exon-5 iGb_3S regardless of their anatomical origin or their GalT status (Fig. 5B). Of note, kidney tissue showed the highest levels of iGb_3S mRNA, whereas liver tissue exhibited clearly lower levels of expression. No apparent differences were found between thymus and spleen iGb_3S mRNA expression. Although these results are not quantitative, β2 microglobulin mRNA was equivalent among all samples tested. In summary, iGb_3S mRNA was broadly expressed in all pig tissues tested whether derived from WT or GalT KO animals.

Discussion

The discovery of iGb_3S that is able to form terminal Galα1,3Gal disaccharides on lipids has been a source of discussion regarding the theoretical alternative expression of αGal epitopes on GalT KO pig cells [10]. This work simultaneously tested all available Ab anti-αGal including polyclonal human Ab, seven different mAb anti-αGal, and two lectins as well as highly sensitive MS techniques to confirm that αGal is not present in GalT KO PAEC, at least not in GalT KO pigs generated by Immerge [11]. Moreover, we report GalT enzymatic activity and iGb_3S gene transcription in WT and GalT KO cells and tissues.

None of the above-mentioned reagents used to determine αGal expression provided positive staining of GalT KO PAEC using flow cytometry and immunofluorescence microscopy, strongly suggesting that at least at this level of detection, there is no αGal expression on the surface of the Immerge GalT KO cells. Noteworthy, flow cytometry of WT PAEC using the 15.101 mAb yielded positive staining in our hands, whereas GalT KO PAEC were not stained by 15.101. This antibody was initially reported to be specific for iGb_3 using human embryonic kidney cells (E293) transfected with rat iGb_3S, and Chinese hamster ovary cells (CHO) transfected with mouse iGb_3S [8,13]. However, subsequently, the 15.101 mAb was demonstrated by Diswall et al. [25] to cross-react to Gb3 and to stain porcine WT intestinal cell extracts in thin-layer chromatography plates while GalT KO tissue remained negative. Taken together, these results suggest that controversial reports regarding the specificity of the 15.101 mAb might depend on discrepancies of the techniques used to demonstrate binding and that the 15.101 mAb might also bind to αGal. Hence, the data obtained with this mAb should be interpreted very carefully.

With regard to the other mAb tested, we did not have access to the mAb GT-21-1-G1.6 and GT-21-1-G1.8 that were reported to stain positive for αGal expression on pig GalT KO fibroblasts by Sharma et al. [26]. The latter mAb (GT-21-1-G1.6 and GT-21-1-G1.8) were subsequently shown to have a lower affinity for the αGal disaccharide in comparison with other IgG mAb developed by the same group, necessitating at least eight disaccharides conjugated to proteins for recognition [27]. However, we obtained the mAb GT4-31 and GT6-27 described in the same study, which both stained exclusively WT PAEC. Thus, to finally reconcile the discrepancy of the specificity of these mAb, we suggest them to be assayed by the "Consortium of Functional Glycomics" (http://www.functionalglycomics.org) to clarify whether they specifically recognize the αGal epitope or also other saccharide chains. In conclusion, it is important to mention that if there is no detectable antibody binding to pig-derived cells/tissues, the risk of antibody-mediated rejection in pig-to-primate xenotransplantation is expected to be negligible.

The glycosphingolipid iGb_3 has been described as a possible source of the αGal epitope in mice and rats [8,9]. It is synthesized by the enzyme iGb_3S using LacCer as precursor [28,29] and characterized by the presence of the αGal in the terminal part [10]. In the second part of our study, we attempted to demonstrate iGb_3 expression in WT and GalT KO PAEC using sophisticated MS techniques. The MS analysis was performed on the neutral membrane extracts of GalT KO PAEC because it is in this fraction where iGb3 can be found and not in the acidic fractions or membrane proteins as reported by others [30,31]. Nevertheless, the highly sensitive ion trap MS analysis identified the presence of Gb_3 at a fmol level [18] and confirmed the lack of the αGal epitope in the lipidic fraction of GalT KO PAEC. Furthermore, iGb_3 was neither detected in WT nor GalT KO PAEC using as much as 100 million cells for the analysis, despite the presence of its precursor LacCer. Similar findings were reported by Diswall et al. [32] analyzing heart and kidney material stemming from Immerge's GalT KO pigs by MS and proton nuclear magnetic resonance (NMR) where neither terminal αGal nor iGb_3 were found in the neutral lipidic fractions. In our analysis of the neutral glycosphingolipids membrane fractions, we detected αGal in the form of Galα1,3Galβ1,4GlcβNAcβ1,3-LacCer in WT, but not in GalT KO PAEC. Similar conclusions were reported after analyzing lipidic fractions of small intestine and pancreas of GalT KO Revivicor animals using thin-layer chromatography and proton NMR by Diswall et al. [33,34]. In conclusion, if there is any iGb_3 expression in WT and GalT KO PAEC, it escapes detection using highly sensitive methods and should, therefore, not be relevant for transplantation.

Regarding the iGb_3-fucosylated structure identified in both cells types, we do not know whether the fucose is terminal or not. From the original work in pig stomach mucosa [35] and later findings of Diswall et al. [33], we presume that Fuc-iGb_3 most likely corresponds to the glycolipid Fucα1,2Galα1,3Galβ1,4Glcβ1,1-Cer, which was also found in both WT and GalT KO pig intestine [33,35]. The fact that the 4F10 mAb does not recognize the Galα1,3(Fuc α1,2)Galβ1,4Glc glycans (Fig. S1) did not help us to directly define the precise position of the fucose, because the level of Fuc-iGb3 present in PAEC is under the detection limit for mAb determinations. On the other hand, the recognition of terminal fucosylated structures in PAEC by UEA-I lectin cannot be explained by the low abundance of the Fuc-iGb_3 (MS signal of 1389 *m/z*). The increased blood group H-type structures revealed by UEA-I staining in GalT KO PAEC has previously been reported and is possibly related to the unmasking of fucose epitopes in the absence of GalT [36]. An alternative pathway leading to a branched fucose epitope in which the fucose is attached to the internal galactose (Galα1,3(Fucα1,2)Galβ1,3Glc-Cer) is independent of the iGb_3 pathway. In this case, LacCer fucosylated by a fucosyltransferase I, a structure that was found in low abundance in WTPAEC membranes (Fig. S3) by MS, might serve as a substrate for additional transferases. In conclusion, the structural characterization of the fucosylated iGb_3

structure which we observed was incomplete, and we can only speculate that it might be synthesized by different fucosyltransferase enzymes such as Fut7 (EC 2.4.1.65) or Fut1 and/or Fut2 (EC 2.4.1.69).

Another point we addressed was the GalT enzymatic activity of PAEC. We used an enzymatic assay based on the incorporation of radioactive UDP-Gal residues into the asialofetuin acceptor substrate [20–22]. GalT acts on βgalactosyl1,4 *N*-acetylglucosaminyl termini, asialo- α1-acid glycoprotein, *N*-acetyllactosamine, and non-reducing terminal *N*-acetyllactosamine residues of glycoproteins but not on 2′-fucosylated-*N*-acetyllactosamine [37]. Our results clearly show that GalT activity is not present in cell lysates from GalT KO PAEC when asialofetuin was used as a substrate. As asialofetuin bears the terminal galactose (Gal β4GlcNAc) in *N*-glycans, we were assaying the α1,3GalT activity toward the terminal galactose. We think that the low and constant activity registered in PAEC WT is due to background because in the case of E293 transfected with rat GalT, the enzymatic activity doubled after 3 h, which is not the case for PAEC WT.

Finally, although we could not detect iGb_3 in the glycolipid neutral fractions by spectrometry, iGb_3S mRNA was present in all pig tissues and cells tested, regardless whether derived from WT or GalT KO animals. The possibility that iGb_3 was further converted into other elongated isoglobo-series glycosphingolipids, such as iGb_4, iGb_5 and iGb_6 glycosphingolipids [38], was excluded by mass spectrometry (data not shown). The mechanism responsible for the absence of the iGb_3S protein in the presence of its mRNA remains to be clarified. It is possible that the iGb_3S lacks a cofactor for enzymatic activity or that there are genetic differences between the source animals. Unpublished data from Christiansen et al. (M. Sandrin, personal communication) support the latter notion as they found differences in iGb_3S exon 5 sequences in different pig sources, revealing a stop codon in exon 5 of iGb_3S in Immerge's breed animals. Importantly, the primer set K2/K3 that proved iGb_3S mRNA expression is located before this stop codon. The discrepancy between our results and Kiernan et al. who did not detect iGb_3S by RT-PCR testing the same animal source material [23] might stem from the different amplification conditions used, in particular the annealing temperature that differed by 7 °C. Nevertheless, the sequence of our amplification products obtained with the K2/K3 primer pair matched the pig iGb_3S gene.

In summary, this work confirms previously published evidence and concludes the earlier controversy about the expression of the terminal αGal disaccharide on the cell surface of GalT KO animals. Neither αGal nor iGb_3 were detected in Immerge's GalT KO breed animals using a panel of different antibodies and a highly sensitive MS method. Most importantly, the lack of αGal antigen recognition by polyclonal human anti-Gal antibodies on GalT KO cells, not even at trace levels, indicates that antibody-mediated delayed xenograft rejection is not related to residual αGal expression. Thus, the xenoantigens responsible for delayed xenograft rejection need to be identified and addressed by novel preventive strategies [7,23,39–44].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

αGal	Galα1,3Gal disaccharide
Fuc	fucose
Gal	galactose
GalT	α1,3galactosyltransferase
Gb_3	globotrihexosylceramide 3 (Galα1,4-Galβ1,4Glcβ1-ceramide)
iGb_3	isoglobotrihexosylceramide 3 (Galα1,3- Galβ1,4Glcβ1-ceramide)
iGb_3S	isoglobotrihexosylceramide 3 synthase
LacCer	lactosylceramide (Galβ1,4Glcβ1-ceramide)
LTQ-ESI-MS	linear ion trap electrospray ionization mass spectroscopy
mAb	monoclonal antibody
MFIR	mean fluorescence intensity ratio
MS	mass spectroscopy
PAEC	pig aortic endothelial cells
RFU	relative fluorescence unit

References

1. Tai HC, Zhu X, Hara H, et al. The pig-to-primate immune response: relevance for xenotransplantation. Xenotransplantation. 2007; 14:227–235. [PubMed: 17489863]
2. Li S, Waer M, Billiau AD. Xenotransplantation: role of natural immunity. Transpl Immunol. 2009; 21:70–74. [PubMed: 18992342]
3. Macher BA, Galili U. The Galalpha1,3Galbeta1,4Glc-NAc-R (alpha-Gal) epitope: a carbohydrate of unique evolution and clinical relevance. Biochim Biophys Acta. 2008; 1780:75–88. [PubMed: 18047841]
4. Galili U, Shohet SB, Kobrin E, Stults CL, Macher BA. Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells. J Biol Chem. 1988; 263:17755–17762. [PubMed: 2460463]
5. Hisashi Y, Yamada K, Kuwaki K, et al. Rejection of cardiac xenografts transplanted from a 1,3-galactosyltransferase gene-knockout (GalT-KO) pigs to baboons. Am J Transplant. 2008; 8:2516–2526. [PubMed: 19032222]
6. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. Nat Med. 2005; 11:32–34. [PubMed: 15619627]
7. Puga YG, Schneider MK, Seebach JD. Immune responses to alpha1,3 galactosyltransferase knockout pigs. Curr Opin Organ Transplant. 2009; 14:154–160. [PubMed: 19300259]

8. Milland J, Christiansen D, Lazarus BD, Taylor SG, Xing PX, Sandrin MS. The molecular basis for galalpha(1,3)gal expression in animals with a deletion of the alpha1,3galactosyltransferase gene. J Immunol. 2006; 176:2448–2454. [PubMed: 16456004]
9. Taylor SG, Mckenzie IF, Sandrin MS. Characterization of the rat alpha(1,3)galactosyltransferase: evidence for two independent genes encoding glycosyltransferases that synthesize Galalpha(1,3)Gal by two separate glycosylation pathways. Glycobiology. 2003; 13:327–337. [PubMed: 12626403]
10. Keusch JJ, Manzella SM, Nyame KA, Cummings RD, Baenziger JU. Expression cloning of a new member of the ABO blood group glycosyltransferases, iGb3 synthase, that directs the synthesis of isoglobo-glycosphingolipids. J Biol Chem. 2000; 275:25308–25314. [PubMed: 10854427]
11. Kolber-Simonds D, Lai L, Watt SR, et al. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. Proc Natl Acad Sci USA. 2004; 101:7335–7340. [PubMed: 15123792]
12. Baumann BC, Schneider MK, Lilienfeld BG, et al. Endothelial cells derived from pigs lacking Gal alpha(1,3)Gal: no reduction of human leukocyte adhesion and natural killer cell cytotoxicity. Transplantation. 2005; 79:1067–1072. [PubMed: 15880045]
13. Milland J, Yuriev E, Xing PX, Mckenzie IF, Ramsland PA, Sandrin MS. Carbohydrate residues down-stream of the terminal Galalpha(1,3)Gal epitope modulate the specificity of xenoreactive antibodies. Immunol Cell Biol. 2007; 85:623–632. [PubMed: 17724458]
14. Baumann BC, Forte P, Hawley RJ, Rieben R, Schneider MK, Seebach JD. Lack of galactose-alpha-1,3-galactose expression on porcine endothelial cells prevents complement-induced lysis but not direct xenogeneic NK cytotoxicity. J Immunol. 2004; 172:6460–6467. [PubMed: 15128838]
15. Nozawa S, Xing PX, Wu GD, et al. Characteristics of immunoglobulin gene usage of the xenoantibody binding to gal-alpha(1,3)gal target antigens in the gal knockout mouse. Transplantation. 2001; 72:147–155. [PubMed: 11468550]
16. Yin D, Zeng H, Ma L, et al. Cutting Edge: NK cells mediate IgG1-dependent hyperacute rejection of xenografts. J Immunol. 2004; 172:7235–7238. [PubMed: 15187097]
17. Li Y, Zhou D, Xia C, Wang PG, Levery SB. Sensitive quantitation of isoglobotriaosylceramide in the presence of isobaric components using electrospray ionization-ion trap mass spectrometry. Glycobiology. 2008; 18:166–176. [PubMed: 18048405]
18. Li Y, Teneberg S, Thapa P, Bendelac A, Levery SB, Zhou D. Sensitive detection of isoglobo and globo series tetraglycosylceramides in human thymus by ion trap mass spectrometry. Glycobiology. 2008; 18:158–165. [PubMed: 18056651]
19. Malissard M, Borsig L, Di Marco S, et al. Recombinant soluble beta-1,4-galactosyltransferases expressed in Saccharomyces cerevisiae. Purification, characterization and comparison with human enzyme. Eur J Biochem. 1996; 239:340–348. [PubMed: 8706738]
20. Borsig L, Katopodis AG, Bowen BR, Berger EG. Trafficking and localization studies of recombinant alpha1, 3-fucosyltransferase VI stably expressed in CHO cells. Glycobiology. 1998; 8:259–268. [PubMed: 9451035]
21. Latemple DC, Henion TR, Anaraki F, Galili U. Synthesis of alpha-galactosyl epitopes by recombinant alpha1,3galactosyl transferase for opsonization of human tumor cell vaccines by anti-galactose. Cancer Res. 1996; 56:3069–3074. [PubMed: 8674064]
22. Leventhal JR, Sun J, Zhang J, et al. Evidence that tilapia islets do not express alpha-(1,3)gal: implications for islet xenotransplantation. Xenotransplantation. 2004; 11:276–283. [PubMed: 15099208]
23. Kiernan K, Harnden I, Gunthart M, Gregory C, Meisner J, Kearns-Jonker M. The anti-non-gal xenoantibody response to xenoantigens on gal knockout pig cells is encoded by a restricted number of germline progenitors. Am J Transplant. 2008; 8:1829–1839. [PubMed: 18671678]
24. Christiansen D, Milland J, Mouhtouris E, et al. Humans lack iGb3 due to the absence of functional iGb3-synthase: implications for NKT cell development and transplantation. PLoS Biol. 2008; 6:e172. [PubMed: 18630988]
25. Diswall M, Gustafsson A, Holgersson J, Sandrin MS, Breimer ME. Antigen-binding specificity of anti-alphaGal reagents determined by solid-phase glycolipid-binding assays. A complete lack of alphaGal glycolipid reactivity in alpha1,3GalT-KO pig small intestine. Xenotransplantation. 2011; 18:28–39. [PubMed: 21342285]

26. Sharma A, Naziruddin B, Cui C, et al. Pig cells that lack the gene for alpha1-3 galactosyltransferase express low levels of the gal antigen. Transplantation. 2003; 75:430–436. [PubMed: 12605105]
27. Xu H, Yin D, Naziruddin B, et al. The in vitro and in vivo effects of anti-galactose antibodies on endothelial cell activation and xenograft rejection. J Immunol. 2003; 170:1531–1539. [PubMed: 12538718]
28. Heissigerova H, Breton C, Moravcova J, Imberty A. Molecular modeling of glycosyltransferases involved in the biosynthesis of blood group A, blood group B, Forssman, and iGb3 antigens and their interaction with substrates. Glycobiology. 2003; 13:377–386. [PubMed: 12626391]
29. Li Y, Thapa P, Hawke D, et al. Immunologic glycosphingolipidomics and NKT cell development in mouse thymus. J Proteome Res. 2009; 8:2740–2751. [PubMed: 19284783]
30. Kim YG, Gil GC, Harvey DJ, Kim BG. Structural analysis of alpha-Gal and new non-Gal carbohydrate epitopes from specific pathogen-free miniature pig kidney. Proteomics. 2008; 8:2596–2610. [PubMed: 18546155]
31. Kim YG, Gil GC, Jang KS, et al. Qualitative and quantitative comparison of N-glycans between pig endothelial and islet cells by high-performance liquid chromatography and mass spectrometry-based strategy. J Mass Spectrom. 2009; 44:1087–1104. [PubMed: 19373860]
32. Diswall M, Angstrom J, Karlsson H, et al. Structural characterization of alpha1,3-galactosyltransferase knockout pig heart and kidney glycolipids and their reactivity with human and baboon antibodies. Xenotransplantation. 2010; 17:48–60. [PubMed: 20149188]
33. Diswall M, Angstrom J, Schuurman HJ, Dor FJ, Rydberg L, Breimer ME. Studies on glycolipid antigens in small intestine and pancreas from alpha1,3-galactosyltransferase knockout miniature swine. Transplantation. 2007; 84:1348–1356. [PubMed: 18049121]
34. Diswall M, Angstrom J, Schuurman HJ, Dor FJ, Rydberg L, Breimer ME. Glycolipid studies in small intestine and pancreas of alpha1,3-galactosyltransferase knockout miniature swine: alpha1,3GALT-KO animals lack alphaGAL antigens and contain novel blood group H compounds. Transplant Proc. 2008; 40:543–546. [PubMed: 18374124]
35. Slomiany BL, Slomiany A, Horowitz MI. Characterization of blood-group-H-active ceramide tetrasaccharide from hog-stomach mucosa. Eur J Biochem. 1974; 43:161–165. [PubMed: 4365813]
36. Hara H, Long C, Lin YJ, et al. In vitro investigation of pig cells for resistance to human antibody-mediated rejection. Transpl Int. 2008; 21:1163–1174. [PubMed: 18764834]
37. Scheer M, Grote A, Chang A, et al. BRENDA, the enzyme information system in 2011. Nucleic Acids Res. 2011; 39(Database issue):D670–D676. [PubMed: 21062828]
38. Zhou D, Levery SB. Response to Milland et al: carbohydrate residues downstream of the terminal Galalpha(1,3)Gal epitope modulate the specificity of xenoreactive antibodies. Immunol Cell Biol. 2008; 86:631–632. author reply 633–634. [PubMed: 18779838]
39. Baumann BC, Stussi G, Huggel K, Rieben R, Seebach JD. Reactivity of human natural antibodies to endothelial cells from Galalpha(1,3)Gal-deficient pigs. Transplantation. 2007; 83:193–201. [PubMed: 17264816]
40. Byrne GW, Stalboerger PG, Davila E, et al. Proteomic identification of non-Gal antibody targets after pig-to-primate cardiac xenotransplantation. Xenotransplantation. 2008; 15:268–276. [PubMed: 18957049]
41. Obukhova P, Rieben R, Bovin N. Normal human serum contains high levels of anti-Gal alpha 1-4GlcNAc antibodies. Xenotransplantation. 2007; 14:627–635. [PubMed: 17991151]
42. Pierson RN III, Dorling A, Ayares D, et al. Current status of xenotransplantation and prospects for clinical application. Xenotransplantation. 2009; 16:263–280. [PubMed: 19796067]
43. Rood PP, Tai HC, Hara H, et al. Late onset of development of natural anti-nonGal antibodies in infant humans and baboons: implications for xenotransplantation in infants. Transpl Int. 2007; 20:1050–1058. [PubMed: 17850234]
44. Zhu A, Hurst R. Anti-N-glycolylneuraminic acid antibodies identified in healthy human serum. Xenotransplantation. 2002; 9:376–381. [PubMed: 12371933]

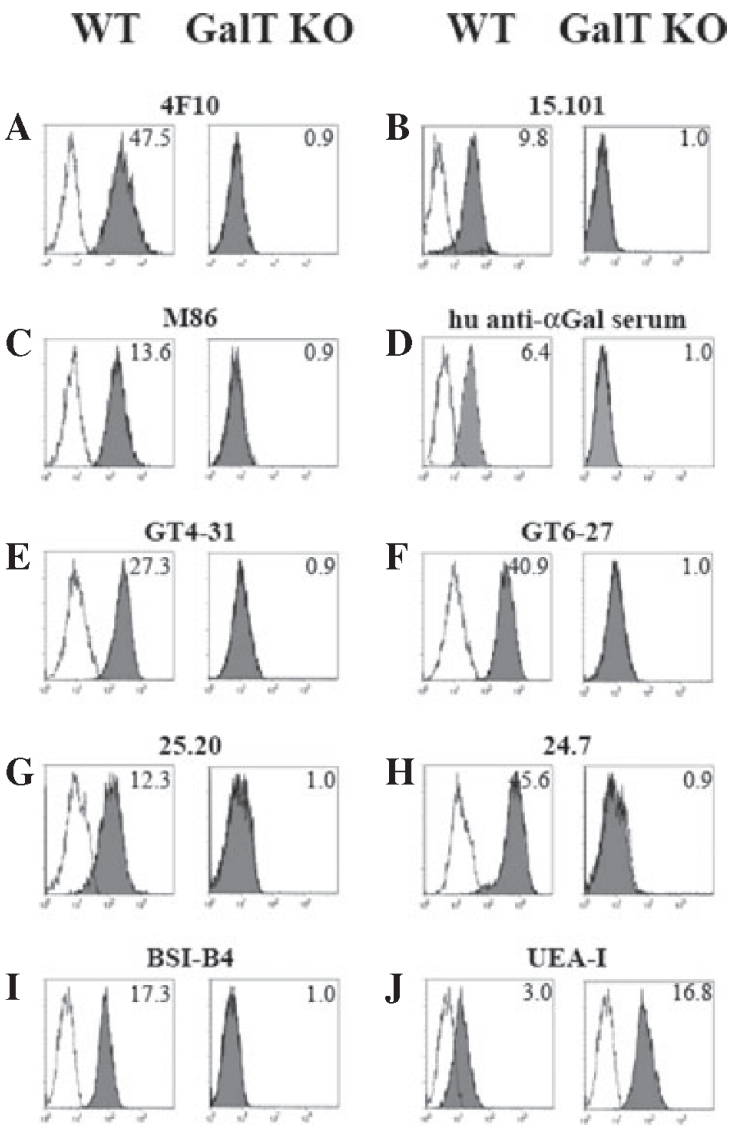

Fig. 1.

Lack of iGb_3 and αGal expression on pig aortic endothelial cells derived from α1,3galactosyltransferase knockout pigs analyzed by flow cytometry. The epitopes iGb_3 and αGal were tested in PAEC cells by indirect flow cytometry. Two mAb recognizing iGb_3: clone 4F10 (A) with specificity for both αGal and iGb_3 and clone 15.101 (B) were assayed in addition to the commercial mAb clone M86 (C); polyclonal purified anti-αGal from human serum (D); others mAb anti-αGal as the clone GT4-31 (E); clone GT6-27 (F); clone 25.20 (G); and clone 24.7 (H). Lectin staining with BSI-B4 (I) which binds to terminal α-D-galactosyl residues present in αGal and the UEA-I (J) which has with affinity for L-Fuc are also shown. Gray-shaded histograms show the staining for the different mAb, human serum, and lectins. Open histograms show the matching isotype controls when mAb were tested, human anti-αGal depleted serum for human serum, and cells incubated only with staining buffer for lectins staining. MFIR are shown in the upper right corner. Representative experiments from 3 to 5 different stainings are shown. BSI-B4, *Bandeiraea simplicifolia* lectin; GalT KO, α1,3galactosyltransferase knockout; iGb_3, isoglobotrihexosylceramide 3; mAb, monoclonal antibodies; MFIR, Mean fluorescence intensity ratio; PAEC, pig aortic endothelial cell; UEA-I, *Ulex europaeus* lectin.

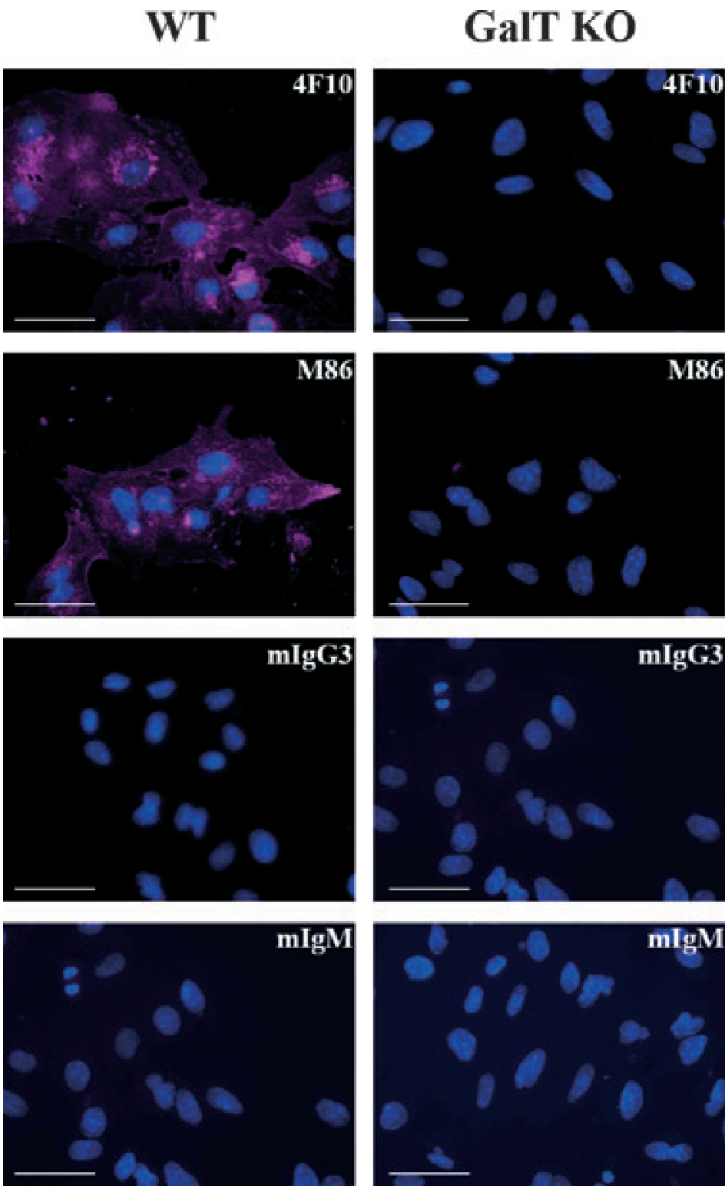

Fig. 2.

Uniform distribution of αGal in wild-type pig aortic endothelial cells. PAEC WT (left column) and GalT KO (right column) were grown directly in 96-well plates; fixed/permeabilized before staining with the 4F10 and M86 mAb or matching isotype controls: IgM and IgG3, respectively, as indicated in the top-right corner of the figures; and analyzed by Olympus fluorescent IX71 microscope fluorescent microscope. Overlay pictures of Evans blue channel in violet and DAPI channel in blue. Bar corresponds to 50 μm. Representative images of four different staining with the different pig cell lines. GalT KO, α1,3galactosyltransferase knockout; mAb, monoclonal antibodies; PAEC, pig aortic endothelial cell; WT, wild-type.

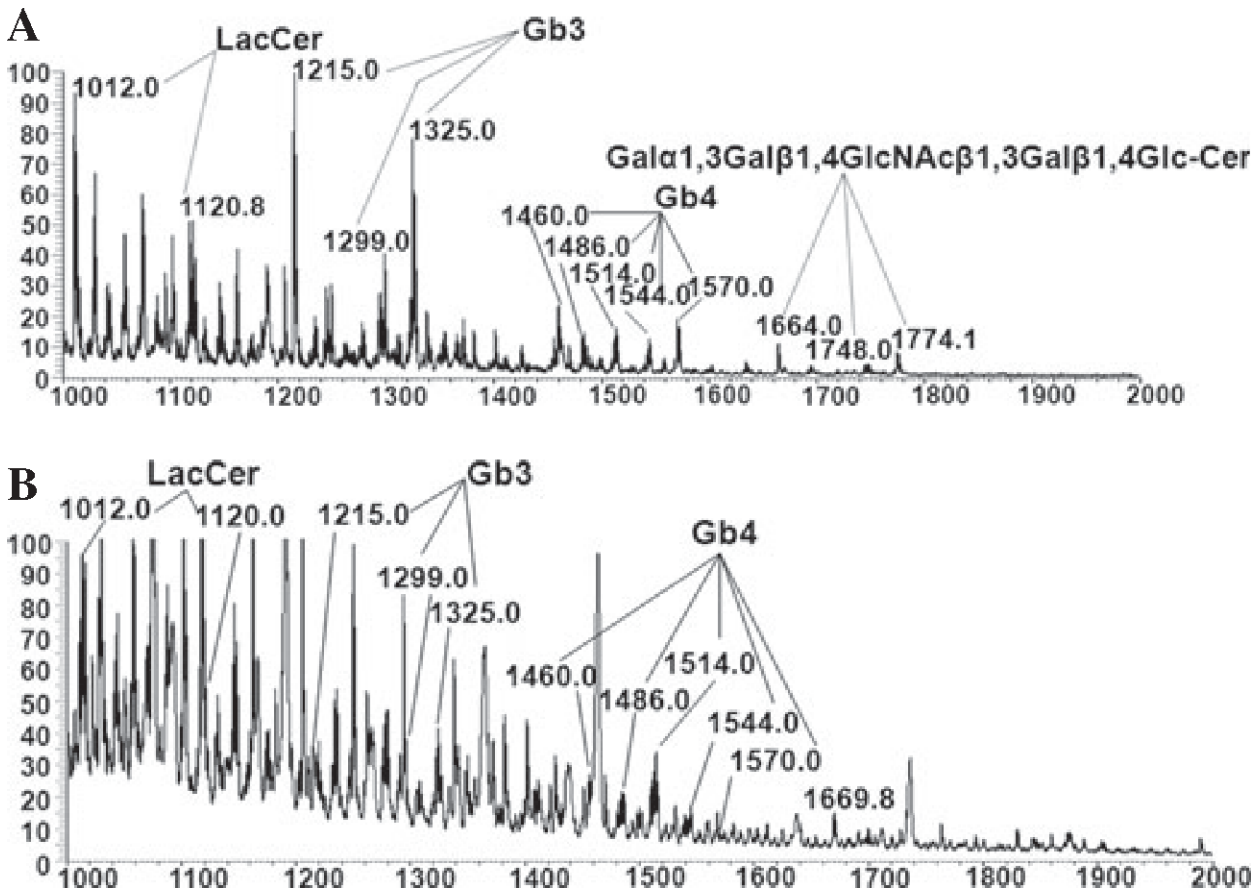

Fig. 3.

Absence of αGal and iGb_3 in pig aortic endothelial cell derived from α1,3galactosyltransferase knockout pigs analyzed by electrospray ionization mass spectroscopy. Neutral glycosphingolipids membranes from PAEC were extracted, permethylated, and analyzed by MS. MS was carried out in positive ion mode on a linear ion trap mass spectrometer using a nano-electrospray source. MS^1 neutral glycolipid fractions of WT (A) and GalT KO (B) PAEC were extracted and analyzed. Molecular ions containing αGal epitope (Galα3nLc4, Galα3Galβ4GlcNAcβ3Galβ4Glc-Cer) are only seen in WT PAEC (1664; 1748; and 1774 *m/z*). The molecular ions representing regioisomers of trihexosyceramides (Gb_3/iGb_3, 1215, 1299, and 1325 *m/z*) present in PAEC were further subjected to MS^4 analysis by ion trap mass spectrometry excluding the presence of iGb_3 [17]. The Gb_3/iGb_3 precursor, LacCer (1012 and 1120 *m/z*), is present in both PAEC Spectra correspond to *x*-axe in *m/z* and *y*-axe relative absorbance. Gb_3, globotriaosylceramide also known as Pk antigen; iGb_3, isoglobotrihexosylceramide; LacCer, lactosylceramide; PAEC, pig aortic endothelial cells; MS, mass spectroscopy.

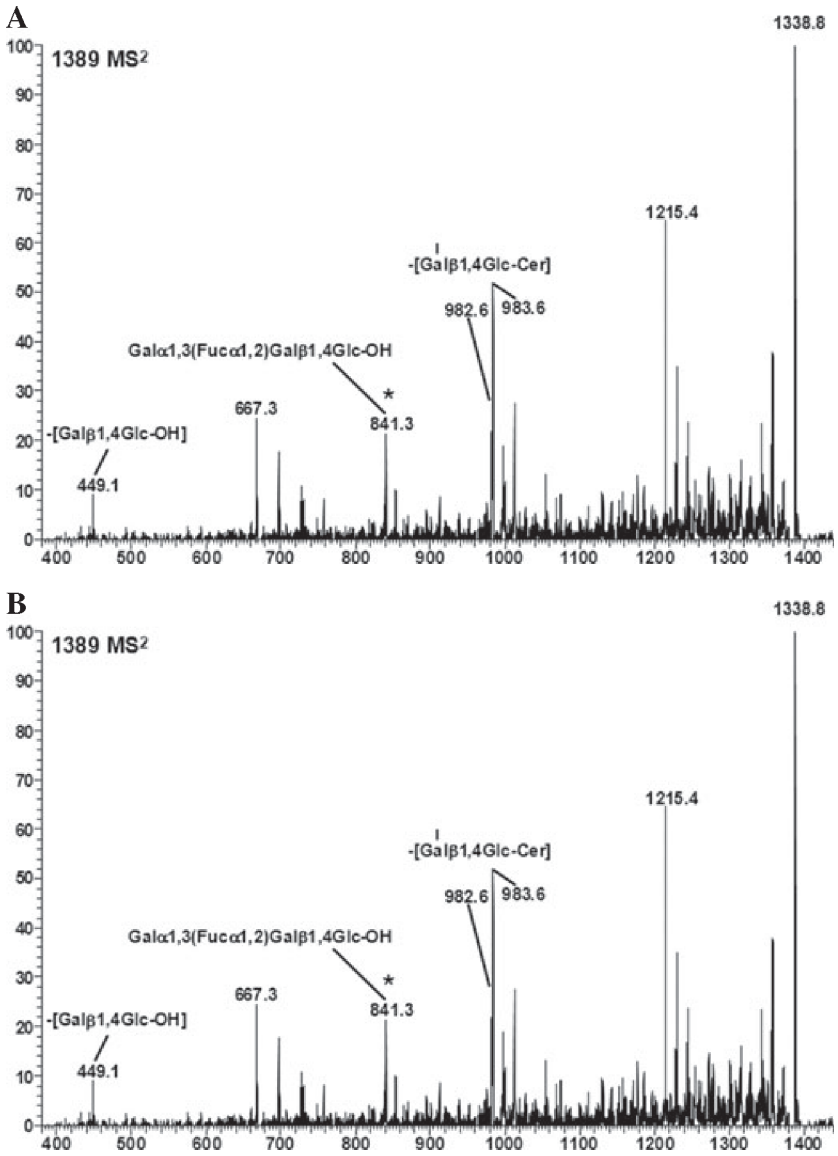

Fig. 4.

Wild-type and α1,3galactosyltransferase knockout neutral pig aortic endothelial cell membranes contain a fucosylated form of iGb_3. Neutral glycosphingolipids membranes from WT and GalT KO PAEC were extracted, permethylated, and analyzed by MS. Molecular ion profiles of neutral glycolipid membrane fractions of WT (A) and GalT KO (B) PAEC obtained by linear ion trap mass spectrometer using the electrospray ionization mass spectroscopic method (LTQ-ESI-MS) are shown. The presence of fucosylated iGb_3 structure was found by precursor ion mapping method (using 841 as the product ion). Candidate structures for the ion are Galα1,3(Fucα1,2) Galβ1,4Glc-Cer, and/or Fucα1,2Galα1,3Galβ1,4Glc-Cer. Spectra *x*-axe in *m/z.* Cer, ceramide; GalT KO, α1,3galactosyltransferase knockout; Gb_3, globotrihexosylceramide 3, iGb_3, isoglobotrihexo sylceramide; MS, mass spectrometry; PAEC, pig aortic endothelial cells; WT, wild-type.

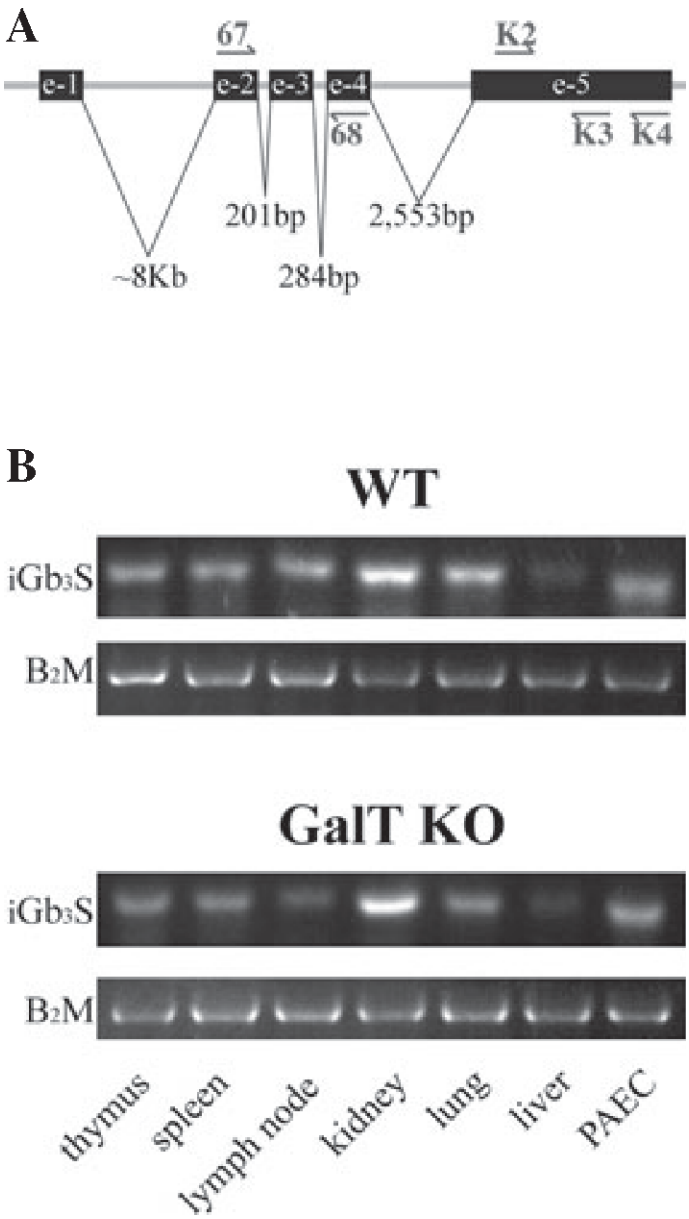

Fig. 5.

Expression of mRNA levels of the isoglobotrihexosylceramide 3 synthase in pig aortic endothelial cells and pig tissues. Different tissues were isolated from just sacrificed animals and snap frozen for RNA extraction and analysis. The mRNA levels for iGb_3S were checked with three different set of primers. Location of primers is shown using the patented WO 2005/047469 sequence as reference (A). Pig β_2-microglobulin was used as a housekeeping gene. RT-PCR products of K2/K3 primers set were run in 2% agarose gel electrophoresis (B). Two different samples from each organ were obtained from a WT animal (#16517, female, AA haplotype, 2.5 yr old) and a GalT KO animal (#16183, female, DD haplotype, 3.5 yr old), respectively. e, exon; GalT KO, α1,3galactosyltransferase knockout; iGb_3S, isoglobotrihexosylceramide 3 synthase; mRNA, messenger ribonucleic acid; PAEC, pig aortic - endothelial cells.

Table 1

α1,3galactosyltransferase activity detected in cell lysates

	1 h		3 h	
	nmol/60 min/150 μg	**mU/g**	**nmol/180 min/150 μg**	**mU/g**
PAEC WT	0.243	0.0263	0.3278	0.0364
PAEC GalT KO	0.014	0.0018	0.0154	0.0017
E293 rat GalT	0.032	0.0031	0.0546	0.0061

GalT, α1,3galactosyltransferase; KO, knock-out; PAEC, pig aortic endothelial cells; WT, wild-type.

One hundred and fifty micrograms of cell lysates from pig aortic endothelial cells from WT and GalT KO animals were incubated in the presence of UDP^{14}[C]-Gal and asialofetuin at 37 °C. Enzymatic reactions were stopped after 1 and 3 h and radioactivity incorporation was quantified. As positive control, human kidney embryonic cells (E293) transfected with rat GalT were used.