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The Possibility of Reducing Xenoantigen Levels with a Novel Gal 3′-Sulfotransferase (GP3ST)[1]

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Glycoprotein-3-sulfotransferase (GP3ST) is a key enzyme in downregulating the expression of Galα1,3Galβ1,4GlcNAc-R (the α-Gal epitope), *via* enzymatic competition with an α1,3 galactosyltransferase (α1,3GT), such as α2,6 sialyltransferase (α2,6ST). In this study, we report the dominance of GP3ST over α1,3GT using transfected pig endothelial cell (PEC) lines. The introduction of the GP3ST gene into PEC suppresses its antigenicity with respect to normal human pooled serum (NHS), including the α-Gal epitope and the Hanganutziu-Deicher (H-D) antigen, and, in addition, reduces the susceptibility to NHS in complement-mediated cell lysis. Western and lectin blot analyses of the products of parental PEC and its transfectants indicated that proteins smaller than 66 kDa have a diminished reactivity with NHS and the IB4 lectin. The levels of the α-Gal epitope in neutral glycosphingolipids were also decreased in the GP3ST transfectants as detected in thin layer chromatography by immunostaining. These data indicate that GP3ST is very effective in reducing xenoepitope levels.

Key words: α2,6ST, glycoantigen, GP3ST, pig endothelial cell, xenotransplantation.

The worldwide shortage of donor organs has led to a revived interest in xenotransplantation. Exposure of pig organs to human blood, however, results in hyperacute rejection, which is mediated by naturally occurring high-titer antibodies and complements in humans The major xenoantigen responsible for this type of rejection has a single carbohydrate structure, Galα1,3Galβ1,4GlcNAc-R (α-galactosyl epitope), which is expressed by the cells of most mammals, except humans and other Old World primates. These species have only a pseudogene for the α1,3 galactosyltransferase (α1,3GT). Consequently, humans acquire natural antibodies that comprise as much as 1% of the total circulating IgG, and which are also found in significant amounts in the form of an IgM antibody (*1–3*). A variety of strategies have been pursued to reduce or eliminate this epitope from pig tissues (*4*). Knocking out α1,3GT using an embryonic stem cell (*5, 6*) or somatic cells in conjunction with nuclear transplantation techniques represents the most reliable way However, at this time, these techniques have not yet been established Other strategies, such as enzyme competition for α1,3GT with other glycosyltransferases (*7, 8*) and/or control of sugar processing by glycosyltransferases (*9*), have also provided new insights into the downregulation of the α-Gal epitope.

A novel sulfotransferase, glycoprotein-3-sulfotransferase (GP3ST), is a type II transmembrane protein composed of 398 amino acid residues It shows sulfotransferase activities toward oligosaccharides and contains nonreducing β-galactosides, such as *N*-acetyllactosamine, lactose, lacto-*N*-tetraose (Lc4), lacto-*N*-neotetraose (nLc4), and Galβ1-3GalNAcα-Bzl (*O*-glycan core 1 oligosaccharide). GP3ST is able to act on both type 1 (Galβ1-3GlcNAc-R) and type 2 (Galβ1-4GlcNAc-R) chains with similar efficiency. Carbohydrate structures with 3′-sulfo β-Gal linkages are ubiquitous in both *N*-glycans and *O*-glycans of glycoproteins in the body (*10, 11*).

In our previous study, we reported the results of an examination of the reduction in the antigenicity of pig endothelial cells (PEC) *via* the overexpression of *N*-acetylglucosaminyltransferase III (GnT-III) (*9*), α1,2 fucosyltransferase (α1,2FT), α2,3 sialyltransferase (α2,3ST; ST3Gal III) (*8*), or α2,6 sialyltransferase (α2,6ST; ST6GalI) (*12*). Among these, α2,6ST was shown to be quite effective in reducing the α-galactosyl epitope levels in both glycoproteins and glycolipids (*13*) Therefore, in this study, the overexpression of GP3ST was examined in terms of the downregulation of the expression of the α-Gal epitope on PEC, as compared with α2,6ST.

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Abbreviations the α-galactosyl epitope, the Galα1,3Galβ1,4GlcNAc-R, α1,3GT, α1,3 galactosyltransferase, GP3ST, glycoprotein-3-sulfotransferase; PEC, pig endothelial cell, GnT-III, *N*-acetylglucosaminyltransferase III, α1,2FT, α1,2 fucosyltransferase, α2,3ST, α2,3 sialyltransferase, α2,6ST, α2,6 sialyltransferase, NHS, normal human pooled serum, FITC, fluorescein isothiocyanate, GSIB4, *Griffonia simplicifolia* I, H-D, Hanganutziu-Deicher; LDH, lactate dehydrogenase, TLC, thin layer chromatography

MATERIALS AND METHODS

Endothelial Cell Cultures—A PEC line, MYP30, was cultured in Dulbecco's modified Eagle's medium (D-MEM) containing 10% FBS with L-glutamine and penicillin/streptomycin (Gibco /BRL) (*14*).

Construction of Plasmids—The cDNAs for human GP3ST and mouse α2,6ST were subcloned into the pCXN2 site (a β-actin promoter and a cytomegalovirus enhancer) (*15*).

Transfection Experiments—The purified plasmids (20 μg) were introduced into MYP-30 by lipid-mediated DNA transfection using the LIPOFECTAMINE™ Reagent (GIBCO/BRL). The transfected MYP-30 cells were maintained in complete medium for several days in an atmosphere of humidified 5% CO_2 at 37°C, and were then transferred to a complete medium containing 0.4 mg/ml G418 (GIBCO/BRL) for selection (*14*). The expressions of the α2,6ST (a gift from Dr. Shuichi Tsuji, Hadano) and GP3ST glycosyltransferases were confirmed by activity assays, as described below.

Enzyme Assays—α2,6ST, GP3ST and α1,3GT activities in each transfectant were assayed as described previously (*8, 10*). For the enzyme activity assays, cells were washed twice with PBS and then centrifuged at 1,500 ×*g* for 10 min. The pelleted cells were resuspended in 100 μl of PBS and then lysed by sonication. The reaction mixture used for α2,6ST was 50 mM cacodylate buffer, 10 mM $MnCl_2$, 0.23% Triton X-100, 5 mM CMP-sialic acid as the donor substrate, and 10 mM LNnT-PA (LNnT-PA, Seikagaku Kogyo) as the acceptor, at pH 6.8 The incubation mixture used for GP3ST contained the following components in a total volume of 25 μl: 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.2), 10 mM $MnCl_2$, 1% Lubrol PX, 1 mM 5′-phosphoadenosine3′-phosphosulfate (PAPS), 0.2 mM PA-Lc4, and enzyme source (7.5 μl). α1,3GT activity was assayed in a reaction mixture containing 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7 2, 20 mM UDP-galactose, 10 mM $MnCl_2$, 33 mM NaCl, 3 mM KCl. The assay mixtures were incubated at 37°C for 3 h, and the reactions were then terminated by heating in a boiling water bath for 5 min, followed by centrifugation of the samples at 15,000 ×*g* for 5 min. The resulting supernatants were injected into a reversed phase HPLC equipped with a TSKgel column, ODS 80TM (4.6 × 250 mm) for α2,6ST and α1,3GT, and a TSKgel SuperQ-5PW column (7 5 × 75 mm, Tosoh) for GP3ST. The products and substrates were eluted with 20 mM ammonium acetate buffer (pH 4.0) containing 0.01% *n*-butanol for α2,6ST and α1,3GT, and a linear gradient of 0–0.2 M ammonium acetate (pH 9.0) for GP3ST. The fluorescence of the column eluate was detected with a fluorescence detector (Shimadzu, model RF-10AXL) using excitation and emission wavelengths of 320 nm and 400 nm, respectively. The specific activity of the enzymes is expressed as picomoles of product produced per h of incubation per mg of protein. Protein concentrations were determined with a BCA protein assay kit (Pierce) using bovine serum albumin as the standard (*10, 13*).

Flowcytometry—Parental PEC and transfectants were incubated with 20% normal human pooled serum (NHS) at 4°C for 1 h, washed, and then incubated with 1.25 μg of fluorescein isothiocyanate (FITC)–conjugated anti–human Ig (Cappel) as a second antibody for 1 h at 4°C. The cell-surface carbohydrate epitopes were also examined with an FITC-conjugated *Griffonia simplicifolia* I (GSIB4) lectin (Honen, Tokyo), which binds the α-Gal epitope. The Hanganutziu-Deicher (H-D) antigen was also detected using a chicken anti–H-D antigen polyclonal antibody (a Gift from Dr. N. Wakamiya, Asahikawa Medical College, Asahikawa) and subsequently stained with FITC-conjugated rabbit anti–chicken IgG (Cappel, West Chester, PA, USA) as a second antibody. Stained cells were analyzed with a FACS Calibure flow cytometer (Becton Dickinson).

Lactate Dehydrogenase (LDH) Assay—This assay was performed according to the manufacturer's recommended protocol using a Kyokuto MTX "LDH" kit The transfected cells were plated at 2×10^4 cells per well in 96-well trays 1 day prior to assay. The next morning, the wells were washed twice in serum-free D-MEM to remove the LDH present in fetal calf serum, and incubated with several concentrations of NHS which had been diluted with D-MEM. The plates were incubated for 2 h at 37°C and the released LDH was then measured. The percent cytotoxicity was calculated using the formula

$$\text{Cytotoxicity} = (E - N - S)/(M - N - S) \times 100$$

Where E is the experimentally observed release of LDH activity from the target PEC, N the LDH activity in each concentration of NHS, S the spontaneous release of LDH activity from target PEC incubated in the absence of NHS, and M the maximal release of LDH activity, as determined by sonication (*16*).

Western Blotting—Total cell lysates (10 μg) from parental or transfected PEC were subjected to 12% SDS/PAGE under reducing conditions using the methods of Laemmli and then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in PBS containing 3% BSA and incubated for 20 min with 0.2% NHS at room temperature After washing, the blots were incubated with horseradish peroxidase–conjugated rabbit Fab to human immunoglobulines (IgG, IgM, IgA) (Cappel) and developed using an ECL detection system (Amersham Pharmacia Bioteck).

Lectin Blotting—Parental and transfected cell products were also tested by lectin blot analysis, using GSIB4. The cell lysate (10 μg) was subjected to 12% SDS/PAGE under reducing conditions and then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The blots were blocked in PBS containing 0 05% Tween 20 and 3% BSA and incubated for 20 min with 10 mg/ml biotinylated GSIB4. After washing, the blots were incubated with horseradish peroxidase–avidin complex (Vector, ABC Reagent) and developed using an ECL detection system (Amersham Pharmacia Bioteck).

Preparation of Glycosphingolipids—Total glycosphingolipid fractions were isolated from PEC (1×10^8 cells) using the Folch method. The following steps involved the use of DEAE-Sephadex A-25-columns (Pharmacia LKB Biotech) to separate the acidic glycosphingolipids from the neutral glycosphingolipids components. Neutral glycosphingolipids were partially purified by mild alkaline treatment.

Thin Layer Chromatography (TLC)—Glycosphingolipids were separated on precoated silica gel plates (Merck 60 HPTLC plates, Merck, Darmstadt, Germany). The solvent

system was chloroform/methanol/water (60:35:8, v/v/v). Neutral glycosphingolipids were visualized by spraying the TLC-plate with orcinol/sulphuric acid reagent (*13*).

TLC Immunostaining—The total neutral glycosphingolipid fractions from parental PEC and transfectants were separated by thin layer chromatography followed by immunostaining with human natural antibody or biotinylated GSIB4 lectin in order to identify the α-Gal epitope. As a second treatment, the NHS treated TLC plate was treated with horseradish-peroxidase–conjugated rabbit Fab to human immunoglobulins (IgG, IgM, IgA) (Cappel). Lectin blot analysis was also performed using the Vector ABC reagent (Vector Laboratories). The ECL detection system (Amersham Pharmacia Bioteck) was employed for the development of both types of staining. Immunostained bands were evaluated by scanning with a Scanning Imager (Molecular Dynamic) (*13*).

Statistics—Data are presented as means ± SD The Student-*t* test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $p < 0\ 05$.

RESULTS

Enzyme Activities of Parental PEC and Transfectants—Several glycosyltransferase clones were established by cDNA transfection. Parental PEC and transfectants showed the classic cobblestone appearance and grew in the same manner. The enzyme activities of these α2,6ST and GP3ST transfectants are shown in Table I. Parental PEC showed no detectable activities corresponding to the transfected enzymes. The intrinsic α1,3GT activity was clearly present in parental PEC. The average α1,3GT activity in the GP3ST transfectants was lower than that in parental PEC, but not greatly changed.

Fluorescence Histograms of Parental PEC and Transfectants with NHS and GSIB4—In the case of GP3ST transfectants, the highest expressing clone, B-12, caused a significant (56.6 and 49.5%) reduction in NHS and GSIB4, respectively, whereas the representative high-expressing α2,6ST clone caused an approximately 75% reduction in both NHS and GSIB4, as evidenced by FACS analysis (Fig. 1, A and B).

H-D Antigen—The remodeling of PEC antigenicity by the overexpression of GP3ST is directed, not only at α-Gal, but also at other unknown epitopes as well. The influence on the H-D antigen in PEC transfectants was then examined. As expected from our previous study, the H-D antigen on PEC was significantly downregulated (Fig. 1C).

LDH Assay of Transfected PEC—The amelioration of complement-mediated lysis by the transfection of glycosyltransferases was determined. In these experiments, NHS was used as a source of natural antibody to the α-Gal epitope and complement Control parental PEC lysis resulting from 20% NHS treatment was found to be 35.0%.

An approximate 73% inhibition in cytotoxicity was observed in the case of the α2,6ST transfectant, and a 71% inhibition was found for the GP3ST transfectant. These results suggest that both glycosyltransferases are quite effective in reducing the α-Gal epitope level in PEC (Fig. 2).

Western and Lectin Blotting—Western and lectin blotting were performed in order to analyze the alteration in the reactivity of glycoproteins with human natural antibodies

TABLE I **Enzyme activities of the α2,6ST and GP3ST transfectants.**

Cell	α2,6ST	GP3ST	α1,3GT
Parental PEC	ND	ND	60 0 ± 3 4
α2,6ST	2,056 ± 395	ND	59 0 ± 2 4
B4 GP3ST	ND	1,993 ± 143	48 8 ± 5 6
B12 GP3ST	ND	2,330 ± 75	41 8 ± 10.0

pmol/h/mg-protein. ND: not detected ($n = 3$)

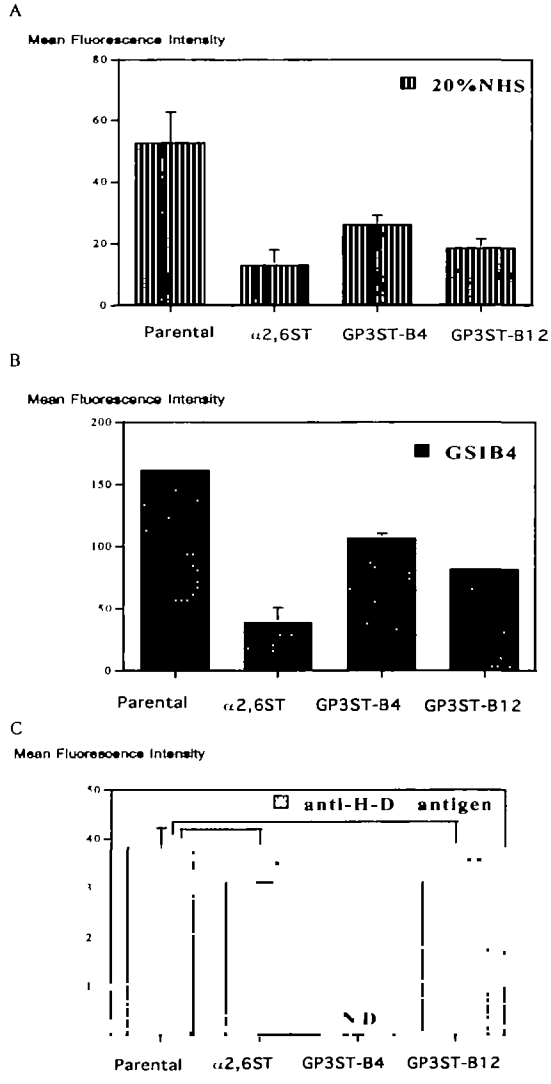

Fig 1 **Flow cytometric analysis of transfectants stained with NHS, IB4 lectin, or anti-H-D antibody.** (A) Parental PEC (control) and stable transfectants, α2,6ST, GP3ST, were treated with 20% NHS as the first antibody and then FITC-conjugated anti–human Ig as a second antibody Each value is expressed as the mean ± SD of three independent experiments. (B) The reduction of the α-Gal epitope on the cell surface was analyzed using GSIB4 lectin Parental PEC and stable transfectants were treated with FITC-conjugated GSIB4 lectin. Each value is expressed as the mean ± SD of three independent experiments. (C) Parental PEC and stable transfectants were treated with chicken anti–H-D polyclonal antibody, and subsequently incubated with FITC-conjugated rabbit anti–chicken IgG N D : not done Each value is expressed as the mean ± SD of seven independent experiments. The asterisks indicate significant differences *vs* parental PEC ($*p < 0\ 05$, $**p < 0\ 01$)

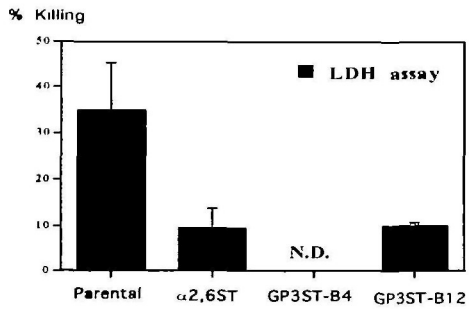

Fig 2 **LDH assay of transfected PEC.** Amelioration of complement-mediated lysis by transfectants and control parental PEC were estimated by 20% NHS, which served as a source of natural antibody and complement The percent inhibition of complement-mediated lysis is indicated N D. not done Each value is expressed as the mean ± SD of five independent experiments

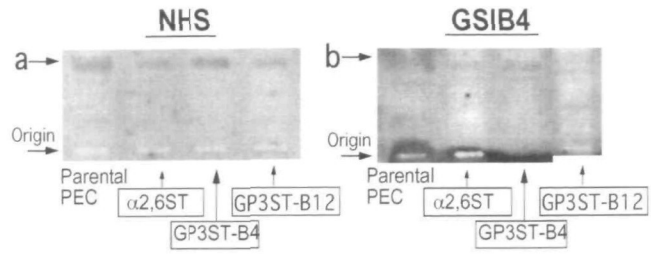

Fig 4 **Orcinol staining of neutral glycosphingolipids and TLC immunostaining with NHS and GSIB4 lectin.** The total neutral glycosphingolipid fractions from parental PEC and transfectants were separated by thin layer chromatography Immunostaining of neutral glycosphingolipids by NHS and GSIB4 were performed in the lower area of the TLC plate, which contain more than five neutral sugar units. The bands indicated by arrows (a) and (b) are the α-Gal epitopes in the neutral glycosphingolipids

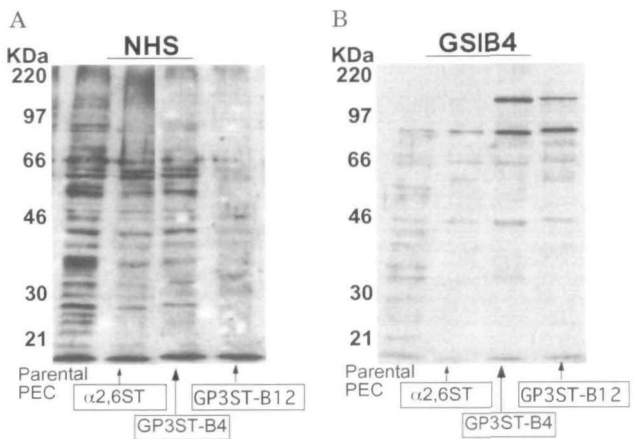

Fig 3 **Western and lectin blotting.** (A) Whole cell lysates from parental PEC and transfectants were separated by 12% SDS/PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with 0 2% of NHS. A typical western blot pattern is shown (B) A lectin blot analysis of whole cell lysates was also performed The blots were probed with biotinylated GSIB4 lectin

and GSIB4 in PEC transfectants. An evaluation of the blot profiles revealed that proteins derived from these transfectants, which have molecular masses less than 66 kDa, are responsible for the reduction in reactivity with NHS as compared with the parental PEC.

Similar to the Western blotting patterns, proteins with molecular masses less than 66 kDa in all transfectants were responsible for the reduction in reactivity with GSIB4, as evidenced by lectin blotting (Fig. 3).

TLC Analysis and Immunostaining—Neutral glycosphingolipids were stained with the orcinol sulphuric acid reagent. A similar staining pattern was observed for parental PEC and all transfectants (data not shown)

The glycosphingolipid fractions were separately developed and stained with NHS and GSIB4. Glycosphingolipids containing the α-Gal epitope were observed in a slow-migrating area. The reactivities with NHS and GSIB4 were reduced in both α2,6ST and GP3ST transfectants (Fig. 4).

DISCUSSION

Trials designed to overcome hyperacute rejection by modification of glycosyltransferase(s) using gene technology are currently underway (*17*). A variety of strategies designed to downregulate the α-Gal epitope have been reported. Since pig ES cells are not available at present, the knock out of α1,3GT is not feasible. In a previous our study, we classified glycosyltransferases with the potential for downregulating the α-Gal epitope into two groups (*13*). Group I includes glycosyltransferases responsible for remodeling the total glyco-antigen of the cell surface from core glycosylation, and includes GnT-III. In addition, we have previously demonstrated the downregulation of xenoantigenicity by this enzyme, using PEC transfectants, as well as transgenic mice and pigs (*18–20*). The disadvantage of this strategy is that GnT-III acts only on *N*-linked sugars of glycoproteins that are considered to have strong antigenicity. Therefore, other epitopes on *O*-linked sugars and glycolipids are basically unregulated by this enzyme.

Group II includes glycosyltransferases that are known to participate in intracellular competition involving terminal glycosylation with α1,3GT for the common acceptor substrate in the trans Golgi stack and network and includes transferases such as α1,2FT, α2,3ST, as well as α2,6ST. Among these, gene transfection with α1,2FT resulted in a remarkable reduction in the levels of the α-Gal epitope. Transgenic pigs that express α1,2FT have been reported by several groups (*21, 22*). However, in our previous study, α2,3ST and α2,6ST were found to dramatically suppress the antigenicity of pig cells to human natural antibodies, and to reduce complement-mediated cell lysis to the same extent or considerably better than the α1,2FT (*13*).

The functional features of GP3ST with the α-Gal epitope place it among the group II enzymes, because the substrate specificities of GP3ST are almost the same as those of α2,6ST. Therefore, the PEC transfectants with GP3ST were compared with a representive α2,6ST transfectant. In the present study, while the features of the downregulation of a xenoantigen, such as the α-Gal epitope and H-D antigen, by GP3ST were very similar to those by α2,6ST. The degree of the reduction of xenoantigen by GP3ST transfection was slightly milder than that by α2,6ST, as much as that by α1,2FT. GP3ST transfection is effective for producing a

downregulation of the α-Gal epitope in both glycoproteins and glycolipids. Since there is no evidence that GP3ST acts on glycolipids, it remains to be solved whether the downregulation of the α-Gal in glycolipids is a direct effect of competition by GP3ST. The GP3ST transfectant showed only about a 50% reduction in reactivity to GSIB4, whereas the percent suppression of cytotoxicity by this molecule was nearly the same as that by α2,6ST (Fig. 2). The data suggest that this molecule changes not only the α-Gal epitope, but also many unknown epitopes that react with human serum.

During the effort to produce transgenic pigs to reduce antigenicity, only transgenic pigs with α1,2FT and GnT-III have been reported. Therefore, it is worth arguing that the producion of transgenic pigs with overexpressed GP3ST is an important issue for future studies.

In the case of α1,2FT, It has been reported that the overexpression of the α1,2FT gene in the presence of endogenous pig α1,3FT activity results in the formation of cell surface Lewis^{Y} (*23*). However, the possibility that new antigens are generated by the overexpression of this GP3ST can be excluded, since the expression of the GP3ST gene in human tissues is ubiquitous with relatively higher expression in the heart, stomach, colon, liver, and spleen (*10*).

The Hanganutziu-Deicher antigen, which contains *N*-glycolylneuraminic acid (NeuGc), is widely distributed in mammalian species, but not in humans. The expression of NeuGc is controlled by a cytidine monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) hydroxylase activity The absence of NeuGc in human glycoconjugates is due to a partial deletion in the gene that encodes CMP-NeuAc hydroxylase (*24–27*). Therefore, this epitope has the potential to become one of the largest epitopes in pig to human xenotransplantation after α1,3GT is knocked out. Fortunately, transfection of the GP3ST and α2,6ST genes reduces the levels of the H-D antigen, although the effect is only about 20%. Therefore, other strategies may be needed to downregulate the H-D antigen more effectively. Our previous study indicated that a group I enzyme, GnT-III, has the potential to reduce H-D antigen levels on PEC, while other group II enzymes, α1,2FT and α2,3ST, have no effect (*28*).

In conclusion, we demonstrate here that GP3ST is quite effective in downregulating α-Gal epitopes in *in vitro* experiments using transfected pig endothelial cells. Further studies, especially using transgenic animals, will be needed to clarify the issue of whether this gene will be useful in clinical xenotransplantation.

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