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## Human alpha galactosidase and alpha 1,2 fucosyltransferase concordantly inhibit xenoreactivity of NIH 3T3 cells with human serum<sup>1</sup>

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**KEY WORDS** alpha-galactosidase; alpha-L-fucosidase; Gal alpha 1,3 Gal; NIH3T3 cell; heterologous transplantation; immunoglobulin M; immunoglobulin G; complement

### ABSTRACT

**AIM:** To study the influence of the expression of human alpha galactosidase and alpha1,2 fucosyltransferase on Gal alpha 1,3 Gal and consequent xenoreactivity in NIH3T3 cells. **METHODS:** The expression levels of G antigen and H antigen and binding of human natural antibodies (IgG and IgM) and complement (C3c) to NIH3T3 cells were analyzed by flow cytometry. Western blot was employed to further determine the expression of glycoproteins of G antigen. Cytolysis assay with normal human serum was performed by MTT assay. **RESULTS:** Western blot showed that glycoproteins with molecular weight of 107 kDa, 98 kDa, 88 kDa, 56 kDa, 40 kDa, and 37 kDa were inhibited and even abrogated totally in alpha galactosidase transfectants and alpha 1,2 fucosyltransferase transfectants. The combined transfection of the two enzymes led to a much stronger inhibition of the glycoproteins. The binding of G<sub>s</sub>-IB<sub>4</sub> was decreased by 57.4 % in alpha galactosidase transfectants, 28.8 % in alpha 1,2 fucosyltransferase transfectants, and 72.1 % in combined transfectants, respectively. In contrast, UEA-1 binding was increased about 6.7-fold, 6.0-fold, and 8.0-fold respectively. The xenoreactivity with human IgG was also reduced by 61.4 %, 67.0 %, and 73.4 %, respectively in the three kinds of transfectants. The resistance to cytolysis mediated by human serum was enhanced by 42.4 % in alpha galactosidase transfectants, 51.9 % in alpha 1,2 fucosyltransferase, and even 65.5 % in the combined transfectants. **CONCLUSION:** Although alpha galactosidase and alpha 1,2 fucosyltransferase had different biochemical properties, they could inhibit the expression of Gal alpha 1,3 Gal synergistically, leading to stronger resistance of xenograft against cytolysis.

### INTRODUCTION

The accelerating advances of organ transplanta-

tion have raised an imbalance between organ supply and demand. The availability of human organs meets only about 5 % of the estimated demand<sup>[1,2]</sup>. Xenotransplantation from pig to human beings is viewed as a potential solution for the acute organ shortage. However, consequent xenorejection prevents xenotransplantation from the clinical application. According to pathology, xenorejection consists of three phases, including hyperacute rejection (HAR), delayed xenograft rejection

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(DXR), and T-cell-mediated rejection<sup>[3]</sup>. Typically, HAR, characterized by interstitial hemorrhage and diffuse thrombosis, begins immediately after transplantation or perfusion with foreign serum. The graft is inevitably destroyed within minutes to a few hours in this process. Some results showed that HAR was assumed to be induced mainly by IgM<sup>[4,5]</sup>, while others indicated that IgG, but not IgM, induced HAR in hepatic xenografting<sup>[6]</sup>. During DXR, the endothelial cell of donor organ was activated and host monocyte and natural killer cell infiltrated into the graft, leading to the intragraft inflammation and thrombosis<sup>[7]</sup>. Finally, the xenorejection ends with T-cell-mediated rejection.

Gal alpha 1,3 Gal (Gal epitope or G antigen), a terminal disaccharide on glycoproteins and glycolipids, is synthesized by Gal $\beta$ 1,4GlcNAc3-alpha-D-galactosyltransferase in *trans*-Golgi and abundantly expresses in pig, mouse, and new world monkey<sup>[8,9]</sup>. It is Gal alpha 1,3 Gal that induces most of xenorejection by interacting with host xenoreactive natural antibodies<sup>[10,11]</sup>. Thus, one of the most attracting attempts to prevent xenorejection is to inhibit the synthesis of Gal alpha 1,3 Gal. The first approach is designed from the treatment with soluble enzyme. *In vitro* treatment of porcine endothelial cells and lymphocytes with green coffee bean alpha galactosidase dramatically decreased the binding of human xenoreactive natural antibodies without any nonspecific immunoglobulin binding sites induced<sup>[12,13]</sup>, but the technical and financial obstacles prevent the soluble enzyme from clinical application. Another approach is raised from gene modification including transgenic expression of human alpha1,2 fucosyltransferase<sup>[14]</sup>, antisense constructs of alpha1,3 galactosyltransferase<sup>[15,16]</sup> or anti-alpha1,3 galactosyltransferase single-chain F<sub>v</sub> antibody, *etc*<sup>[17,18]</sup>, and the gene knock-out of alpha1,3 galactosyltransferase by homologous recombination<sup>[19,20]</sup>. Although the transgenic expression procedures prolong the survival of xenografts, xenorejection can not be abrogated totally by single procedure.

The purpose of this paper was to investigate the influence of combination of human alpha galactosidase and alpha1,2 fucosyltransferase transfectants on Gal alpha 1,3 Gal and consequent xenoreactivity in NIH3T3 cells.

## MATERIALS AND METHODS

**Plasmid construction** Human alpha galactosidase and alpha 1,2 fucosyltransferase cDNA were ob-

tained respectively from human hepatic cell and lymphocyte by reverse transcription (RT)-PCR. The cDNA were verified by sequencing and separately cloned into *EcoR*I-*Not* I site and *EcoR*I-*Xho* I site of the eukaryotic expression vector pcDNA3 under the control of the CMV promoter and termed pcDNA3-alpha-galase and pcDNA3-FT.

**Cell culture and transfections** NIH 3T3 cells were incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % heat-inactivated fetal calf serum, and were transfected with pcDNA3-alpha-galase, pcDNA3-FT, or combination of them with lipofectamine (Gibco). The transfectants of the empty vector were used as control. Stable transfectants were selected with G418 at the concentration of 1 g/L.

**Reverse transcription (RT)-PCR** Total RNA was extracted with Trizol reagent (Gibco) according to the manufacturer's instructions. RNA 0.5  $\mu$ g was used to synthesize cDNA. PCR was performed as follows: 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 80 s for 30 cycles. The corresponding primers for alpha galactosidase were P1 (5' GCGAATTCATGCAGCTGAGG-AACCCAGAACTACA 3') and P2 (5' GGCGGCCGCTT-AAAGTAAGTCTTTTAATGACATCTGCAT 3'). The primers for alpha 1,2 fucosyltransferase were P1 (5' CGGGAATTCAGCCATGCTGGTTCGTTTCAG 3') and P2 (5' CAACTCGAGCTATTAGTGCTTGAGTAAGG 3'). GAPDH was used as an internal standard and co-amplified with primers P1 (5' ACGACCCCTTCATTG-ACC 3') and P2 (5' AGACACCAGTAGACTCCACG 3').

**Cell surface antigen assay** G antigen was detected with fluorescein isothiocyanate (FITC)-conjugated *Griffonia simplicifolia* isolectin B<sub>4</sub> (G<sub>S</sub>-IB<sub>4</sub>) (Sigma), which is specific for G antigen<sup>[18]</sup>. H antigen (the universally tolerated O blood group antigen) was detected with FITC-conjugated *Ulex europaeus*-I (UEA-I) lectin (Sigma), which is specific for H antigen<sup>[18]</sup>. Cells 1 $\times$ 10<sup>6</sup> were washed two times with PBS, then incubated with lectin at 4 °C for 30 min, and flowcytometric analysis was performed with a Becton Dickso FACScan cytometry. Data were collected on 1 $\times$ 10<sup>4</sup> cells. Fluorescence intensity was statistically analyzed with SigmaPlot.

**Immunological assay** To detect the binding of the human natural antibody, 1 $\times$ 10<sup>6</sup> cells were washed two times with PBS and then incubated with 10 % normal human serum at 4 °C overnight. After washing with PBS twice, cells were incubated with FITC-con-

jugated anti-human IgG or IgM antibody, or C3c complement at 37 °C for 30 min. Then fluorescence intensity was detected and analyzed.

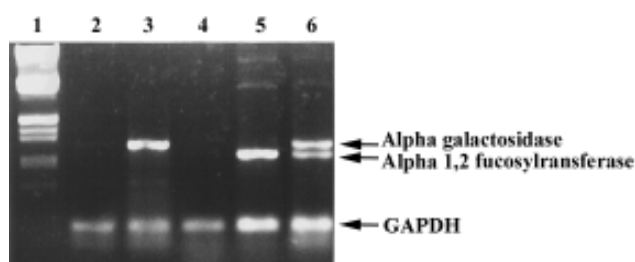
**Western blot** Cell membrane was prepared as follows<sup>[19]</sup>. Cells were pelleted by centrifugation and resuspended in lysis buffer (Tris-HCl, edetic acid, egtazic acid 5 mmol/L, respectively). Large cellular debris and cells were removed by centrifugation at 500×g for 5 min. Finally, the cell membranes in supernatant were pelleted by centrifugation at 10 000×g for 30 min and solved in loading buffer.

Samples were run on a 10 % SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were first incubated in blocking buffer (10 % fat-free milk in Tris-buffered saline) at 25 °C for 30 min, and subsequently with normal human serum (1:10 dilution) for 2 h. After washing with TBS containing 0.05 % Tween-20, the membranes were incubated with HRP-conjugated rabbit anti-human IgG antibody (1:500) at 25 °C for 2 h. Finally the membranes were developed with a chemiluminescent detection kit (ECL Western blot Detection kit).

**Cytolysis assay with MTT** Cytolysis assay with normal human serum was performed as follows<sup>[20]</sup>. Briefly, 1×10<sup>4</sup> cells transfected with pcDNA3-alpha-galase, pcDNA3-FT or combination of them were planted in 96-well plates, and 48 h later, media were removed. Cells were mixed with 50 μL normal human serum (in serial dilution), incubated at 37 °C for 30 min, and then normal human serum were substituted with 200 μL DMEM and 15 μL MTT. Four hours later, 50 μL of solubilization buffer (10 % SDS-5 % isobutanol-HCl 0.01 mol/L) was added into each well. Formazan crystals were dissolved at 37 °C overnight. The plate was read on microplate reader at a wavelength of 570 nm/630 nm.

## RESULTS

**Xenoreactivity induced by human xenoreactive natural antibodies** RT-PCR showed that alpha galactosidase and alpha 1,2 fucosyltransferase were both expressed at high levels in NIH3T3 cells (Fig 1). To determine whether the expression of alpha galactosidase, alpha 1,2 fucosyltransferase or combination of them influences the xenoreactivity of xenoreactive natural antibodies with Gal alpha 1,3 Gal and what the difference among them is, transfectants of empty vector or interest genes were analyzed by immunological assay as de-



**Fig 1.** Expression of human alpha galactosidase and alpha 1,2 fucosyltransferase in NIH3T3 cells by semi-quantitative RT-PCR. Lane 1, 2, 3, 4, 5, and 6 were DNA marker, the transfectants of mock, pcDNA3-alpha-Galase, mock, pcDNA3-FT and double transfectants, respectively.

scribed previously. After the transfection of pcDNA3-alpha-galase, pcDNA3-FT or combination of them, the IgG binding was decreased by 61.4 %, 67.0 %, and 73.4 % respectively (Fig 2A). The difference between the control and alpha galactosidase, alpha 1,2 fucosyltransferase or combined transfectant was significant ( $P<0.01$ ). IgM binding was decreased by 22.3 %, 28.9 %, and 36.6 % respectively (Fig 2B). A 47.8 % reduction both in alpha galactosidase and alpha 1,2 fucosyltransferase transfectants and a 60.1 % reduction in combined transfectants were observed (Fig 2C).

**Alpha galactosidase and alpha 1,2 fucosyltransferase differentially inhibited Gal alpha 1,3 Gal expression on NIH3T3 cells** In cell surface antigen assay, the lectin G<sub>S</sub>-IB<sub>4</sub> which specifically recognizes G antigen and lectin UEA-I which specifically recognizes H antigen were used to detect the change of cell surface antigen. The G<sub>S</sub>-IB<sub>4</sub> binding was decreased by 57.4 %, 28.8 %, and 72.1 % in alpha galactosidase transfectants, alpha 1,2 fucosyltransferase transfectants, and double transfectants, respectively (Fig 2D). In contrast, UEA-I binding was increased about 6.7-fold, 6.0-fold, and 8.0-fold in alpha galactosidase transfectants, alpha 1,2 fucosyltransferase transfectants, and double transfectants, respectively (Fig 2E).

Western blot further proved the inhibitory effects of the two enzymes on cell surface glycoproteins (Fig 3). In the samples from alpha galactosidase transfectants, glycoproteins equal to 98 kDa and 56 kDa were abrogated totally, glycoproteins equal to 40 kDa and 37 kDa were greatly decreased and glycoproteins equal to 107 kDa and 88 kDa were also decreased. In the samples from alpha 1,2 fucosyltransferase transfectants, glycoproteins equal to 98 kDa and 56 kDa were also abrogated whereas glycoproteins equal to 107

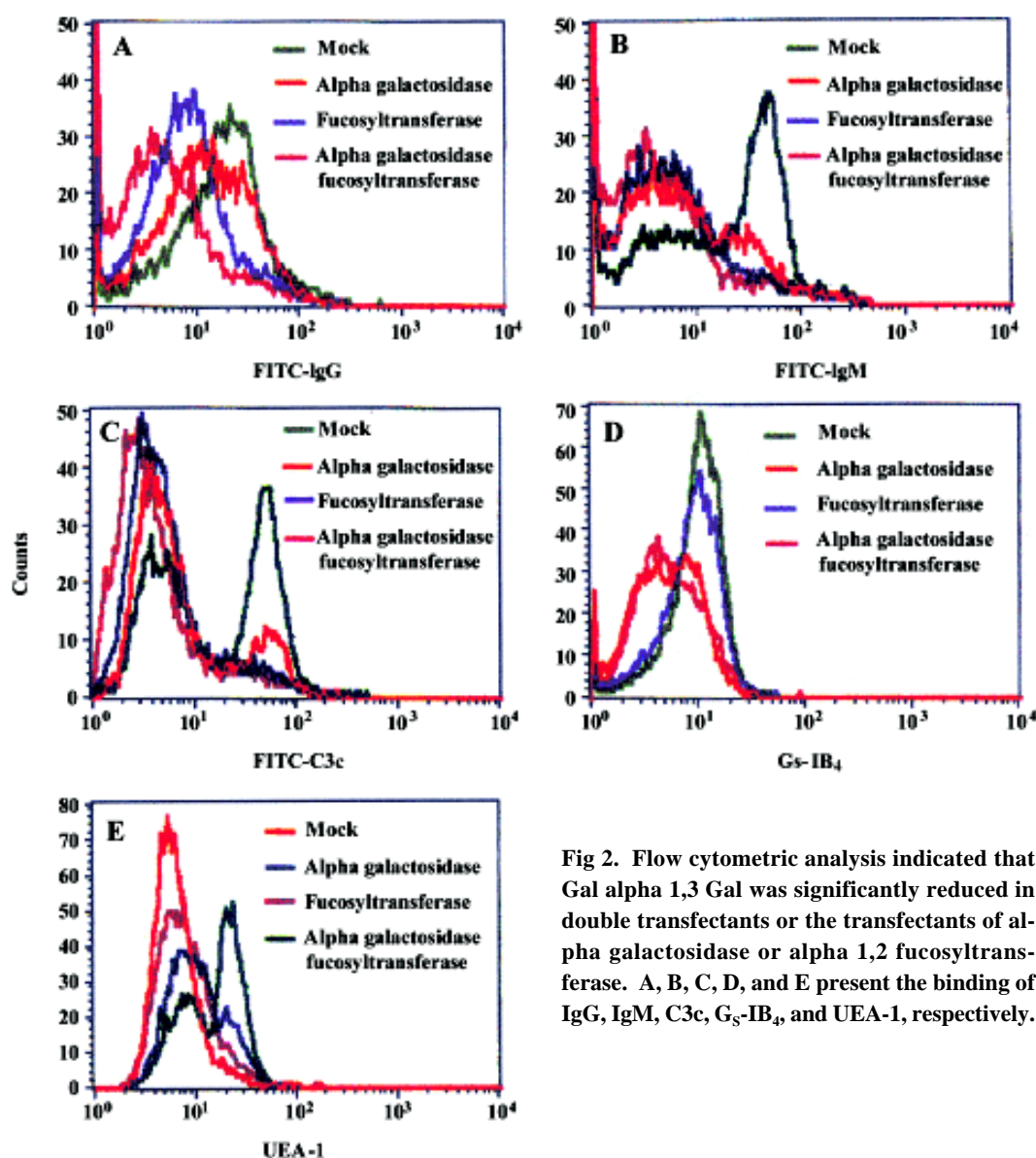


Fig 2. Flow cytometric analysis indicated that Gal alpha 1,3 Gal was significantly reduced in double transfectants or the transfectants of alpha galactosidase or alpha 1,2 fucosyltransferase. A, B, C, D, and E present the binding of IgG, IgM, C3c, G<sub>s</sub>-IB<sub>4</sub>, and UEA-1, respectively.

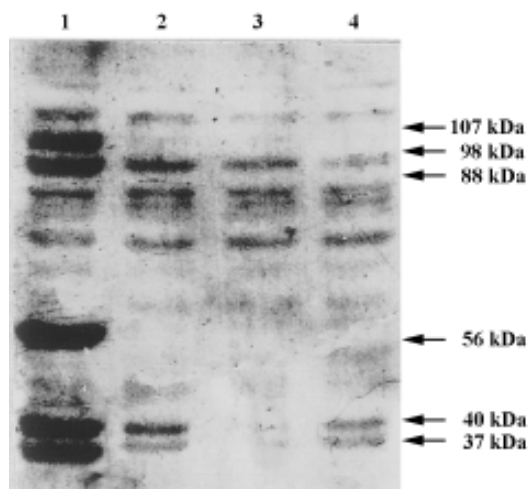
kDa, 88 kDa, 40 kDa, and 37 kDa were decreased. In the samples from combined transfectants, glycoproteins equal to 107 kDa and 88 kDa were decreased to much lower levels and glycoproteins equal to 98 kDa, 56 kDa, 40 kDa, and 37 kDa were abrogated.

**Increase in resistance to cytolysis** Cytolysis of combined transfectants was markedly decreased by 65.5 %, higher than those induced by alpha galactosidase transfectants (42.4 %) and alpha 1,2 fucosyltransferase (51.9 %) alone (Fig 4).

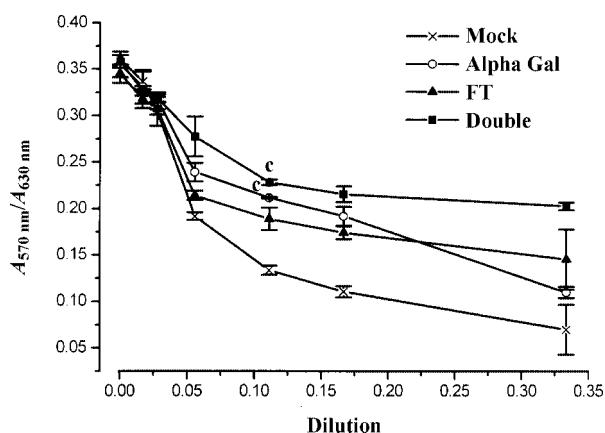
## DISCUSSION

Human alpha galactosidase and alpha 1,2 fucosyltransferase are two of the most competent candidates for the gene modification of Gal alpha 1,3

Gal<sup>[17,24]</sup>. In agreement with the previous studies, the two enzymes significantly inhibited the expression of Gal alpha 1,3 Gal in the present study. However, Western blot showed that alpha galactosidase and alpha 1,2 fucosyltransferase differently influenced the expression of glycoprotein in NIH3T3 cells. In the cells transfected with alpha galactosidase, glycoproteins with molecular weight of 98 kDa and 56 kDa were abrogated totally, those of 40 kDa and 37 kDa were reduced to much lower levels and glycoproteins with molecular weight of 107 kDa and 88 kDa were inhibited. Whereas glycoproteins with molecular weight of 98 kDa, 56 kDa were diminished completely, and those with molecular weight of 107 kDa, 88 kDa, 40 kDa, and 37 kDa were greatly reduced in cells transfected with alpha 1,2 fucosyltransferase. It was consistent



**Fig 3.** Western blot indicates some protein bands (arrow) diminished or decreased in the transfectants of alpha galactosidase, alpha 1,2 fucosyltransferase or double transfectants. Lane 1, 2, 3, and 4 were samples from mock, alpha galactosidase, double transfectants, and alpha 1,2 fucosyltransferase, respectively.



**Fig 4.** Inhibition of human serum-mediated cell lysis by alpha galactosidase and alpha 1,2 fucosyltransferase. \**P*<0.01 vs mock.

with the results of cell surface antigen assay that Gal alpha1, 3 Gal was reduced by 57.4 % and H antigen increased about 6.7-fold in cells transfected with alpha galactosidase, while Gal alpha 1,3 Gal reduced by only 28.8 % and H antigen increased 6-fold in cells transfected with alpha 1,2 fucosyltransferase. The results may be due to the biochemical properties of the two enzymes. According to results of biochemical studies, alpha galactosidase is a lysosomal enzyme that hydrolyses the alpha galactosyl residues from glycosphingolipids and glycoproteins<sup>[25]</sup>, whereas alpha 1,2 fucosyltransferase competes with alpha 1,3 galactosyltransferase

for the common substrate *N*-acetylactosamine and caps it with the nonimmunogenic fucose to synthesize the universal donor H antigen<sup>[14,26]</sup>.

As viewed from the xenoreactivity with human natural antibodies, alpha 1,2 fucosyltransferase was more potential than alpha galactosidase. The xenoreactivity with human IgG was reduced by 67.0 % after transfection with alpha 1,2 fucosyltransferase, higher than the result from the transfection of alpha galactosidase (61.4 %) and the resistance to cytolysis in cells transfected with alpha 1,2 fucosyltransferase is significantly stronger than that obtained from alpha galactosidase.

Combined transgenic expression of alpha galactosidase and alpha 1,2 fucosyltransferase has been presumed as an optimal strategy for gene modification of Gal alpha 1, 3 Gal. Since degalactosylation results in the exposure of *N*-acetylactosamine residue which is a new xenoreactive epitope, alpha galactosidase alone is unlikely to overcome xenorejection<sup>[17,24]</sup>. Coexpression with alpha 1,2 fucosyltransferase may enhance the reduction of Gal alpha 1,3 Gal and mask the new xenoreactive epitope by fucosylation, which leads to an optimal inhibition of xenoreactivity with natural antibodies. The previous study to examine the assumption was carried out on COS cell which was transfected with alpha 1,3 galactosyltransferase and subsequently expressed Gal alpha 1,3 Gal<sup>[27]</sup>. However, Gal alpha 1,3 Gal-positive COS cell expressing alpha 1,3 galactosyltransferase, alpha galactosidase, and alpha 1,2 fucosyltransferase showed negligible cell surface staining and was not susceptible to lysis by human serum containing antibodies and complements. In agreement with the previous study, our results proved the assumption in NIH3T3 cells. As indicated by cell surface antigen assay and immunological assay, the combined transfection with alpha galactosidase and alpha 1,2 fucosyltransferase led to a greater decrease in the expression of Gal alpha 1,3 Gal (about 72.1 %) and in the xenoreactivity with human IgM (36.6 %) than the transfection with alpha galactosidase or alpha 1,2 fucosyltransferase alone. Subsequently, the resistance to cell lysis was increased to a higher level, about 65.5 %. What is more, Western blot proved the concordant inhibitory effects of the two enzymes on the synthesis of glycoproteins with molecular weight of 107 kDa, 98 kDa, 88 kDa, 56 kDa, 40 kDa, and 37 kDa in the present study.

However, in contrast with the previous study, our results showed that coexpression of alpha galactosi-

dase and alpha (1,2) fucosyltransferase did not abrogate xenoreactivity with human natural antibodies. It may be due to the difference of cells. In the present study, all experiments were carried out on NIH3T3 cell which is mouse fibroblast with strong endogenous expression of Gal alpha 1,3 Gal, while the previous study employed COS cell and artificially induced the expression of Gal alpha 1,3 Gal by transfection with alpha 1,3 galactosyltransferase<sup>[27]</sup>. In the view of this point, the present study may embody the *in vivo* function of alpha galactosidase and alpha 1,2 fucosyltransferase more actually.

In conclusion, although alpha galactosidase and alpha 1,2 fucosyltransferase maintain different biochemical properties, they could concordantly inhibit the expression of Gal alpha 1,3 Gal, resulting in the stronger resistance of xenograft to cell lysis mediated by natural antibodies and complements. It implies a practical significance of combined transgenic strategy with alpha galactosidase and alpha 1,2 fucosyltransferase in xenotransplantation.

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