

## Adenovirus-mediated expression of human secretor type $\alpha(1,2)$ fucosyltransferase reduces level of $\text{Gal}\alpha(1,3)\text{Gal}$ epitop<sup>1</sup>

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**KEY WORDS** human adenoviruses; fucosyltransferases; gene expression; antigens; epitopes

### ABSTRACT

**AIM:** To test the potential of human secretor type  $\alpha(1,2)$  fucosyltransferase [ $\text{Sea}(1,2)\text{FT}$ ] to downregulate the expression of  $\text{Gal}\alpha(1,3)\text{Gal}$  epitope (gal epitope) in cultured cell lines. **METHODS:** Expression of  $\text{Sea}(1,2)\text{FT}$  was mediated by human adenoviral vector. Flow cytometric analysis was used to compare the expression level of H blood group antigen or gal epitope. MTT was employed to assess the susceptibility of mouse NIH3T3 cells to human natural antibody and complement mediated lysis. **RESULTS:** A recombinant replication-deficient adenovirus (rAdv) containing human  $\text{Sea}(1,2)\text{FT}$  cDNA (Ad5hSeFT) was designed and successfully constructed. Flow cytometric analysis showed that after mock infection, Ad5null infection, and Ad5hSeFT infection, the mean fluorescence intensity (MFI) values for the binding of *Ulex europaeus* I (UEA-I) lectin to NIH3T3 cells were  $2.3 \pm 0.6$ ,  $2.1 \pm 1.0$ , and  $36.5 \pm 5.9$ , respectively; MFI values for the binding of *Griffonia simplicifolia* isolectin B4 (GS-IB4) lectin to NIH3T3 cells were  $167 \pm 23$ ,  $170 \pm 19$ , and  $100 \pm 14$ , respectively; MFI values for the binding of human natural IgG and IgM antibodies to NIH3T3 cells were  $31 \pm 3$ ,  $32 \pm 4$ , and  $22 \pm 4$ , respectively. **CONCLUSION:** H blood group antigen was detected on NIH3T3 cells after Ad5hSeFT infection and resulted in more than 40 % reduction in the level of gal epitope on the cell surface. This reduction increased the resistance of NIH3T3 cells to lysis by normal human serum.

### INTRODUCTION

One of major obstacles to xenotransplantation of pig organs into humans is the occurrence of hyperacute rejection (HAR) caused by the binding of natural human IgG and IgM antibodies to the antigenic molecules on the membrane of pig cells, particularly endothelial cells in vascularized organs, followed by the complement cascade reaction. The major antigenic molecule is carbohydrate epitope  $\text{Gal}\alpha(1,3)\text{Gal}$  (gal epitope) synthesized by  $\alpha(1,3)$ galactosyl transferase [ $\alpha(1,3)\text{GT}$ ]<sup>[1]</sup>. It has been reported that the downregulation or knockout of  $\alpha(1,3)\text{GT}$  gene can suppress HAR<sup>[2,3]</sup>. Gal epitope is present in pigs, mice, and New World monkeys, but is replaced with H blood group antigen in humans, apes, and Old World monkeys due to lack of a functional  $\alpha(1,3)\text{GT}$  gene<sup>[4,5]</sup>. H blood group antigen is synthesized by  $\alpha(1,2)$  fucosyltransferase [ $\alpha(1,2)\text{FT}$ ]<sup>[6]</sup>. In human tissues there are at least two distinct  $\alpha(1,2)\text{FT}$ , one is H type and another is secretor type<sup>[7]</sup>. For downregulation of gal epitope expression in xenotransplantation, the H type  $\alpha(1,2)\text{FT}$  has been examined by several groups<sup>[3,8-11]</sup>. This study is to test the secretor type  $\alpha(1,2)\text{FT}$  for the same purpose.

### MATERIALS AND METHODS

**Cell lines** The 293 cells (provided by Prof XUE Jing-Lun, Institute of Genetics, Fudan University, China) and mouse NIH3T3 cells [Type Culture Collection of Chinese Academy of Sciences (TCCAS)] were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) containing 10 % heat-inactivated newborn bovine serum (NBS). Chinese Hamster Ovary (CHO) cells (TCCAS) were maintained in RPMI-1640 medium (Gibco, BRL) supplemented with 10 % NBS.

**PCR amplification** PCR amplification was performed in 50  $\mu\text{L}$  reaction mixture containing 10 pmol of each primer, Taq DNA polymerase 1.5 units, dNTP 400  $\mu\text{mol/L}$ , and  $1 \times$  Taq reaction buffer. The temperature profile was as follows: one cycle consisted of denatura-

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tion at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min; 30 cycles for PCR and 35 cycles for RT-PCR.

**RNA extraction and RT-PCR** Total RNA was extracted using TRIzol RNA isolation reagent (Life Technologies) from human peripheral blood lymphocytes. One strand of the cDNA was then synthesized with Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies). The cDNA corresponding to the open reading frame (ORF) of human *Sea*(1,2) FT<sup>(7)</sup> was obtained by RT-PCR, using the following primers: P1 5'-CGG GAA TTC AGC CAT GCT GGT CGT TCA G-3' (28-mer, 59-78, containing EcoR I site); P2 5'-CAA CTC GAG CTA TTA GTG CTT GAG TAA GG-3' (29-mer, 1079-1096, containing Xho I site). The PCR product of 1.0 kb in length was cloned into pCDNA3 and sequenced, then subcloned into adeno virus shuttle plasmid pAdCMV (S)-BGHpA to construct pAd-SeFT.

**Viral vectors** The recombinant replication-deficient type 5 adenoviral vectors (rAdvs) Ad5null, Ad5 $\beta$ gal, and Ad5hSeFT were used in this study. Viruses were produced by homologous recombination of the pJM17 containing the dl309 genome<sup>(12)</sup> with shuttle plasmids pAdCMV (S)-BGHpA, pAd-lacZ, and pAd-SeFT, respectively, in 293 cells. Viruses from the resulting plaques were plaque-purified thrice. Ad5null contains no exogenous gene in its genome and was used as a control. Ad5 $\beta$ gal contains the *E coli lacZ* gene encoding  $\beta$ -galactosidase, under the transcriptional control of a human cytomegalovirus (CMV) promoter. High titers of viruses were prepared, titered, and stored according to the methods described earlier<sup>(13)</sup>.

**Adv infection** Bacterial  $\beta$ -galactosidase ( $\beta$ -gal) gene expression was used as a marker of viral infection efficiency. CHO and NIH3T3 cells were tested using Ad5 $\beta$ gal containing *E coli lacZ* gene. Exponentially growing cells were seeded in duplicate in 24-well tissue culture plates at a density of  $4 \times 10^4$  cells/well and infected with Ad5 $\beta$ gal virus at different multiplicities of infection (MOI) at the same time. After 48 h cells were fixed with 0.5 % glutaraldehyde and then stained with X-gal solution (Sigma). Positive cells infected with Ad5 $\beta$ gal expressed  $\beta$ gal activity and developed blue color. The results were analyzed qualitatively by visualization and captured by photomicrography.

**Lectin binding assay**<sup>(2)</sup> The cells detached from the tissue culture flasks 48 h after infection with rAdvs were fixed in 4 % paraformaldehyde, washed, and stained with fluorescein isothiocyanate (FITC)-conjugated lectins (Sigma) [*Griffonia simplicifolia* isolectin B4

(GS-IB4) specific for gal epitope; *Ulex europaeus* I (UEA-I) specific for H blood group antigen<sup>(3)</sup>] for 1 h at 25 °C and then subjected to flow cytometric analysis. For CHO cells maintained in monolayer, the fluorescence of cell surface carbohydrate epitope was also examined directly with FITC-UEA-I lectin.

**Human IgG and IgM preparations**<sup>(14,15)</sup> Normal human serum samples were obtained from 5 healthy blood donors with blood group B and stored at -70 °C. IgG and IgM were purified from human sera using sequential ammonium sulfate precipitations and polyethylene glycol precipitations, respectively. Final protein pellets were dissolved in phosphate-buffered saline (PBS, pH 7.4) containing 0.01 % Na<sub>3</sub>. Igs were quantified by OD readings (at 280 nm), with purity monitored by silver staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Flow cytometry** NIH3T3 cells were detached from the tissue culture flasks by brief treatment with edetic acid 5 mmol/L for 48 h after infection with rAdvs, fixed in 4 % paraformaldehyde, incubated with polyclonal human IgG and IgM antibodies at 37 °C for 1.5 h, washed, and then stained by FITC-conjugated rabbit anti human IgG and IgM antibodies (DAKO) at 37 °C for 1 h. Stained cells were analyzed with a Becton Dickinson FACScan cytometer.

**MTT** According to previously described method<sup>(16)</sup> with some modifications NIH3T3 cells were grown in 96-well plates, two days after rAdvs infection, the cells were treated for at 37 °C 30 min with normal human sera at varying concentrations. A stock solution of MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide 5 g/L in PBS 0.01 mol/L, NaCl 0.14 mol/L, pH 7.2] was prepared. This solution 20  $\mu$ L was added to each well of a 96-well plate. Cells were incubated at 37 °C for 4 h. MTT was converted into an insoluble purple formazan via cleavage of the tetrazolium ring by mitochondrial dehydrogenase<sup>(17)</sup>. After that, the medium was exhausted and the cells were lysed with 100  $\mu$ L Me<sub>2</sub>SO to release the purple product from the cells. The cells were incubated for a further 10 min at 25 °C with gentle shaking and absorbance readings were recorded by a computer controlled micro-plate analyser. Cell survival was expressed as the amount of dye reduction based on the absorbance at 490 nm (reference at 630 nm).

**Statistical analysis** Data were expressed as  $\bar{x} \pm s$  and analyzed by *t*-test.

## RESULTS

**Efficiency of rAdv infection** Following infec-

tion with Ad5 $\beta$ gal, CHO and NIH3T3 cells exhibited different efficacies of rAdv infection. However, more than

80 % of infectivity was achievable for both cell lines at different optimum MOI (Fig 1). The optimum MOI

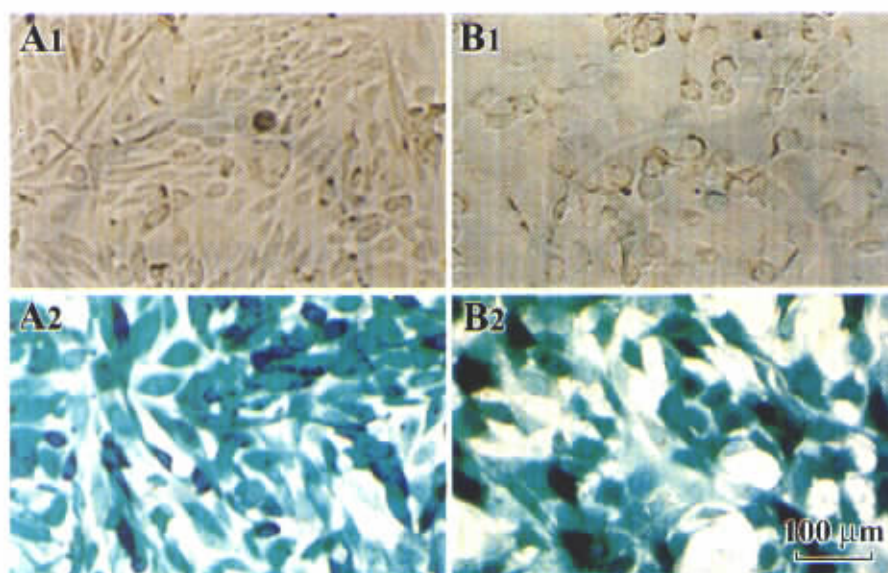


Fig 1.  $\beta$ Gal gene expression 48 h after infection with Ad5 $\beta$ gal in CHO cells. (A) at 50 MOIs as well as in NIH3T3 cells; (B) at 200 MOIs (1. Mock infection. 2. Ad5 $\beta$ gal infection).  $\times 100$ .

was 40 – 50 for CHO cells, 150 – 200 for NIH3T3 cells.

**Characterization of adenoviral vector expressing human  $Se\alpha(1,2)FT$**  After double digestion of recombinant plasmid pAd-SeFT with Hind III and Xba I, a DNA fragment of about 1.0 kb in length was released. This result indicated that adenoviral shuttle vector containing  $Se\alpha(1,2)FT$  cDNA was what we needed (Fig 2A). Cotransfection of 293 cells with pAd-SeFT and the pJM17 resulted in the generation of Ad5hSeFT. After subcloning of the virus pool, individual viral plaques were obtained. DNA extracted from three viral plaques were examined with PCR analysis and a DNA fragment of about 1.0 kb in length was developed by PCR amplification from Ad5hSeFT DNAs, but not from Ad5null and 293 cell genomic DNA (Fig 2B). These results showed that the  $Se\alpha(1,2)FT$  cDNA had been inserted into the adenoviral genome and the recombinant adenovirus expressing human  $Se\alpha(1,2)FT$  had been successfully constructed (Fig 3).

CHO cells have been successfully used to investigate the effects of newly introduced human H type  $\alpha(1,2)FT$  on glycoconjugate biosynthesis<sup>[18]</sup>. In this study, after infection with Ad5hSeFT we also detected the H blood group antigens on CHO cell surface (Fig 4) due to the expression of  $Se\alpha(1,2)FT$ . This verified the expression of human  $Se\alpha(1,2)FT$  cDNA integrated in Ad5hSeFT genome.

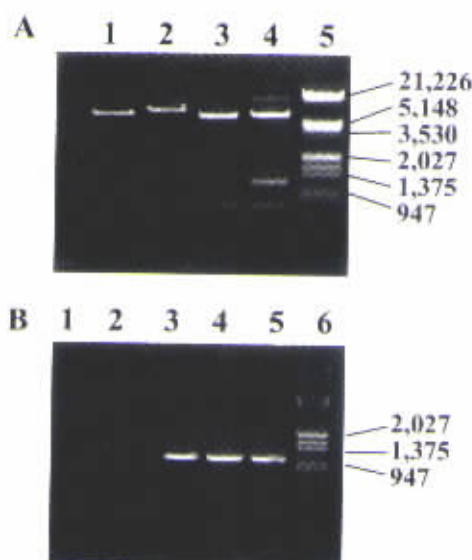
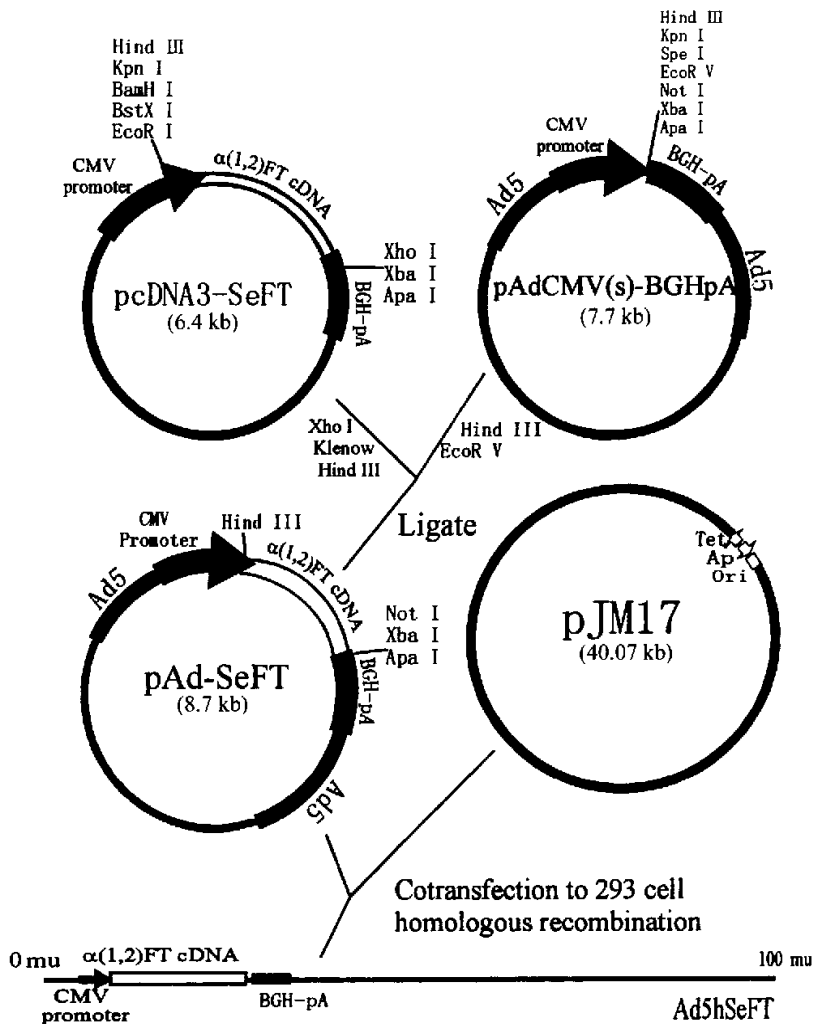


Fig 2. (A) Restriction analysis of recombinant plasmid. 1) pAdCMV (S)-BGHpA/Hind III. 2) pAd-SeFT/Hind III. 3) pAdCMV (S)-BGHpA/Hind III + Xba I. 4) pAd-SeFT/Hind III + Xba I. 5)  $\lambda$ DNA/EcoR I + Hind III. (B) PCR determination of integration of  $Se\alpha(1,2)FT$  cDNA into Ad5hSeFT genome. 1) 293 cell DNA. 2) Ad5null DNA. 3), 4), 5) Ad5hSeFT DNA. 6)  $\lambda$ DNA/EcoR I + Hind III.

**Reduction of  $Gal\alpha(1,3)Gal$  expression on NIH3T3 cell membrane after infection with Ad5hSeFT** To determine whether expression of  $Se\alpha(1,2)FT$  would downregulate the gal epitope on cell



**Fig 3. Construction of Ad5hSeFT expressing human *Sea*(1,2)FT.** Human *Sea*(1,2)FT cDNA obtained by RT-PCR was cloned into pcDNA3 to construct the recombinant plasmid pcDNA3-SeFT and then was subcloned into a shuttle vector pAdCMV (S)-BGHpA which harbored a cytomegalovirus promoter (CMV) and a BGH poly A signal. The resulting plasmid pAd-SeFT was cotransfected with a virus-rescuing vector pJM17 into 293 cells and then the recombinant adenovirus Ad5hSeFT expressing human *Sea*(1,2)FT was generated.

membranes, the mouse NIH3T3 cells, which express high levels of gal epitope<sup>(19)</sup>, were used as a model system. Flow cytometric analysis demonstrated that Ad5hSeFT-infected NIH3T3 cells highly expressed H antigen on cell membrane (Tab 1), whereas the level of gal epitope on the cell surface was significantly decreased when compared with the control (Tab 2). After Ad5hSeFT infection, the mean fluorescence intensity (MFI) value of H blood group antigen on the surface of NIH3T3 cells was increased by about 18-fold, and that

of gal epitope was reduced by about 40 %.

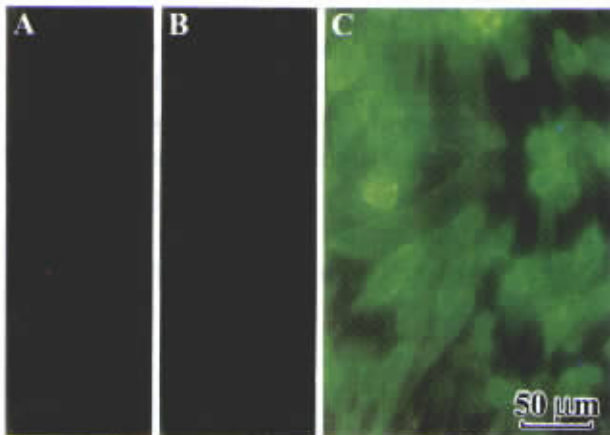
**Tab 1. Flow cytometric MFI values for the binding of FITC-UEA-I lectin specific for H blood group antigen to NIH3T3 cells infected with rAdvs.** n = 3 experiments.  $\bar{x} \pm s$ . \*P > 0.05, \*P < 0.01 us control (mock infection).

Control	Ad5null	Ad5hSeFT
2.3 ± 0.6	2.1 ± 1.0 <sup>a</sup>	36.5 ± 5.9 <sup>a</sup>



**Tab 2.** Flow cytometric MFI values for the binding of FITC-GS-IB4 lectin specific for gal epitope to NIH3T3 cells infected with rAdvs.  $n = 3$  experiments.  $\bar{x} \pm s$ .  $^aP > 0.05$ ,  $^cP < 0.01$  vs control (mock infection).

Control	Ad5null	Ad5hSeFT
167 ± 23	170 ± 19 <sup>a</sup>	100 ± 14 <sup>c</sup>



**Fig 4.** FITC-UEA-I lectin staining of the surface of CHO cells after: A) mock infection, B) Ad5null infection, and C) Ad5hSeFT infection. Results are representatives of at least 3 experiments.  $\times 200$ .

#### Adenovirus-mediated $Se\alpha(1,2)$ FT expression results in decrease in natural human antibody binding to gal epitope and makes NIH3T3 cells resistant to lysis by normal human serum

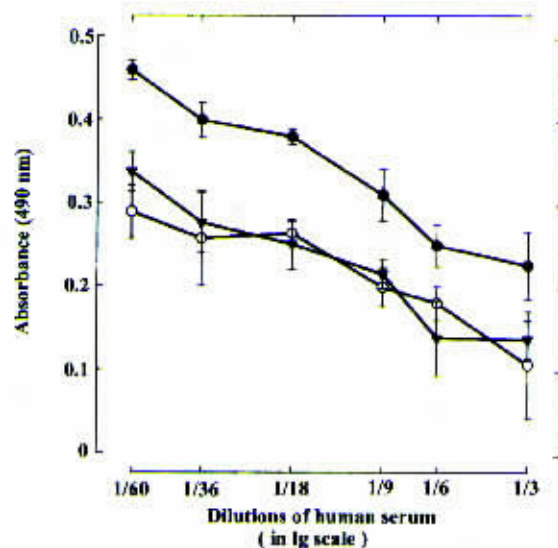
To examine the effect of decrease in gal epitope expression on the binding of natural human antibodies to cells, NIH3T3 cells were analyzed with flow cytometry for their ability to bind natural human antibodies 48 h after infection with rAdv. After Ad5hSeFT infection, the NIH3T3 cells showed a 30 % reduction in binding to natural human IgG and IgM antibodies when compared with parental and Ad5null-infected NIH3T3 cells (Tab 3).

**Tab 3.** Flow cytometric MFI values for the binding of human natural IgG and IgM antibodies to NIH3T3 cells infected with rAdvs.  $n = 3$  experiments.  $\bar{x} \pm s$ .  $^aP > 0.05$ ,  $^cP < 0.01$  vs control (mock infection).

Control	Ad5null	Ad5hSeFT
31 ± 3	32 ± 4 <sup>a</sup>	22 ± 4 <sup>c</sup>

To assess the functional significance of this ob-

served decrease in binding of natural human antibodies to cells, rAdv-infected NIH3T3 cells were used to detect their susceptibility to normal human serum mediated lysis by virtue of MTT method. The results indicated that at dilutions of human serum in the range of 1/60 to 1/3, the susceptibility of Ad5hSeFT-infected NIH3T3 cells to human antibody-mediated lysis was decreased when compared with that of parental cells and Ad5null-infected cells (Fig 5). Overall, the results showed that the expression of  $Se\alpha(1,2)$  FT in NIH3T3 cells had effects in eliminating gal epitope on NIH3T3 cells.



**Fig 5.** Cytotoxicity of normal human serum to NIH3T3 cells after rAdvs infection.  $\blacktriangledown$  mock infection,  $\circ$  Ad5null infection,  $\bullet$  Ad5hSeFT infection. Data is representative of at least 4 experiments.  $\bar{x} \pm s$ .

## DISCUSSION

Because the major target of natural human antibodies in the hyperacute rejection of discordant xenotransplantation is the gal epitope<sup>[1]</sup>, strategies to prevent anti-Gal $\alpha(1,3)$ Gal antibody reactivity are designed to remove the gal epitope. One strategy to down-regulate the gal epitope is to knock out the  $\alpha(1,3)$ GT gene using embryonic stem cells. But this system is not yet available in the pig due to the unavailability of porcine embryonic stem cells although inactivation of  $\alpha(1,3)$ GT gene by knockout techniques has been successful in mice<sup>[2,20,21]</sup>. Another strategy for down-regulating the gal epitope is to take advantage of enzymatic competition between  $\alpha(1,3)$ GT and  $\alpha(1,2)$ FT for a common acceptor substrate, *N*-acetyllactosamine (Gal $\beta$ 1-4GlcNAc-R)<sup>[22]</sup>. Cells

transfected with human H type  $\alpha(1,2)$  FT cDNA expressed high level of the H blood group antigen and a markedly reduced expression of the gal epitope<sup>[22]</sup>. The current study demonstrated that human  $Se\alpha(1,2)$  FT was as efficient as H type  $\alpha(1,2)$  FT in downregulation of the gal epitope expression. In addition, the recombinant adenoviral vector containing  $Se\alpha(1,2)$  FT cDNA, Ad5hSeFT, could be used to directly transfer  $Se\alpha(1,2)$  FT gene into pig endothelium without embryonic manipulation.

Since human and other Old World primates lack the gal epitope, they are not immunotolerant to it and produce anti-gal epitope antibody (anti-gal) throughout life in response to antigenic stimulation by gastrointestinal bacteria. In natural human antibodies, anti-gal is found in all four IgG subclasses, and also in IgM and IgA isotypes. In general, the IgM isotype of anti-gal fixes complement and induces complement-mediated lysis of the xenograft cells. Furthermore, the binding of anti-gal IgG to gal epitopes expressed on glycolipids and glycoproteins of xenograft cells results in antibody-dependent cell-mediated cytotoxicity (ADCC)<sup>[23]</sup>. So we examined the binding of both IgG and IgM human antibody isotypes to NIH3T3 cells after rAdv-mediated expression of human  $Se\alpha(1,2)$  FT and the results revealed a reduction in human immunoglobulin deposition compared with control. However, significant deposits of both IgG and IgM were still evident. This residual antibody reactivity represents human Ig binding to non-gal epitopes as well as gal epitopes which can not be replaced with H blood group antigen. Despite the residual natural human antibody reactivity, Ad5hSeFT-transduced NIH3T3 cells showed a decrease in cytotoxicity on exposure to human antibody and complement when compared with parental and Ad5null-transduced NIH3T3 cells.

Sandrin *et al*<sup>[22]</sup> reported that expression of human H type  $\alpha(1,2)$  FT resulted in an approximately 70 % decrease in the gal epitope expression in pig epithelial PLL-K1 cells. However, Sepp *et al*<sup>[11]</sup> observed that H type  $\alpha(1,2)$  FT resulted in only a 40 % decrease in the gal epitope expression in PLECT cells. Here, the data relative to  $Se\alpha(1,2)$  FT are in good agreement with the latter study. These differences can be explained as follows: (1) Reduction of gal epitope expression that occurs through competition with  $\alpha(1,2)$  FT and which is likely to be cell type-specific as described by Chen *et al*<sup>[10]</sup> and can be determined by the individual protein repertoires of different cells. (2)  $Se\alpha(1,2)$  FT may cause a different type of glycosylation from that developed by H-type  $\alpha$

(1,2) FT<sup>[18]</sup>. (3) Different level of endogenous  $\alpha(1,3)$  GT activity in different cells.

In conclusion, the transduction of the human  $Se\alpha(1,2)$  FT gene by an adenoviral vector is effective to inhibit gal epitope expression *in vitro*. This suggests that it would be an alternative method for prevention of HAR in xenotransplantation.

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### 腺病毒载体介导的人分泌型 $\alpha$ (1,2)岩藻糖基转移酶的表达降低 Gal $\alpha$ (1,3)Gal 抗原表位水平<sup>1</sup>

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**关键词** 人腺病毒; 岩藻糖基转移酶类; 基因表达; 抗原; 表位

**目的:** 检测人分泌型  $\alpha$ (1,2)岩藻糖基转移酶降低 Gal $\alpha$ (1,3)Gal 表位(gal 表位)水平. **方法:** 以人腺病毒载体表达人分泌型  $\alpha$ (1,2)岩藻糖基转移酶. 流式细胞术比较 H 血型抗原和 gal 表位的表达水平. MTT 法分析小鼠 NIH3T3 细胞对人天然抗体和补体介导的细胞裂解作用的敏感性. **结果:** 设计并构建了表达人分泌型  $\alpha$ (1,2)岩藻糖基转移酶基因的复制缺陷型重组腺病毒载体 Ad5hSeFT. 流式细胞分析结果表明, NIH3T3 细胞及 Ad5null 和 Ad5hSeFT 感染后的 NIH3T3 细胞, UEA-I 凝集素结合细胞的平均荧光强度 (MFI) 分别为  $2.3 \pm 0.6$ ,  $2.1 \pm 1.0$  和  $36.5 \pm 5.9$ ; GS-IB4 凝集素结合细胞的 MFI 值分别为  $167 \pm 23$ ,  $170 \pm 19$  和  $100 \pm 14$ ; 人天然 IgG 和 IgM 抗体结合细胞的 MFI 值分别为  $31 \pm 3$ ,  $32 \pm 4$  和  $22 \pm 4$ . **结论:** NIH3T3 细胞被 Ad5hSeFT 感染后在细胞表面表达了 H 血型抗原, 导致细胞表面 gal 表位的表达下降了 40%, 并且这种下降增强了细胞对正常人血清裂解作用的抵抗力.

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