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Adenovirus-mediated expression of antisense RNA transcripts complementary to pig α(1,3) galactosyltransferase mRNA inhibits expression of Gal α(1,3) Gal epitope[1]

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KEY WORDS human adenoviruses; antisense RNA; galactosyltransferases; fucosyltransferases; gene expression; epitopes

ABSTRACT

AIM: To examine the effects of the expression of antisense RNA transcripts complementary to the pig α(1,3) galactosyltransferase [α(1,3)GT]mRNA on the expression of Gal α(1,3) Gal structure (gal epitope) in cultured cell lines. **METHODS**: Human adenoviral vectors were used to mediate the expression of antisense RNA. The expression levels of H blood group antigens and gal epitopes were analyzed by flow cytometry using FITC-UEA-I and FITC-GS-IB4 lectins, respectively. **RESULTS**: Recombinant adenoviruses, Ad5anti-sGT600 and Ad5-anti-sGT1100, which express antisense RNA complementary to different regions of the pig α(1,3) GT mRNA, were constructed and used to infect cell line of NIH3T3. The results showed about 30 % reduction in the expression level of gal epitopes on the surface of NIH3T3 cells. In addition, co-expression of human secretor type α(1,2) fucosyltransferase [α(1,2)FT]cDNA and antisense RNA complementary to the pig α(1,3) GT mRNA led to a further reduction in the gal epitope level. **CONCLUSION**: Recombinant adenoviruses, Ad5anti-sGT600 and Ad5anti-sGT1100, are effective to down-regulate the gal epitope expression.

INTRODUCTION

Galα(1,3) Gal epitope (gal epitope) is synthesized in the trans-Golgi by UDP-Gal: βl − 4GlcNAcα 1,3-galactosyltransferase [α(1,3) GT; EC 2.4.1.51], which catalyzes the addition of galactose to an *N*-acetyllactosamine (*N*-lac) core, and plays a very important role in complement-dependent hyperacute rejection (HAR) as well as complement independent delayed xenograft rejection (DXR) in the xenograft of a vascularized organ between discordant species, such as swine and human[1].

It would be clearly advantageous to develop a pig model that did not express gal epitope. However, embryonic stem cells have not yet been established for the construction of α(1,3) GT knockout pig. Hayashi *et al*[2] proposed that adenovirus-mediated gene transfer might be an excellent strategy for *in vivo* gene transduction to remove the gal epitope from swine endothelium. At present, recombinant adenoviruses (rAdv) expressing different exogenous genes, such as antisense ribozyme for α(1,3) GT gene and α(1,2) fucosyltransferase gene, have been constructed and tested *in vitro*[2-4].

In this study, we constructed two rAdv expressing antisense RNA complementary to the different regions of swine α(1,3) GT mRNA and examined their inhibitory effects on the expression of gal epitope in NIH3T3 cells.

MATERIALS AND METHODS

Cells The 293 cells, BEL-7404 cells (human liver cancer cell line), and NIH3T3 cells were all from Type Culture Collection of Chinese Academy of Sciences and were maintained in Dulbecco's modified Eagle's medium (Gibco, BRL) with 10 % heat-inactivated newborn bovine serum. The 293 cells were used for adenovirus transfection, amplification, and titration.

PCR amplification PCR was performed in 50 μL reaction mixture containing 10 pmol of each primer, Taq DNA polymerase 1.5 u (Sino-American Biotechnology Co), dNTP 400 μmol/L, and 1 × Taq reaction

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buffer. The temperature profile was as follows: 1 cycle of denaturation at 94 ℃ for 1 min; 30 cycles of 94 ℃ for 1 min, 44 ℃ for 1 min, 72 ℃ for 1 min; 1 cycle of extension at 72 ℃ for 10 min. PCR products were analyzed by 0.7 % agarose gel electrophoresis and stained with ethidium bromide.

RNA extraction and RT-PCR Total RNA was extracted using TRIzol total RNA isolation reagent according to the manufacturer's instructions (Life Technologies). The first-strand cDNAs were then synthesized by Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies) at 37 ℃ in the presence of dNTP 200 μmol/L, DTT 0.01 mol/L, RNase 20 u with random hexamers in a total volume of 20 μL. The pig cDNA corresponding to the open reading frame (ORF) of α1,3GT mRNA[5] was obtained by RT-PCR from pig spleen total RNA using the following primers: P1 5′-GGC TCG AGC ATG AGG AGA AAA TAA TGA ATG-3′ (30-mer, 1-22, containing Xho I site); P2 5′-GGG AAT TCT GGA GAA GTA GCC AGA GTA ATA (30-mer, 1193-1170, containing EcoR I site); P3 5′-GGG AAT TCT GGT CTT CAT GCG CAT CAT G (28-mer, 640-621, containing EcoR I site). The cDNA corresponding to ORF of mouse α(1,3) GT mRNA could also be amplified by RT-PCR using these primers. RT-PCR produced a 1.19 kb DNA fragment using P1 and P2, and yielded a 0.6-kb DNA fragment using P1 and P3. These two fragments were cloned into pcDNA3 and sequenced, then subcloned into adenovirus shuttle plasmid pAdCMV (S)-BGHpA to construct the recombinant plasmids pAd-sGT1100 and pAd-sGT600.

Northern blot analysis Briefly, total RNA 30 μg was separated on a 1.2 % agarose formaldehyde gel and transferred onto a Hybond nylon membrane (Amersham). Blots were then hybridized in a buffer containing [α-^{32}P]dATP-labeled DNA probe for pig α(1,3) GT cDNA and autoradiographed.

Viral vectors The rAdv, Ad5null, Ad5hSeFT, Ad5anti-sGT1100, and Ad5anti-sGT600 were used in this study. Viruses were produced by homologous recombination of the pJM17 containing the dl309 genome[6] with shuttle plasmids pAdCMV (S)-BGHpA, pAd-SeFT, pAd-sGT1100, and pAd-sGT600 in 293 cells, respectively. Viruses from the resulting plaques were plaque-purified for thrice. Ad5null does not contain exogenous gene in its genome and was used as a control. Ad5hSeFT expressing human secretor type α(1,2) fucosyltransferase [α(1,2)FT] which determines the production of human blood group H antigen is described in detail in another paper[4]. Viruses of Ad5anti-sGT1100 and Ad5anti-sGT600 were characterized by PCR analysis. High titers of viruses were prepared, titered, and stored according to the methods described earlier[7].

Flow cytometric analysis The cells detached from the tissue culture flasks 48 h after transduction with rAdv were fixed in 4 % paraformaldehyde for 10 min, washed, and stained with fluorescein isothiocyanate (FITC)-conjugated lectins (Sigma) [Two kinds of lectins were used, *Griffonia simplicifolia* isolectin B4 (GS-IB4)[8] specific for gal epitope and *Ulex europaeus* Ⅰ (UEA-Ⅰ)[8] specific for H blood group antigen] at 25 ℃ for 1 h. After washing, the stained cells were analyzed with a Becton Dickinson FACScan cytometer.

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

RESULTS

Construction and characterization of recombinant adenoviruses Ad5anti-sGT600 and Ad5-anti-sGT1100 After double digestion of recombinant plasmid pAd-sGT1100 with Hind Ⅲ and Xba Ⅰ, a DNA fragment of about 1.19 kb in length was released (Fig 1A). pAd-sGT600 was characterized by the occurrence of a 1.3-kb DNA fragment after digestion with Xba I (Fig 1B). The results indicated that adenoviral shuttle vectors containing α(1,3) GT cDNA fragments placed in an antisense orientation under the control of human cytomegalovirus (CMV) promoter had been constructed.

Two rAdv, Ad5anti-sGT600 and Ad5anti-sGT1100, were generated by cotransfection of pJM17 with pAd-sGT600 and pAd-sGT1100 into 293 cells respectively (Fig 2). After subcloning of the virus pool, individual viral plaques were obtained. DNA extracted from viruses were analyzed by PCR using pig α(1,3) GT cDNA specific primers (P1, P2, and P3) and the DNA fragments of 1.19 kb and 0.6 kb in length were detected in DNA from Ad5anti-sGT1100 and Ad5anti-sGT600 viruses, respectively (Fig 3). The results showed that α(1,3) GT cDNA fragments had been inserted into the adenoviral genomes, and that recombinant adenoviruses Ad5anti-sGT600 and Ad5anti-sGT1100 had been successfully constructed.

BEL-7404 is a human tumor cell line and does not express the gal epitope. Moreover, we could not detect any mRNA transcribed in this cell line by RT-PCR using primers P1, P2, and P3. Therefore, BEL-7404 was

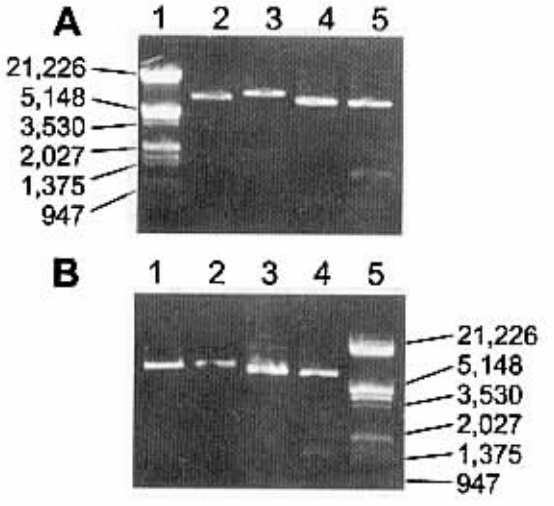

Fig 1. Restriction analysis of recombinant plasmids: (A) 1) λDNA/EcoR Ⅰ + Hind Ⅲ; 2) pAdCMV(S)-BGHpA/Hind Ⅲ; 3) pAd-sGT1100/Hind Ⅲ; 4) pAd-CMV(S)-BGHpA/Hind Ⅲ + Xba Ⅰ; 5) pAd-sGT1100/Hind Ⅲ + Xba Ⅰ. (B) 1) pAdCMV(S)-BGHpA/Hind Ⅲ; 2) pAd-sGT600/Hind Ⅲ; 3) pAdCMV(S)-BGHpA/Xba Ⅰ; 4) pAd-sGT600/Xba Ⅰ; 5) λDNA/EcoR Ⅰ + Hind Ⅲ.

used to determine whether antisense RNA could be transcribed from CMV promoter located in Ad5anti-sGT600 and Ad5anti-sGT1100 genomes. Forty-eight hours after transduction with rAdv, RNA was extracted from BEL-7404 cells and treated with RNase free DNase (Boehringer Mannheim) at 37 ℃ for 30 min, and then RT-PCR and Northern hybridization were performed. The RT-PCR results showed that 1.19 kb and 0.6 kb DNA fragments were developed in RNA from Ad5anti-sGT1100- and Ad5anti-sGT600- transduced BEL-7404 cells, respectively, and these results did not result from rAdv genomic DNA contamination because no PCR product was produced from the RNA without doing reverse transcription (RT) (Fig 4). Northern hybridization also detected the strong signals in RNA isolated from BEL-7404 cells transduced with Ad5anti-sGT1100 and Ad5anti-sGT600, respectively, but not with control vector Ad5null (Fig 5).

Expression of antisense RNA from Ad5anti-sGT1100 and Ad5anti-sGT600 results in the reduction in the expression level of gal epitope More than 80 % NIH3T3 cells were infected by human rAdv at 200 MOIs (multiplicities of infection) as described earlier[4]. Here, NIH3T3 cells were analyzed by flow cytometry using FITC-GS-IB4 lectin 48 h after transduction with rAdv at 100 and at 200 MOIs. The cells transduced with Ad5anti-sGT600 and Ad5anti-sGT1100 at 100 MOIs showed reduction in lectin binding up to 12 % and 14 % compared with control, respectively. And at 200 MOIs, the reduction reached to 28 % and 27 %, respectively (Tab 1). These results demonstrated that antisense RNA transcripts from Ad5anti-sGT600 and Ad5anti-sGT1100 partly inhibited the expression of gal epitope.

Tab 1. Flow cytometric MFI (mean fluorescence intensity) values for the binding of FITC-GS-IB4 lectin to NIH3T3 cells transduced with rAdv. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Treatment group	MFI
Control (no virus)	443 ± 12
Ad5null (200 MOIs)	444 ± 15[a]
Ad5anti-sGT600 (100 MOIs)	386 ± 18[c]
Ad5anti-sGT600 (200 MOIs)	318 ± 21[c]
Ad5anti-sGT1100 (100 MOIs)	381 ± 10[c]
Ad5anti-sGT1100 (200 MOIs)	323 ± 19[c]
Control (Ad5null, 400 MOIs)	162 ± 40
Ad5hSeFT (200 MOIs) + Ad5anti-sGT600 (200 MOIs)	77 ± 16[c]
Ad5hSeFT (200 MOIs) + Ad5anti-sGT1100 (200 MOIs)	72 ± 15[c]

Co-expression of antisense RNA and human secretor type α(1,2) FT leads to further reduction in gal epitope level Recently we constructed a recombinant adenovirus Ad5hSeFT expressing human secretor type α(1,2) FT and found that its transduction resulted in 40 % reduction in gal epitope level on the surface of NIH3T3 cells. In this study, HIN3T3 cells were co-transduced with Ad5hSeFT plus Ad5anti-sGT600 or with Ad5hSeFT plus Ad5anti-sGT1100. Forty-eight hours after transduction, the cells were subjected to flow cytometric analysis. Flow cytometric analysis using FITC-UEA-I lectin showed that H blood group antigen was displayed on the surface of NIH3T3 cells due to adenovirus-mediated expression of α(1,2) FT (Fig 5), and using FITC-GS-IB4 lectin demonstrated that the reduction in GS-IB4 lectin binding to gal epitope on the cells reached up to 52 % and 55 %, respectively (Tab 1). Therefore, synergic effects between α(1,2) FT and antisense RNA transcripts complementary to α(1,3) GT mRNA on the gal epitope expression were detected.

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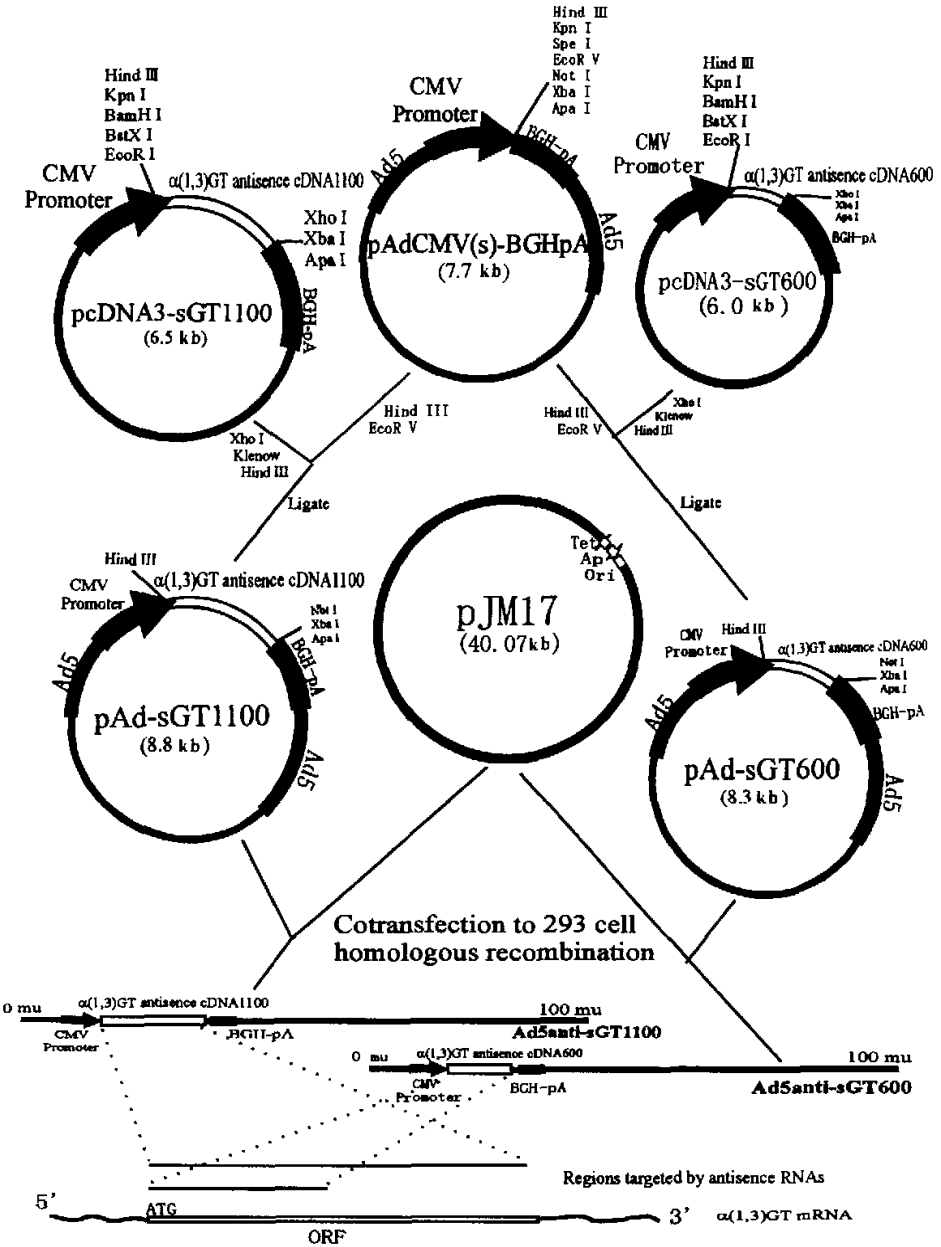

Fig 2. Construction of recombinant adenoviruses Ad5anti-sGT600 and Ad5anti-sGT1100 which can express antisense RNA complementary to pig α(1,3) GT mRNA. The DNA fragments of pig α(1,3) GT cDNA obtained by RT-PCR from pig spleen RNA were cloned into pcDNA3 to construct the recombinant plasmids pcDNA3-sGT600 and pcDNA3-sGT1100, and then were inserted into a shuttle vector pAdCMV(S)-BGHpA in an antisense orientation under the control of CMV promoter. The resulting plasmids pAd-sGT600 and pAd-sGT1100 were cotransfected with a virus-rescuing vector pJM17 into 293 cells and then the recombinant adenoviruses Ad5anti-sGT600 and Ad5anti-sGT1100 were generated respectively.

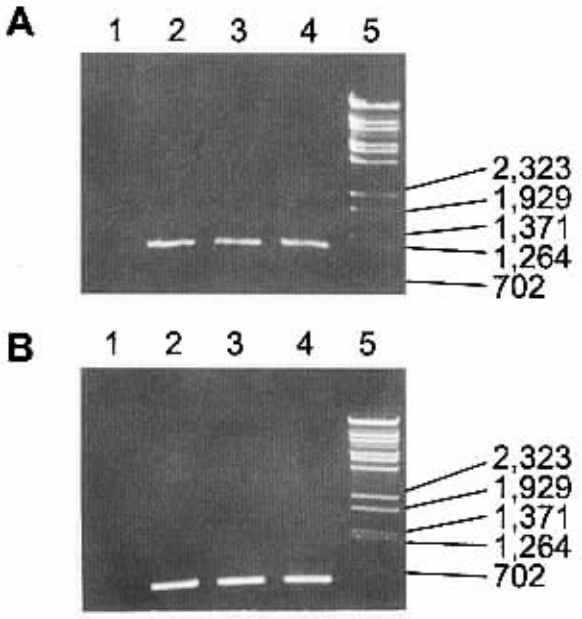

Fig 3. PCR analysis of rAdv DNAs using pig α(1,3) GT cDNA specific primers (A; P1 + P2; B; P1 + P3). (A) 1) 293 cell DNA; 2,3,4) Ad5anti-sGT1100 DNA; 5) λDNA/Eco91 Ⅰ; (B) 1) 293 cell DNA; 2,3, 4) Ad5anti-sGT600 DNA; 5) λDNA/Eco91 Ⅰ.

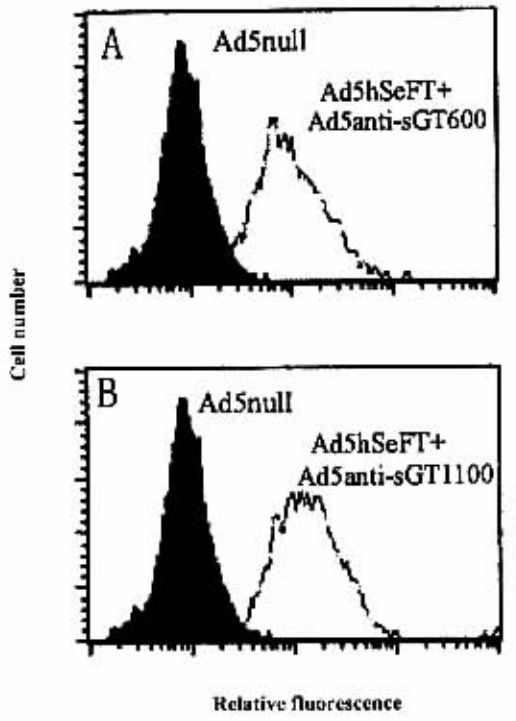

Fig 5. Expression of H blood group antigen on the surface of NIH3T3 cells after transduction with Ad5hSeFT plus Ad5anti-sGT600 (A) and Ad5hSeFT plus Ad5anti-sGT1100 (B). Cells were stained with FITC-UEA-I lectin.

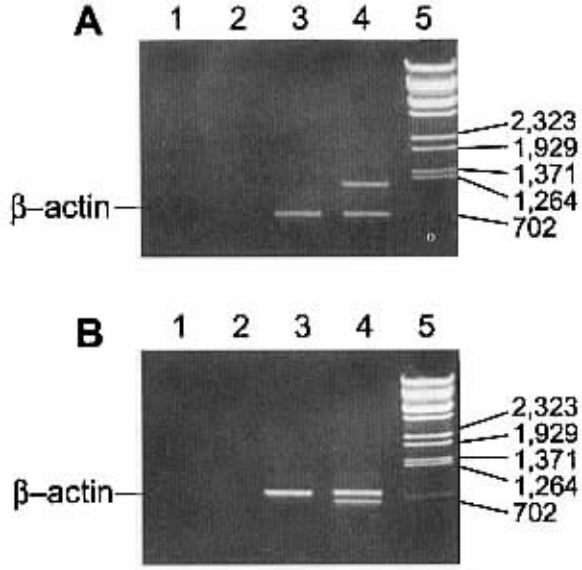

Fig 4. RT-PCR analysis of transcription of antisense RNA in BEL-7404 cells after rAdv transduction using pig α(1,3) GT cDNA specific primers (A, P1 + P2; B, P1 + P3). 1,3 mock transduction; A2, 4 Ad5anti-sGT1100; B2,4 Ad5anti-sGT600 (1,2 without RT reaction; 3,4 with RT reaction); 5 λDNA/Eco91 Ⅰ.

DISCUSSION

Regulation of gene expression by antisense RNA occurs naturally in both prokaryotes[9] and eukaryotes[10]. Antisense-based gene therapy has been a major strategy for downregulation of gene expression and can be approached in two ways, viz, synthetic antisense oligonucleotide and plasmid constructs expressing antisense RNA within the cells. To prevent the expression of the gal epitope, we construct two recombinant adenoviruses expressing antisense RNA complementary to different regions of the pig α(1,3) GT mRNA. The major advantage of using recombinant adenovirus as expression vector is that antisense RNA can be generated within the cells in relatively large quantities.

Because no cultured cells of pig origin were at hand and the ORF of pig α(1,3) GT shares 79 % identity with that of mouse α(1,3) GT mRNA[11] in nucleotide sequence, NIH3T3[12] cell from mouse was used as a model to test the inhibitory effects of antisense RNA transcripts on the expression of gal epitope. The results showed that these two kinds of antisense RNA (from Ad5anti-sGT600 and Ad5anti-sGT1100, respectively),

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decreased the level of gal epitope on the NIH3T3 cells to different degrees. In general, the effectiveness of an antisense sequence depends upon not only its ability to recognize the intended target, but also its ability to form stable duplexes with the complementary template, and its ability to remain in a non self-complementary conformation. Because antisense sequence inhibits mRNA and protein systhesis in a sequence specific as well as dose-dependent manner, the somewhat small reduction in gal epitope level by the antisense RNA in this study could partly come from the difference in the nucleotide sequence of the α(1,3) GT mRNA between pig and mouse.

Antisense-based inhibition of gene expression is through RNA-RNA duplex formation and subsequent degradation of the double-stranded transcripts. Human α(1,2) FT downregulates the expression of gal epitope through its competition with α(1,3) GT for the common substrate acceptor-*N*-acetyllactosamine to influence the formation of Gal α(1,3) Gal structure. As described above, when α(1,2) FT and antisense RNA directed against α(1,3) GT mRNA functioned together, their synergic effects on the expression of gal epitope occurred and an additive reduction in the gal epitope level on NIH3T3 cells was observed.

In conclusion, the recombinant adenoviruses Ad5anti-sGT600 and Ad5anti-sGT1100 are potential therapeutic agents for suppressing the expression of gal epitope *in vivo*.

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以腺病毒为载体表达猪 α(1,3)半乳糖基转移酶反义 RNA 抑制 Galα(1,3)Gal 抗原表位的表达[1]

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关键词 人腺病毒；反义 RNA；半乳糖基转移酶类；岩藻糖基转移酶类；基因表达；表位

目的：尝试以反义 RNA 的方法抑制 Galα(1,3) Gal 抗原表位(gal 抗原)的表达. **方法**：以人腺病毒载体表达猪 α(1,3)半乳糖基转移酶基因的反义 RNA. 流式细胞术比较 H 血型抗原和 gal 抗原的表达水平. **结果**：构建了表达反义 RNA 的重组腺病毒载体 Ad5anti-sGT600 和 Ad5anti-sGT1100. 反义 RNA 的表达使 NIH3T3 细胞表面的 gal 抗原表位下降约 30 %. 另外，反义 RNA 与人分泌型 α(1,2)岩藻糖基转移酶的共同作用可使 gal 抗原表位的水平进一步下降. **结论**：重组腺病毒 Ad5anti-sGT600 和 Ad5anti-sGT1100 可有效降低 gal 抗原表位的表达.

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