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ORIGINAL ARTICLE

Enhanced expression of the β4-galactosyltransferase 2 gene impairs mammalian tumor growth

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Altered *N*-glycosylation of membrane proteins is associated with malignant transformation of cells. We found that the expression of the β4-galactosyltransferase 2 (β4GalT2) gene is decreased markedly during the transformation. Here, we examined whether the tumor growth activity of B16-F10 mouse melanoma cells can be reduced by the enhanced expression of the β4GalT2 gene. We isolated a clone, B16-β4GalT2, showing its β4GalT2 transcript 2.5 times higher than a control clone, B16-mock, by transducing its cDNA, and transplanted them subcutaneously into C57BL/6 mice to examine their tumor growth activity. The results showed that the average size of tumors formed with B16-mock cells is 13.1 ± 0.76 mm, whereas that of tumors formed with B16-β4GalT2 cells is 5.1 ± 1.13 mm ($P < 0.01$) 2 weeks after transplantation. Immunohistochemical analyses showed that the apoptosis and the suppression of angiogenesis are induced in the tumors upon transduction of the β4GalT2 gene. To pursue a clinical usefulness of the β4GalT2 gene for suppressing human tumor growth, we injected adenoviruses carrying the human β4GalT2 cDNA into HuH-7 human hepatocellular carcinomas developed in severe combined immunodeficient mice, and observed marked growth retardation of the tumors. The enhancement of the β4GalT2 gene expression in tumors is one of the promising approaches to suppress human tumor growth.

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INTRODUCTION

Upon malignant transformation of cells, the structures of *N*-glycans attached to proteins have been shown to change dramatically (reviewed in Kobata A[1]). One of the most prominent changes of the structures is the increase in the amount of highly branched *N*-glycans with the Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Man branch.[2–4] This branch is formed by *N*-acetylglucosaminyltransferase V,[5] whose activities were also shown to increase by the malignant transformation of cells.[6–9] The correlation of the increased amount of highly branched *N*-glycans with metastatic potential of tumors has been well documented.[10–12] However, the biological significance of this high branching of *N*-glycans in tumors has not been well established.

It is of interest to note that many carbohydrate determinants involved in the cell adhesion processes such as Lewis X, sialyl Lewis X, poly-*N*-acetyllactosamine, polysialic acid and HNK-1 carbohydrate are expressed on the outer branches of *N*-glycans, the Galβ1→4GlcNAc group, which is synthesized by β4-galactosyltransferase (β4GalT) (reviewed in Furukawa and Clausen[13]). Now there are seven β4GalTs, and they form the β4GalT family. The β4GalT1 and β4GalT2 are involved in the galactosylation of *N*-glycans, β4GalT3 and β4GalT4 in the biosynthesis of lacto-series glycolipids, β4GalT5 and β4GalT6 in the biosynthesis of lactosylceramide, and β4GalT7 in the galactosylation of the xylose residue at the linkage region between core proteins and glycosaminoglycans (reviewed in Furukawa and Clausen[13]). Among seven members of the β4GalT family, it has been shown that β4GalT1 and β4GalT5 are involved in the development of multidrug resistance of human leukemia cells,[14] β4GalT2 in p53- or cisplatin-mediated apoptosis in HeLa cells,[15,16] and β4GalT5 in arsenic trioxide-mediated apoptosis of human glioma cells,[17] indicating that the abnormal expression of β4GalTs is critical to progression and development of malignant properties of tumors.

Our previous studies showed that the expression level of the β4GalT2 gene is decreased markedly upon malignant transformation of NIH3T3 cells without a change in the β4GalT activities towards *p*-nitrophenyl-*N*-acetyl-1-thio-β-glucosaminide between the normal and transformed cells.[18] Analysis of the expression levels of the β4GalT1, β4GalT2, β4GalT3, β4GalT4, β4GalT5 and β4GalT6 genes in several human cancer cell lines revealed that the decreased expression of the β4GalT2 gene is conversely related with the increased expression of the *N*-acetylglucosaminyltransferase V gene.[19] As β4GalT2 is involved in the biosynthesis of *N*-glycans,[20,21] such a change presumes to affect the galactosyl patterns of *N*-glycans, which is important for the subsequent expression of the onco-fetal antigens including poly-*N*-acetyllactosamine and Lewis X antigen on branched side chains of *N*-glycans in tumors.[22,23]

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To elucidate biological significance of the decreased expression of the β4GalT2 gene in tumors, the expression of the β4GalT2 gene was enhanced by the transduction of its cDNA into B16-F10 mouse melanoma cells, a highly tumorigenic cell line, and the tumor growth activity was examined *in vivo* in the present study. Furthermore, HuH-7 human hepatocellular carcinomas developed in severe combined immunodeficient (SCID) mice were adenovirally transduced with the human β4GalT2 cDNA, and the tumor growth was monitored with a possible clinical application by its gene transfer.

MATERIALS AND METHODS

Animals and chemicals

B16-F10 mouse melanoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal calf serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin at 37 °C in a humidified 5% CO_2 incubator. HuH-7 human hepatocellular carcinoma cells[24] and HEK293 cells obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI1640 medium containing 10% fetal calf serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Six-week-old male C57BL/6 mice and female BALB/c Slc-*scid/scid* mice were obtained from local animal suppliers (Japan SLC, Hamamatsu, Japan, and Japan CLEA, Tokyo, Japan, respectively). Mice were grown under a specific pathogen-free condition in environmentally controlled animal facilities at Nagaoka University of Technology and Chiba University. Peroxidase-conjugated concanavalin A (Con A), *Ricinus communis* agglutinin-I (RCA-I), leukoagglutinating phytohemeagglutinin (L-PHA) and peanut agglutinin were purchased from Honen Oil (Tokyo, Japan) and EY-Lab. (San Mateo, CA, USA). Diplococcal β-galactosidase and *N*-glycanase, and sialidase from *Arthrobacter ureafaciens* were from Roche Diagnostics (Mannheim, Germany) and Nacalai Tesque (Kyoto, Japan), respectively.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the Japanese Society for Biochemistry. The protocols were approved by the Committees on the Ethics of Animal Experiments of Nagaoka University of Technology and of Chiba University, respectively. All surgery was performed under ether anesthesia, and all efforts were made to minimize suffering.

Constructs of the pcDNA3.1/β4GalT2 and Ad/β4GalT2

A pGEM-T Easy vector (Promega, Madison, WI) containing a full length of the mouse β4GalT2 cDNA was digested with Sph I and Sal I. The fragment was blunted, and then ligated into the EcoR V site of a mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) containing the neomycin-resistant gene, which is referred to as pcDNA3.1/β4GalT2. The E1 region-deleted recombinant type 5 adenovirus containing human β4GalT2 cDNA (referred to as Ad/β4GalT2) or adenovirus carrying no cDNA (referred to as Ad/mock) was prepared according to the method described previously.[25] In brief, the Ad/β4GalT2 prepared with pHMCMV6, pAdHM4 DNAs and human β2GalT2 cDNA corresponding to 1–1119 bp (accession number: AB024434) was transducted into HEK293 cells, and Ad/mock was produced likewise. The recombinant Ad/β4GalT2 and Ad/mock were propagated by repeated infection into HEK293 cells. Titers of Ad/β4GalT2 and Ad/mock were calculated with 50% tissue culture infectious dose and expressed as plaque-forming unit.[26]

Cloning of B16-F10 cells with the pcDNA3.1/β4GalT2 or pcDNA3.1(+)

B16-F10 cells (2×10^5 cells) were plated in a ϕ35-mm dish a day before transduction. Each 2 μg of the vector pcDNA3.1/β4GalT2 or pcDNA3.1(+) (mock) were co-transfected with a FuGENE 6 Transfection Reagent (Roche Diagnostics) into the plated cells. Transfection was performed as suggested by manufacture's protocol. After incubation for 48 h, medium was exchanged to DMEM containing 1 mg ml^{-1} geneticin (G418 sulfate, Sigma-Aldrich, St Louis, MO, USA) and cultured continuously for 2 weeks. The geneticin/neomycin-resistant colonies were isolated by limited dilution.

Analysis of the gene expression levels by RT-PCR and quantitative real time RT-PCR

In order to screen clones showing higher expression levels of the β4GalT2 transcript, reverse transcription-polymerase chain reaction (RT-PCR) was performed. In brief, total RNA preparations were isolated from each clone with Sepasol RNA I (Nacalai Tesque) and reverse-transcribed into cDNAs using 1 μg total RNA, oligo(dT) primers and a Reverse-iT1St Strand Synthesis kit (ABgene, Surrey, UK). Amplification was carried out in a DNA programmable thermal controller (PTC-200, MJ Research, Cambridge, MA, USA) for 32 cycles at 63 °C for the β4GalT2 gene and 61 °C for the β4GalT1 gene using Thermo-Start PCR Master Mix (ABgene) with primers specific for mouse β4GalT2 cDNA corresponding to 53–453 bp (accession number: AB019541) (forward primer 5′-TTCTCTGCCTGCTGCACTTCC-3′ and reverse primer 5′-CCGGTGTCTAAAGGGGATGAT-3′) and for mouse β4GalT1 cDNA corresponding to 339–781 bp (accession number: J03880) (forward primer 5′-CTTGTCTTCGTTGCCAGTGC-3′ and reverse primer 5′-AGGCATTACGGTCGTCCATC-3′). The amplified products were analyzed by 2% agarose gel electrophoresis, as described previously.[18] Quantitative real time RT-PCR was performed according to the method described previously.[27]

Analysis of tumor growth activity of B16-F10 clones

Cloned cells cultured in DMEM containing 0.5 mg ml^{-1} geneticin were harvested by 0.25% trypsin treatment, and viable 2×10^5 cells suspended in 50 μl of serum-free DMEM were transplanted subcutaneously into 6-week-old male C57BL/6 mice. The long diameters of tumors formed were measured periodically. The results are expressed as the means ± s.e.'s of data obtained from 10 or 8 animals. The statistical significance was determined by Student's *t*-test, and $P < 0.05$ was considered significant.

Transduction of the Ad/β4GalT2 into human tumor grown in animals

HuH-7 cells (5×10^6 cells) were subcutaneously transplanted into SCID mice. When tumors were grown up to 5–6 mm long in diameter (with a volume of 40–50 mm^3), the Ad/β4GalT2 or Ad/mock (8×10^7 plaque-forming unit, 0.1 ml per tumor) was injected into tumors, and tumor volumes were monitored periodically according to the formula ($1/2 \times \text{length} \times \text{width}^2$).

Histological and immunohistochemical analyses of tumors

The tumors were removed from the animals and fixed with phosphate-buffered saline (pH 7.4) containing 4% formaldehyde. The paraffin sections in 10 μm thickness were stained with Haematoxyline-Eosin solution. Tumors developed in mice were sliced to prepare cryosections and the specimens were stained with anti-CD31 antibody (Ab) (BD Bioscience, San Jose, CA, USA), anti-human Ki-67 Ab (Life Technologies, Carlsbad, CA, USA) or anti-mouse Ki-67 Ab (Nichirei Biosciences, Tokyo, Japan) followed by peroxidase-conjugated second Ab (Nichirei Biosciences). The sections were visualized by a staining kit (Histofine) (Nichirei Biosciences) and counter-stained with Haematoxylin solution. Apoptosis was detected with an *in situ* cell death detection kit based on a terminal deoxynucleotidyl transferase dUTP nick end labeling technology[28] (Roche Applied Science, Mannheim, Germany). The statistical significance of numbers of positively stained cells was determined by one-way analysis of variance, and $P < 0.05$ was considered significant.

Lectin blot analysis of membrane proteins

Membrane protein samples were prepared from the tumors formed with B16-mock (2) cells or B16-β4GalT2 (4) cells. They were solubilized in a sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (7.5%). Proteins were transferred to polyvinylidene difluoride filter (Immobilon-P, Millipore , Bedford, MA, USA), and lectin blot analysis using Con A, RCA-I, L-PHA and peanut agglutinin was conducted. In some experiments, a blot equivalent to one sample lane was blocked with bovine serum albumin and then treated with sialidase, diplococcal β-galactosidase or *N*-glycanse before incubation with lectin according to the method described previously.[29,30] The β4GalT activity was determined by the method described previously.[31]

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RESULTS

Cloning of B16-F10 cells showing the higher expression of the β4GalT2 gene

In order to enhance the β4GalT2 activity, mouse β4GalT2 cDNA as described in Materials and Methods was cloned into the pcDNA3.1 vector, and it was transducted into B16-F10 cells. Five clones were obtained after selection with G418, and the gene expression levels were determined by RT-PCR analysis. The results showed that one clone, B16-β4GalT2 (4), expresses the β4GalT2 transcript by 2.5 times higher than B16-mock (2), one of the control clones transducted with the pcDNA3.1(+) vector, whose expression level was the same as the parental cells (data not shown) and used as a control, whereas other clones (B16-β4GalT2 (1), (2), (3) and (5)) express 1.2–1.5 times higher than B16-mock (2) (Figure 1a), which was estimated by NIH image J analysis. To confirm this further, quantitative real time RT-PCR was performed using total RNA preparations from B16-mock (2) cells and B16-β4GalT2 (4) cells. The results showed that the expression level of the β4GalT2 transcript in B16-β4GalT2 (4) cells increases by 2.4 folds higher than that of B16-mock (2) cells (Figure 1b). Therefore, in the present study, we used B16-β4GalT2 (4) cells, and B16-mock (2) cells as a control. That the transduction does not affect the expression of the β4GalT1 gene was also confirmed (data not shown).

Morphological appearances and proliferation rates of B16-mock (2) cells and B16-β4GalT2 (4) cells

When proliferation rates of B16-mock (2) cells and B16-β4GalT2 (4) cells were determined with a Cell Proliferation Assay kit (Promega), they showed similar proliferation rates (Figure 2a). In a growing phase, there was no difference in morphological appearances (data not shown) but at the sub-confluence, partial cellular interaction among cells appeared in B16-β4GalT2 (4) cells, which was not observed in B16-mock (2) cells (Figure 2b). The results suggest that cell surface property is slightly changed upon enhancing the expression of the β4GalT2 gene in B16-F10 cells. The consistency of this phenomenon has to be confirmed in several lines of tumor cells with different expression levels of the β4GalT2 gene after its transduction.

Lectin blot analysis of membrane proteins from B16-mock (2) cells and B16-β4GalT2 (4) cells

Membrane protein samples were prepared from B16-mock (2) cells and B16-β4GalT2 (4) cells, and they were subjected to lectin

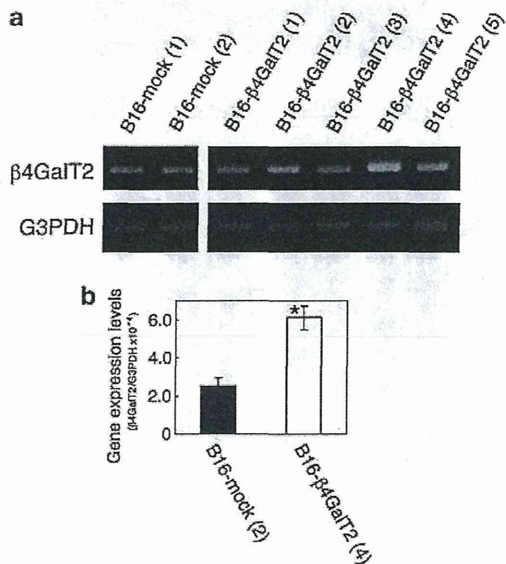

Figure 1. The expression levels of the β4GalT2 gene in the B16-F10 clones. (**a**) The expression levels of the β4GalT2 transcript in B16-β4GalT2 (1), (2), (3), (4) and (5) cells transducted with pcDNA3.1/β4GalT2 and in B16-mock (1) and (2) cells transducted with pcDNA3.1(+) were determined by RT-PCR as described in the text. (**b**) The expression levels of the β4GalT2 transcript in B16-mock (2) cells and B16-β4GalT2 (4) cells relative to that of G3PDH transcript. Quantitative real time RT-PCR was performed, and the results show means ± s.e. of three separate experiments. $*P<0.01$ refers to that of B16-mock (2) cells.

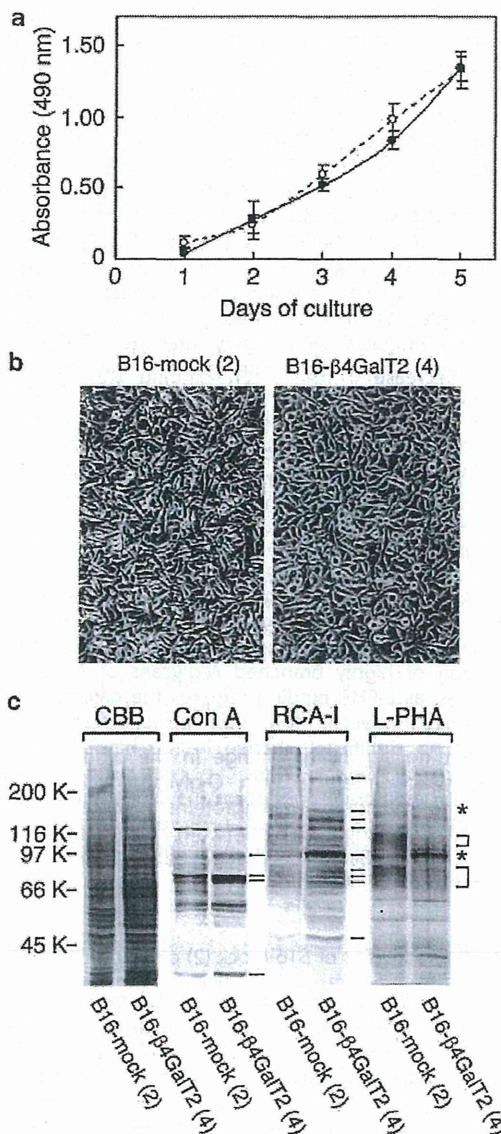

Figure 2. Light microscopic and biochemical analyses of B16-mock (2) cells and B16-β4GalT2 (4) cells. (**a**) Growth curves of B16-mock (2) cells and B16-β4GalT2 (4) cells. Values indicate means ± s.e. of triplicate assays. Closed and open circles indicate B16-mock (2) cells and B16-β4GalT2 (4) cells, respectively. (**b**) Morphological appearances of B16-mock (2) cells and B16-β4GalT2 (4) cells. Cells were grown to sub-confluence in DMEM containing 10% fetal calf serum, and their morphological appearances of cells are shown (×80). (**c**) Lectin blot analysis of membrane proteins of B16-mock (2) cells and B16-β4GalT2 (4) cells. Blots were stained with CBB or incubated with Con A, RCA-I or L-PHA after blocking. Lectin-bound proteins were visualized by the method described in the text.

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blot analysis as described in Materials and Methods. When blots were stained with Coomassie Brilliant Blue (CBB), two samples showed similar protein components (lanes B16-mock (2) and B16-β4GalT2 (4) of Figure 2c—CBB). In order to examine whether or not *N*-glycosylation of the B16-F10 cells is altered by the gene transduction, lectin blot analysis was performed. When the blot was incubated with Con A, which binds mainly to high mannose-type glycans,[32] binding of the lectin toward protein bands with molecular weights of 35, 72, 75 and 95 K increased in B16-β4GalT2 (4) cells (indicated with arrows at the right side of lane B16-β4GalT2 (4)) as compared with B16-mock (2) cells (Figure 2c—Con A). In the case of RCA-I, which interacts with glycans terminating with the Galβ1→4GlcNAc/Gal group,[33] the blots were initially treated with sialidase to remove sialic acid residues attached to galactose residues if any, and then incubated with the lectin. A significant increase in the lectin binding was observed for several proteins with molecular weights of 50, 70, 75, 80, 95, 130, 145, 160 and 220 K in B16-β4GalT2 (4) cells (indicated with arrows at the right side of lane B16-β4GalT2 (4)) as compared with B16-mock (2) cells (Figure 2c—RCA-I). The lectin binding was nullified with digestion of the blot with diplococcal β-galactosidase, which cleaves the Galβ1→4GlcNAc/Glc linkage but not the Galβ1→3GlcNAc/Glc linkage,[34,35] prior to incubation with the lectin (data not shown), indicating that galactose residues are attached to glycans in a β-1,4-linkage. When incubated with L-PHA, which interacts with highly branched *N*-glycans,[36] a significant decrease in the lectin binding was observed for protein bands with molecular weights of 68–85, 95 and 105–110 K (indicated with arrows at the right side of lane B16-β4GalT2 (4)) but not 95 and 165 K protein bands, which showed a significant increase in the lectin binding in B16-β4GalT2 (4) cells (indicated with asterisks at the right side of lane B16-β4GalT2 (4)) as compared with B16-mock (2) cells (Figures 2c—L-PHA). All lectin bindings disappeared upon treatment of the blots with *N*-glycanase prior to incubation with lectins (data not shown). These results indicate that the transduction of the β4GalT2 cDNA stimulates not only the β4-galactosylation of *N*-glycans but also the high mannose-type glycosylation of proteins, and inhibits the galactosylation of highly branched *N*-glycans of several membrane proteins, as L-PHA binding requires the galactosylation of highly branched *N*-glycans.[36] In addition, our preliminary study showed that no significant change in the binding of peanut agglutinin, which interacts with *O*-glycans with the core 1 structure,[37] to membrane glycoproteins is detected between B16-mock (2) cells and B16-β4GalT2 (4) cells (data not shown), indicating that β4GalT2 is predominantly involved in the β4-galactosylation of *N*-glycans.

Tumor growth activities of B16-mock (2) cells and B16-β4GalT2 (4) cells

In order to examine whether the enhanced expression of the β4GalT2 gene affects tumor growth activities of the B16-F10 cells *in vivo*, B16-mock (2) cells and B16-β4GalT2 (4) cells showing >95% viability were transplanted subcutaneously into 10 C57BL/6 mice each. Tumor growth in the animals was monitored periodically, and sizes of tumors were measured. The results showed that the average diameter formed with B16-mock (2) cells is 13.1 ± 0.76 mm, whereas that of tumors formed with B16-β4GalT2 (4) cells is 5.1 ± 1.13 mm ($P<0.01$), respectively (Table 1). It is noteworthy that two of the animals failed to develop tumors when B16-β4GalT2 (4) cells were transplanted. Tumors formed with B16-mock (2) cells and B16-β4GalT2 (4) cells in the animals are shown in Figure 3a. This study was repeated by using another 10 animals, and the same results were obtained. These results showed that the transduction of the β4GalT2 cDNA reduces tumor growth activity of the B16-F10 cells and suppresses the subsequent tumor growth.

Table 1. Tumorigenic potentials of B16-mock (2) cells and B16-β4GalT2 (4) cells.

Clones	*Tumors formed no. of the animals (%)*	*Tumor size (mm) (n)*	*P-value*[a]
B16-mock (2)	10/10 (100%)	13.1 ± 0.76 (10)[b]	
B16-β4GalT2 (4)	8/10 (80%)	5.1 ± 1.13 (8)	<0.01

[a]*P*-value refers to a comparison between the average sizes of tumors formed with B16-mock (2) cells and those with B16-β4GalT2 (4) cells. [b]Values given are means ± s.e.'s (*n*).

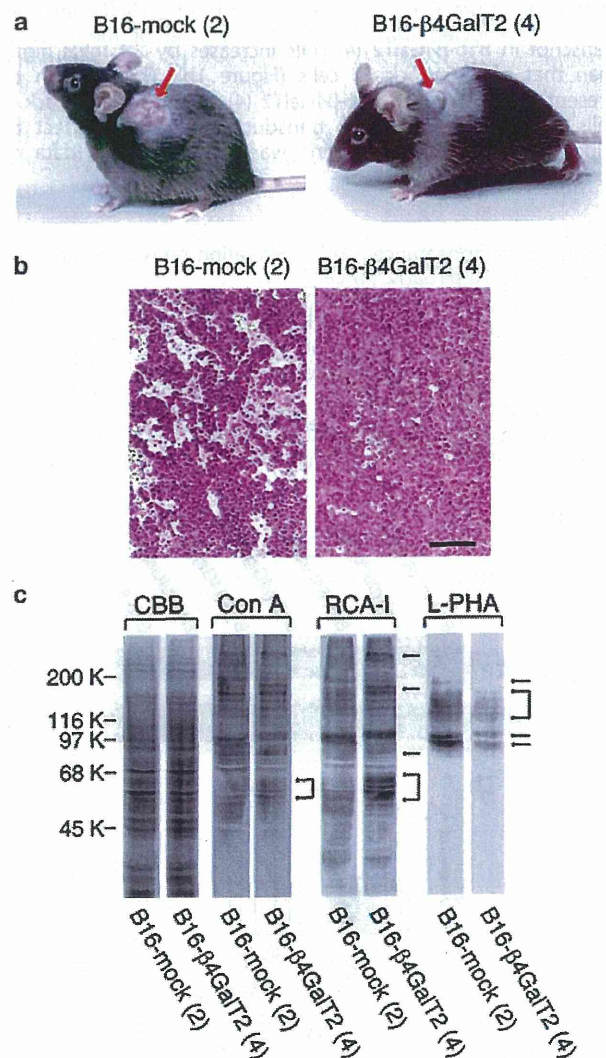

Figure 3. Tumors formed with B16-mock (2) cells and B16-β4GalT2 (4) cells in mice. (**a**) The cells were transplanted subcutaneously into C57BL/6 mice and tumors formed 2 weeks after transplantation are shown. (**b**) Paraffin sections of tumor tissues stained with Haematoxylin-Eosin solution. Bar indicates 100 μm. (**c**) Lectin blot analysis of membrane proteins of tumors formed with B16-mock (2) cells and B16-β4GalT2 (4) cells. Blots were stained with CBB or incubated with Con A, RCA-I or L-PHA after blocking. Lectin-bound proteins were visualized by the method described in the text.

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A histochemical study was conducted for the tumors formed. In the case of the tumors formed with B16-β4GalT2 (4) cells, cells were arranged compactly with less intercellular cavities probably owing to partially recovered cellular interactions, whereas tumor cells of B16-mock (2) origin were sparsely distributed with many cavities (Figure 3b).

Lectin blot analysis of membrane proteins from the melanomas

Membrane protein samples were prepared from the tumors formed with B16-mock (2) cells and B16-β4GalT2 (4) cells, and they were subjected to lectin blot analysis. The blots were stained with CBB, and two samples showed similar protein components (Figure 3c—CBB). When the blots were incubated with Con A, a slight increase in the lectin binding was observed for protein bands with molecular weights of 53–58 K in B16-β4GalT2 (4)-derived tumors (indicated with arrows at the right side of lane B16-β4GalT2 (4)) as compared with B16-mock (2)-derived tumors (Figure 3c—Con A). Similarly to the analysis using cells, the blots were treated with sialidase, and then incubated with RCA-I. A remarkable increase in the lectin binding was observed for several proteins with molecular weights of 50–60, 80, 180 and 240 K in B16-β4GalT2 (4)-derived tumors (indicated with arrows at the right side of lane B16-β4GalT2 (4)) as compared with B16-mock (2)-derived tumors (Figure 3c—RCA-I). The lectin binding was nullified with digestion of blots with diplococcal β-galactosidase prior to incubation with lectin (data not shown), indicating that galactose residues are attached to glycans with a β-1,4-linkage. When incubated with L-PHA, a remarkable decrease in the lectin binding was observed for protein bands with molecular weights of 90, 97, 116–160, and 180 K in B16-β4GalT2 (4)-derived tumors (indicated with arrows at the right side of lane B16-β4GalT2 (4)) as compared with B16-mock (2)-derived tumors (Figures 3c—L-PHA). All lectin bindings disappeared upon treatment of the blots with *N*-glycanase prior to incubation with lectins (data not shown). These results indicate that the transduction of the β4GalT2 cDNA stimulates strongly the galactosylation of *N*-glycans of some membrane glycoproteins, and inhibits strongly the L-PHA binding to several membrane glycoproteins that reacted with this lectin before the gene transduction. Bindings of RCA-I and L-PHA to individual membrane glycoproteins were not always identical between B16-β4GalT2 (4) cells and its tumors, and this could be due to differences in a growth condition between *in vitro* and *in vivo* or between two-dimensional and three-dimensional circumstances.

Transduction of the Ad/β4GalT2 into human solid tumor

As the transduction of the β4GalT2 cDNA into B16-F10 cells appeared effective to suppress the tumor growth in the animals, whether or not growth of human tumors developed in animals can be suppressed by the transduction of the β4GalT2 cDNA was examined with an aspect of clinical application. For this purpose, the human β4GalT2 cDNA was cloned into an adenovirus vector, and HuH-7 human hepatocellular carcinoma cells[24] were allowed to grow as big as 5–6 mm in a diameter in SCID mice. Then the Ad/β4GalT2 or Ad/mock was injected into the tumors directly, and subsequent tumor growth was monitored periodically. Murine cells but not human cells are resistant to type 5 Ad-mediated transduction due to poor expression of coxsackie adenovirus receptor, a major cellular receptor.[38] The reason why we did not use human melanomas in this study was that melanoma cells generally express a very low level of the coxsackie adenovirus receptor, but HuH-7 cells express relatively high levels of the cellular receptor, which is suitable for Ad-mediated transduction. A marked growth retardation was observed for the tumors injected with the Ad/β4GalT2 as compared with those injected with the Ad/mock (Figure 4a). Untreated tumors grew almost similarly to the tumors injected with the Ad/mock (data not shown). The representative tumors injected with the Ad/β4GalT2 or Ad/mock are shown in Figure 4b.

When tumors were excised and the expression of the β4GalT2 transcript was determined by RT-PCR analysis, about five times more transcript was detected in the tumors injected with the Ad/β4GalT2 when compared with those injected with the Ad/mock (Figure 4c) as determined by NIH image J analysis. In the case of β4GalT activity, tumors injected with the Ad/β4GalT2 showed 1.4 time higher activity toward 1 mM *p*-nitrophenyl-N-acetyl-1-thio-β-glucosaminide than the control tumors (Figure 4d). Furthermore, lectin blot analysis of membrane proteins showed that no significant change is found in Con A-binding (Figure 4e—Con A) but a significant increase in RCA-I-binding is observed for several protein bands with molecular weights of 100–120, 160, 180, 200, 220 and 240 K (indicated with arrows at the right side of lane Ad/β4GalT2) from the tumors injected with the Ad/β4GalT2 (Figure 4e—RCA-I). No lectin-reactive bands were detected in the samples upon digestion of blots with diplococcal β-galactosidase or *N*-glycanse (data not shown). Owing to the limited amounts of the samples available, lectin blot analysis using L-PHA was not performed. These results indicate that the β4GalT2 cDNA injected into the human tumors is translated into an active enzyme that enhances the β4-galactosylation of *N*-glycans of several membrane proteins. These results clearly demonstrated that the enhanced expression of the β4GalT2 gene leads to the suppression of tumor growth *in vivo*.

Immunohistochemical analyses

We pursued possible biological mechanisms of antitumor effects induced by the enhanced expression of the β4GalT2 gene with immunohistochemical analyses of apoptotic cells, CD31-positive vessels for angiogenesis[39] and Ki-67-positive cells for proliferating cells[40] (Table 2). When apoptosis was examined *in vivo* with the terminal deoxynucleotidyl transferase dUTP nick end labeling assay,[28] B16-β4GalT2 (4)-derived melanomas showed numbers of apoptotic cells greater than those of B16-mock (2)-derived tumors (terminal deoxynucleotidyl transferase dUTP nick end labeling in Figure 5; Table 2 $P = 0.03$), but no significant difference was observed between HuH-7 hepatocellular carcinomas transduced with the Ad/mock and those with the Ad/β4GalT2 (Table 2 $P = 0.21$). B16-β4GalT2 (4)-derived melanomas showed decreased numbers of CD31-positive vessels when compared with those of B16-mock (2)-derived tumors (CD31 in Figure 5; Table 2 $P = 0.02$). However, no significant difference in the numbers of CD31-positive vessels between HuH-7 hepatocellular carcinomas transduced with the Ad/mock and those with the Ad/β4GalT2 (Table 2 $P = 0.76$). Numbers of Ki-67-positive cells that indicate proliferation activities in the tumors remained the same in both B16-F10 melanomas (Ki-67 in Figure 5; Table 2 $P = 0.53$) and HuH-7 hepatocellular carcinomas (Table 2 $P = 0.74$) regardless of the β4GalT2 gene expression. These results collectively suggest that the growth retardation of B16-β4GalT2 (4)-derived melanomas is partly attributed to augmented apoptosis and inhibited angiogenesis. Nevertheless, the mechanism of the growth retardation of HuH-7 hepatocellular carcinomas after the Ad/β4GalT2 transduction remains currently unknown although the Ad/β4GalT2-transduced tumors showed increased numbers of apoptotic cells and minimally decreased numbers of CD31-positive vessels in comparison with the Ad/mock-transduced tumors (Table 2).

DISCUSSION

Our previous studies showed that the expression of the β4GalT2 gene is decreased upon malignant transformation of NIH3T3 cells with the constant expression of the β4GalT1 gene[18], in spite of the involvement of both β4GalT1 and β4GalT2 in the β4-galactosylation of *N*-glycans.[20,21] As the altered *N*-glycosylation

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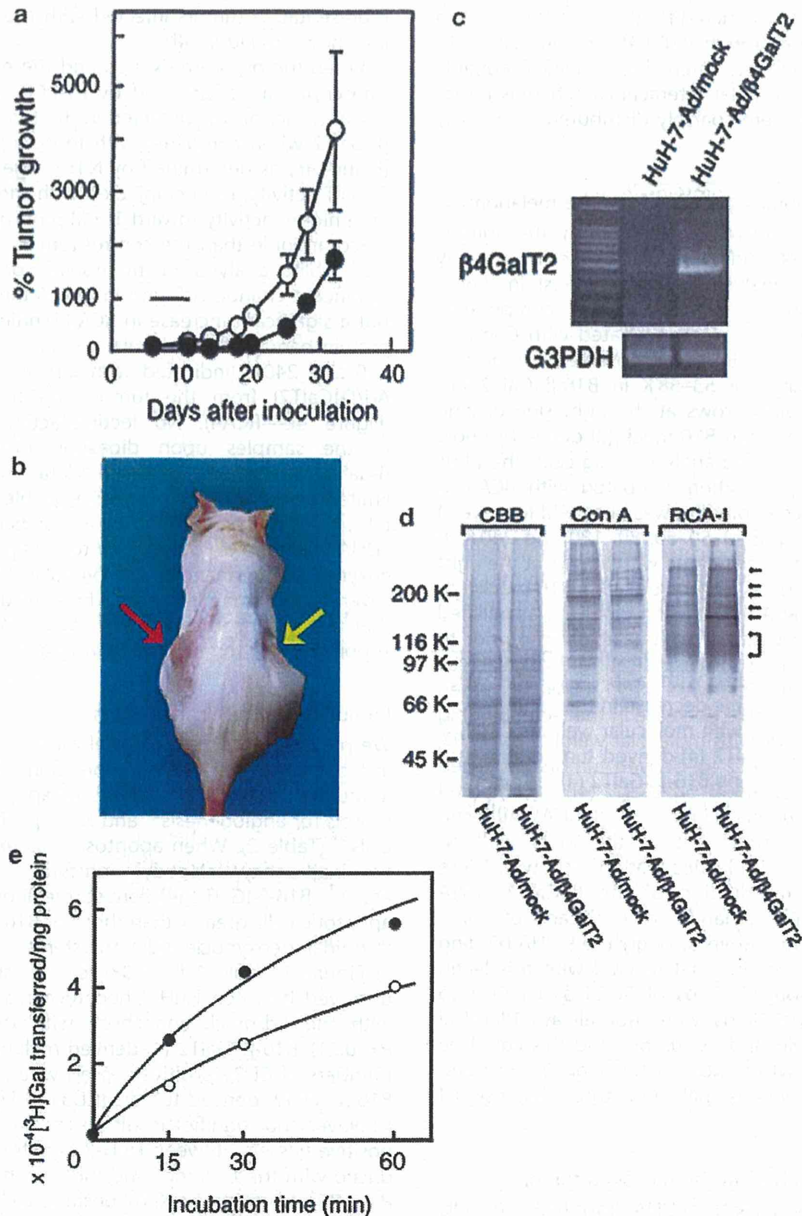

Figure 4. Tumor growth of HuH-7 hepatocellular carcinoma cells injected with the Ad/β4GalT2. (**a**) Tumors formed with HuH-7 cells transplanted subcutaneously into SCID mice ($n = 7$ per group) were injected with the Ad/β4GalT2 (closed circles) or Ad/mock (open circles), and tumor volumes were measured periodically as described in the text. Averages of tumor volumes with s.e. (bars) are shown. The significance of the differences in tumor volumes by the injection with the Ad/β4GalT2 or the Ad/mock was as follows: Day 11, $P < 0.0187$; Day 14, $P < 0.0044$; Day 18, $P < 0.0013$; Day 20, $P < 0.0039$; Day 25, $P < 0.0142$; Day 28, $P < 0.0367$; and Day 32, $P < 0.1161$, respectively. (**b**) A photo picture of a representative SCID mouse carrying tumors, which were injected intratumorally with the Ad/mock indicated with a red arrow (left flank) and Ad/β4GalT2 indicated with a yellow arrow (right flank), respectively. (**c**) RT-PCR analysis of the β4GalT2 transcript in tumors injected with the Ad/mock and with the Ad/β4GalT2. (**d**) β4GalT activities toward *p*-nitrophenyl-*N*-acetyl-1-thio-β-glucosaminide in tumors injected with the Ad/mock (open circle) and with the Ad/β4GalT2 (closed circle). (**e**) Lectin blot analysis of membrane proteins from tumors injected with the Ad/mock and with the Ad/β4GalT2. Blots were stained with CBB or incubated with Con A or RCA-I followed by visualization as described in the text.

of cell surface proteins relates with malignant properties of tumors,[11,12] we examined an effect of the enhanced expression of the β4GalT2 gene in B16-F10 melanoma cells on their tumor growth activities. The present study clearly demonstrated that the enhanced expression of the β4GalT2 gene in B16-F10 cells increases the β4-galactosylation of *N*-glycans of the membrane proteins, changes morphological appearances with partial recovery of cellular interactions, and finally suppresses tumor growth when the cells were transplanted subcutaneously into C57BL/6 mice. In addition, B16-β4GalT2 (2) cells with the 1.5 times higher expression of the β4GalT2 gene than B16-mock (2) cells also showed growth retardation of the tumors upon subcutaneous transplantation into the mice (data not shown). However, its growth inhibition level was much lower than B16-β4GalT2 (4) cells

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with the 2.5 times higher expression of the β4GalT2 gene, indicating that the growth inhibition level of the tumors relates with the expression levels of the β4GalT2 gene, which has to be determined with further studies.

As the transduction of the β4GalT2 cDNA into B16-F10 cells to enhance the β4-galactosylation of the *N*-glycans appeared promising to suppress the tumor growth, we then introduced the human β4GalT2 cDNA into the human solid tumor developed in immune compromised mice to pursue practical application of this therapeutic strategy. For this purpose, we injected the Ad/β4GalT2 into HuH-7 human hepatocellular carcinomas, confirmed its transfer by the results showing the increased expression of the β4GalT2 transcript, the increased β4GalT activity toward *p*-nitrophenyl-*N*-acetyl-1-thio-β-glucosaminide, and the increased β4-galactosylation of *N*-glycans of the several membrane proteins, and finally observed marked growth retardation of the HuH-7 carcinomas. The continuous growth observed in these tumors could be due to the presence of the untransduced tumor cells. In this way, there are several factors that affect Ad-mediated gene transfer into human tumors such as the expression level of the virus receptors on the tumor cells and the penetration of Ad into tumor tissues, and, therefore, the development of enhanced vector delivering systems will be a key issue for feasible clinical application of this reagent.

Table 2. Immunohistochemical analyses of the B16-F10 mouse melanomas and HuH-7 human hepatocellular carcinomas

Stainings	*Tumors formed with*	*Numbers of positive cells*[a]
TUNEL	B16-mock (2)	3.7 ± 1.20
	B16-β4GalT2 (4)	8.3 ± 0.88[b]
	HuH-7-Ad/mock	7.3 ± 0.67
	HuH-7-Ad/β4GalT2	13.7 ± 4.18
CD31	B16-mock (2)	95.0 ± 9.66
	B16-β4GalT2 (4)	60.0 ± 1.53[c]
	HuH-7-Ad/mock	30.0 ± 4.73
	HuH-7-Ad/β4GalT2	27.3 ± 6.77
Ki-67	B16-mock (2)	98.0 ± 18.5
	B16-β4GalT2 (4)	83.7 ± 9.95
	HuH-7-Ad/mock	35.3 ± 6.34
	HuH-7-Ad/β4GalT2	32.0 ± 7.03

Numbers of positively stained cells per arbitrary areas were counted in respective tumor specimens ($n = 3$).
[a]Values given are means ± s.e.'s ($n = 3$).
[b]$P < 0.05$; TUNEL staining, B16-β4GalT2 (4)-derived melanomas relative to B16-mock (2)-derived melanomas.
[c]$P < 0.05$; CD31-staining, B16-β4GalT2 (4)-derived melanomas relative to B16-mock (2)-derived melanomas.

In order to pursue the biological mechanisms how the tumor growth is retarded upon transduction of the β4GalT2 gene, we conducted a series of immunohistochemical analyses for the B16-F10 mouse melanomas and HuH-7 human hepatocellular carcinomas using a terminal deoxynucleotidyl transferase dUTP nick end labeling staining for detecting apoptotic cells,[28] an anti-CD31 antibody for detecting angiogenesis,[39] and an anti-Ki-67 antibody for detecting proliferating cells.[40] These results indicate that the enhanced expression of the β4GalT2 gene induces apoptosis to the melanomas significantly and the hepatocellular carcinomas slightly. In the case of the melanomas, angiogenesis was also suppressed significantly, however, no such effect was observed for the hepatocellular carcinomas, which could be due to the less efficient transduction of the β4GalT2 gene, and to the complex interactions between mouse extracellular matrices and human tumors as well as immune responses generated against human cells. Concerning to the analysis of the Ki-67-positive cells, there was no difference in the both tumors before and after the trasduction of the β4GalT2 gene, which is quite consistent to the *in vitro* growth activities of B16-mock (2) cells and B16-β4GalT2 (4) cells. These appear to be the possible reasons why the tumors transduced with the β4GalT2 gene show significant growth retardation.

The decreased binding of L-PHA by transduction of the β4GalT2 cDNA was observed in several protein bands with molecular weights of 68–85 K and 105–110 K in B16-β4GalT2 (4) cells and of 90, 97, 116–160 and 180 K in B16-β4GalT2 (4)-derived tumors, and such protein bands may include lysosome-associated membrane

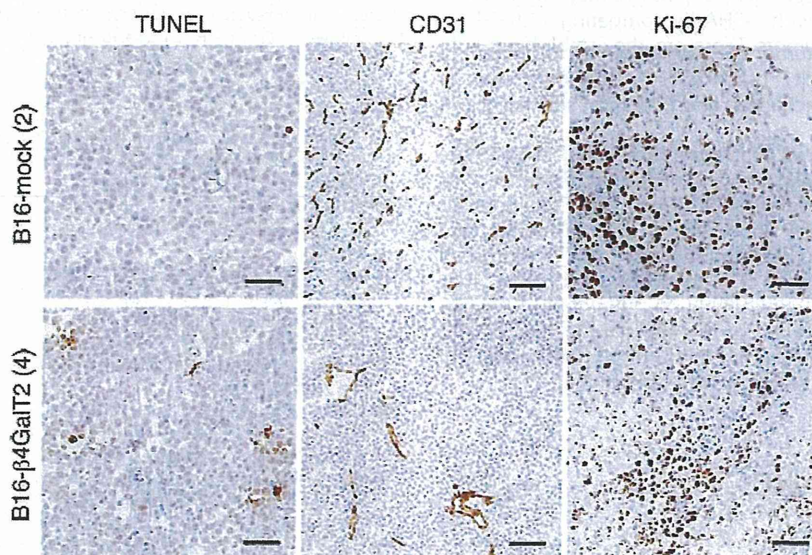

Figure 5. Immunohistochemical analyses of B16-F10 mouse melanomas. The tumor cryosections were stained with the TUNEL assay (bar: 70 μm) or incubated with anti-CD31 antibody (bar: 200 μm) and anti-mouse Ki-67 antibody (bar: 70 μm), and visualized with a staining kit as described in Materials and Methods. Upper panels indicate B16-mock (2)-derived tumors and lower panels indicate B16-β4GalT2 (4)-derived tumors, respectively.

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proteins with molecular weights of 110–120 K (LAMPs), and a β1-integrin with molecular weights of 130–140 K, which are involved in cell growth and adhesion, and carry L-PHA-positive N-glycans in tumors.[12,41,42] Therefore, another reason of the growth retardation *in vivo* can be attributed to restored functions of individual membrane proteins such as LAMPs and β1-integrin in B16-β4GalT2 (4)-derived tumors by changing their N-glycan structures not to bind to L-PHA. Previous studies demonstrated that the disappearance of L-PHA-reactive N-glycans by the treatment of the cells with swainsonine results in reduced colonization of the melanoma cells to the lungs,[43] and that α5β1-integrin with highly branched N-glycans shows decreased binding to fibronectin.[44–47] A similar decrease in L-PHA binding might have occurred to the membrane proteins of the growth-retarded HuH-7 tumors by the transduction of the Ad/β4GalT2, although it was not established in the present study owing to limited amount of the materials available. It is ideal to determine structures of the N-glycans attached to LAMPs and β1-integrin molecules from B16-β4GalT2 (4) cells and their tumors to show the structural backgrounds for the lectin bindings. Alternatively, the expression pattern of terminal galactose residues on N-glycans could be also important for the growth suppression by interacting with a variety of galectins, some of which are involved in growth inhibition of cells.[48,49] Therefore, the detailed molecular mechanisms based on the N-glycans also have to be elucidated further.

As β4GalT2 has been shown to be involved in mouse neural development[50] and in medaka (*Oryzias latipes*) gastrulation,[51] the β4-galactosylation of N-glycans by β4GalT2 must be quite important for animal embryonic development and growth. In fact, the β4GalT2-deficient mice showed a significant decrease in the amount of the HNK-1 carbohydrate antigen expressed on the Galβ1→4GlcNAc group of N-glycans in the brain,[50] and its antigen has been shown to be involved in the neural development.[52] Quite interestingly, this antigen is expressed normally in the β4GalT1-deficient mice,[53] which indicates that the β4-galactosylation of N-glycans by β4GalT2 is different from that by β4GalT1. In accordance with this, β4GalT1 and β4GalT2 show slightly different acceptor specificities towards branched N-glycans,[21] and our preliminary studies show that β4GalT2 has a preference to galactosylate the GlcNAcβ1→2Manα1→3Man branch to the GlcNAcβ1→2Manα1→6Man branch as compared with β4GalT1 (data shown). This acceptor specificity of β4GalT2 may bring about the N-glycans not to react with L-PHA by competing with other glycosyltransferases in the tumors. Therefore, the establishment of the fine acceptor specificity of β4GalT2 is the third way to elucidate the molecular mechanism and biological significance of the present study.

It has recently been shown that the β4GalT1 and β4GalT5 are involved in the hedgehog signaling and multidrug resistance.[14] As the enhanced expression of the β4GalT2 gene induced apoptosis and suppressed angiogenesis significantly in the melanomas, and as the hedgehog signaling regulates angiogenesis,[54] apoptosis,[55] and cell cycle progression,[56] the hedgehog signaling may be involved in the growth retardation of the β4GalT2 gene-transduced tumors. Examination of this signaling in the β4GalT2 gene-transduced tumors will lead to the further understanding of the molecular mechanism of the growth retardation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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