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Molecular characterization of pig α2,3-Gal-β1,3-GalNAc-α2,6-sialyltransferase (pST6GalNAc IV) gene specific for Neu5Acα2-3Galβ1-3GalNAc trisaccharide structure

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Abstract Sialic acids of glycoconjugates play crucial roles in various biological processes, such as cell-cell communication and cell-substrate interaction. A sisalyltransferase, ST6GalNAc IV (Neu5Ac-α2,3-Gal-β1,3-GalNAc-α2,6-sialyltransferase), catalyzes the formation of α2-6-linkages onto GalNAc residues of O-glycosidically linked Ser/Thr of proteins. In this study, we cloned the pig ST6GalNAc IV (pST6GalNAc IV) and investigated its functional characterization. pST6GalNAc IV cDNA has been isolated from pig liver tissues and it contains an entire open reading frame (ORF, 906 bp) coding for 302 amino acid residues. Entire ORF of pST6GalNAc IV containing sialylmotif 'L'—(Large), 'S'—(Small) and '—VS' (Very small) has a high degree of sequence similarity with *Homo sapiens* (90%), *Pan troglodytes* (91%) and *Mus musculus* (87%). Expression of pST6GalNAc IV mRNA in various pig tissues was identified by reverse transcription polymerase chain reaction (RT-PCR) analysis. pST6GalNAc IV mRNA was highly expressed in tongue, muscle and heart, whereas it was not expressed in pancreas. For functional characterization of pST6GalNAc IV gene in pig kidney PK15 cells, we have also established pST6GalNAc IV-transfected PK15 cells, which are stably expressing the pST6GalNAc IV gene. The glycosylation pattern of pST6GalNAc IV-transfected PK15 cells was detected by flow cytometry and immunofluorescence analysis with *Maackia amurensis* agglutinin (MAA), *Maackia amurensis* hemagglutinin (MAL II), *Sambucus nigra* agglutinin (SNA) and *peanut* agglutinin (PNA) lectins. The specific carbohydrate structures of Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc tetrasaccharide or Neu5Acα2-6GalNAc disaccharide recognized by MAL-II and SNA were revealed to be newly synthesized by pST6GalNAc IV. From the results, it was suggested that the pig pST6GalNAc IV gene is capable of synthesizing Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc tetrasaccharide structures on *O*-glycoproteins.

Keywords Neu5Ac-α2,3-Gal-β1,3-GalNAc-α2,6-sialyltransferase · Neu5Ac-α2,3-Gal-β1,3(Neu5Acα2,6) GalNAc · Tissue expression · MAA · MAL II · SNA · PNA

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Abbreviations

NeuAc	*N*-acetylneuraminic acid
ST6GalNAc IV	Neu5Ac-α2,3-Gal-β1,3-GalNAc-α2, 6-sialyltransferase
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
RT	Reverse transcriptase
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
FITC	Fluorescein isothiocyanate
DAPI	Fluorchrome 4, 6-diamino-2-phenylidol
ORF	Open reading frame
MAA	*Maackia amurensis* agglutinin
MAL II	*Maackia amurensis* hemagglutinin
SNA	*Sambucus nigra* agglutinin
PNA	*peanut* agglutinin

Introduction

Sialic acids are generally located at the non-reducing terminal position of the glycoproteins and glycolipids, and bound to α2-3-sialic acids for polysialylation reaction, α2-3- or α2-6-linked to a β-D-galactopyranose residue, or a lateral position α2-6-linked to a GalNAc or a GlcNAc residue [1, 2]. These sialic acids of the glycoconjugates play crucial roles in a variety of biological reactions, such as cell-cell communication, cellular signaling, viral infection and cell-substrate interaction. The sialic acids functions as ligand components for carbohydrate binding proteins such as lectins, hormones, antibodies and inorganic cations [2–4].

It is well defined that the sialyltransferase family, belonging to type II transmembrane glycosyltransferases, catalyzes transfer of sialic acid to glycoconjugates [5, 6]. The sialyltransferase family was classified as four different subfamilies, ST3Gal, ST6Gal, ST6GalNAc and ST8Sia, according to the carbohydrate linkages synthesized. Sialyltransferase family has four conserved sialyl motifs such as 'L' (Large), 'S' (Small), 'VS' (Very small), 'III' [7–9]. These motifs contribute to recognition of donor (L), acceptor substrates (VS) or both (S) [8, 10]. Furthermore, it was suggested that sialyl motifs functioned as hallmarks for the identification of eukaryotic sialyltransferase [7].

So far, six types of GalNAc α2,6-sialyltransferase including ST6GalNAc I, II, III, IV, V and VI have been reported [3, 11–15]. Among these GalNAc α2,6-sialyltransferase family, the cDNA of ST6GalNAc IV has been isolated from human and mouse [3, 11]. It was demonstrated that ST6GalNAc IV has restricted substrate specificity which utilizes Neu5Acα2-3Galβ1-3GalNAc trisaccharides found on *O*-glycosylated proteins [1, 3]. At present, there is no any report on the pig ST6GalNAc IV enzyme and cDNA, although the pig ST6GalNAc IV cDNA sequence has been deposited in the NCBI gene bank database without any information on its structure and functional characterization.

Therefore, in this study, we have cloned the pig pST6GalNAc IV gene and checked the level of pST6GalNAc IV mRNA expression in various pig tissues. Finally, we have further investigated the functional characterization of pST6GalNAc IV gene by establishment of pST6GalNAc IV-transfected PK15 cells using flow cytometry or fluorescent microscope analysis with *Maackia amurensis* agglutinin (MAA), *Maackia amurensis* hemagglutinin (MAL II), *Sambucus nigra* agglutinin (SNA) and *peanut* agglutinin (PNA) lectins (Table 1). Our result shows that binding of MAA, MAL II and SNA lectins were increased in the pST6GalNAc IV-transfected PK15 cells, while binding of PNA lectin was decreased in pST6GalNAc IV-transfected PK15 cells compared with mock control. These results suggested that the Neu5Acα2-3Galβ1-3(Neu5Acα2-6) GalNAc tetrasaccharide structures on *O*-glycoproteins were increased in the pST6GalNAc IV-transfected PK15 cells, indicating that the pST6GalNAc IV gene is functional in the pig PK15 cells.

Materials and methods

Cell line and culture condition

Porcine kidney cells (PK15 cells) were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and incubated at 37°C, 0.5% CO_2 incubator condition.

Cloning of the pST6GalNAc IV gene and construction of pST6GalNAc IV gene expression vector

For cloning of the pST6GalNAc IV cDNA, following primers were designed on basis of pST6GalNAc IV gene sequence (GenBank accession number EU643700) from NCBI gene bank database; AA GGTACCATGAAGCC TCCGGGT primer (sense) and AACTCGAGCTGGGTCT TCCACGA primer (anti-sense). Total RNAs were isolated from pig liver tissue and cDNAs were synthesized by reverse transcriptase (RT) with oligo dT primer using RT premix (Super-bio Co., Ltd, South Korea). The pST6GalNAc IV cDNA was amplified with polymerase chain reaction (PCR) system using the following primers; 95°C for 5 min; 35 cycle of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min.

For the construction of pST6GalNAc IV gene expression vector, the amplified PCR products were digested by *Kpn* I

Table 1 Carbohydrate specificities using various lectins

Lectin	Carbohydrate Specificity	Reference
Maackia amurensis leukoagglutinin (MAL, MAL I)	Neu5Acα2-3Galβ1-4GlcNAc	[19–21]
Maackia amurensis hemagglutinin (MAH, MAL II)	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc	[19–21]
Sambucus nigra agglutinin (SNA)	Neu5Acα2-6GaINAc/Gal	[16–18]
Peanut agglutinin (PNA)	terminal Gal-β1-3-GaINAc	[16–22]

(TaKaRa) and *Xho* I (TaKaRa) and then inserted into the *Kpn* I and *Xho* I site of expression vector pcDNA 3 using a T4 DNA ligase (Fermentas). The recombinant expression vector, the ligated pST6GalNAc IV cDNA was transformed into *E. coli* DH5α. The clones were selected on LB agar plates with 100 μg/ml ampicillin. Plasmid was isolated from positive colonies using plasmid DNA purification kit (DNA spin, Intron). The recombinant expression vectors were confirmed by enzyme digestion and sequencing.

Establishment of pST6GalNAc IV gene-transfected PK15 cells

Transfection of pig pST6GalNAc IV gene cloned into pcDNA3 vector was performed in PK15 cells using WelFect EXTM Plus transfection reagent (WelGENE Inc). 2.0×10^4 PK15 cells were seeded in 6-Well culture test plate one day before transfection. When PK15 cells were cultured approximately 50∼60% in 6-Well cluture test plate, PK15 cells were transfected with 5 μg plasmid DNA using WelFect EXTM Plus protocol. Stable selection was performed with culture medium and G418 (800 μg/ml) for 3 weeks.

pST6GalNAc IV mRNA expression in various pig tissues

Total RNAs from various pig tissues were isolated using Corezol reagent (Welgene, Inc) according to manufacturer's protocol. One μg of each total RNA was used to RT-PCR using the above-mentioned RT-PCR analysis method. The pST6GalNAc IV cDNAs from various pig tissues were amplified by the above-mentioned PCR analysis method. Pig β-actin primers were used as an internal control in equal amount to that of mRNA used. Primer sequences for pig β-actin were CACGCCATCCTGCGTCTGG A primer (sense) and TCTGCATCCTGTCGGCGATG primer (anti sense), resulting in a 427 bp RT-PCR product.

Flow cytometry analysis

To identify alteration of glycosylation patterns of pST6GalNAc IV-transfected PK15 cells, we performed flow cytometry analysis with MAL II, PNA (Vector Laboratories, Inc.), MAA and SNA (E.Y Laboratories.) lectins. The transfected cells were seeded at 6-Well culture testplate one day before assay. The cells were washed twice with phosphate buffer saline (PBS) and harvested by scraping. Then, the cells were separately reacted with MAA (2 mg/ml) (1:250 diluted by 1% bovine serum albumin (BSA)/PBS), MAL II (2 mg/ml) (1:500 diluted by 1% BSA/PBS), SNA (2 mg/ml) (1:250 diluted by 1% BSA/PBS) and PNA (5 mg/ml) (1:1000 diluted by 1% BSA/PBS). After 2 h at room temperature, the cells were washed twice with PBS, then reacted with Alexa Fluor conjugated streptavidin (Invitrogen, USA) (1:4000 diluted in 1% BSA/PBS) for 30 min at 4°C dark incubator. The cells were washed twice with PBS, analyzed with a FACSCalibur (Becton Dickinson). Reactions without lectins were used as a negative control.

Immunofluorescence microscopy analysis

The pST6GalNAc IV transfected PK15 cells were seeded at 6-Well culture testplate with clean slide glass one day before assay for immunofluorescence microscopy analysis. The cells on slide glass were fixed with PBS containing 3.7% formaldehyde and then washed twice with PBS. The washed cells were reacted with MAL II lectin (2 mg/ml) (1:500 MAL II diluted by PBS/1% bovine serum albumin (BSA)). After 30 min at room temperature, the cells were washed twice with PBS and then reacted with alexa fluor conjugated streptavidin (1:4000 diluted by PBS/1% BSA) for 30 min. The cells were washed twice with PBS and then stained DNA specific fluorchrome 4, 6-diamino-2-phenylidol (DAPI). The stained cells were observed with the fluorescence microscope (Olympus, Tokyo, Japan).

Result

Isolation and sequence analysis of pST6GalNAc IV cDNA from pig liver tissue

As we have previously deposited the sequence of pig pST6GalNAc IV, the nucleotide sequence of pig pST6GalNAc IV gene (GenBank accession number EU643700) from the NCBI Genebank database was used to generate the gene specific primer, 3'-AA GGTACCATGAAGCCTCCGGGT-5' primer (sense) and 3'-AACTCGAGCTGGGTCTTC

CACGA-5' primer (anti-sense). Using total RNAs isolated from pig liver tissue, a cDNA library was synthesized, as described in Materials and Methods. The open reading frame (ORF) of pST6GalNAc IV cDNA containing 909 bp, encoding 302 amino acids, was then amplified with the designed primers. Multiple alignment analysis reveals that the amino acid sequence was highly conserved in sialyl motif 'L', 'S' and 'VS', and has high homology with the ST6GalNAc IV of *Homo sapiens* (90%), *Pan troglodytes* (91%) and *Mus musculus* (87%) (Fig. 1a). The hydropathy plot analysis of pST6GalNAc IV cDNA-deduced amino acid sequence showed a hydrophobic segment of 23 amino acids in N-terminal region, predicting that pST6GalNAc IV has type II transmembrane topology characteristic of many other glycosyltransferases cloned to date (Fig. 1b).

Expression of pST6GalNAc IV mRNA in pig tissues

To investigate the expression of pST6GalNAc IV mRNA in various pig tissues, the RT-PCR analysis has been performed using the designed primers. As shown in Fig. 2, the pST6GalNAc IV mRNA was constitutively expressed in most tissues including rectum, bladder, stomach, tongue, muscle, spleen, small intestine, testicle, kidney, liver, spinal cord, heart and colon. Especially,

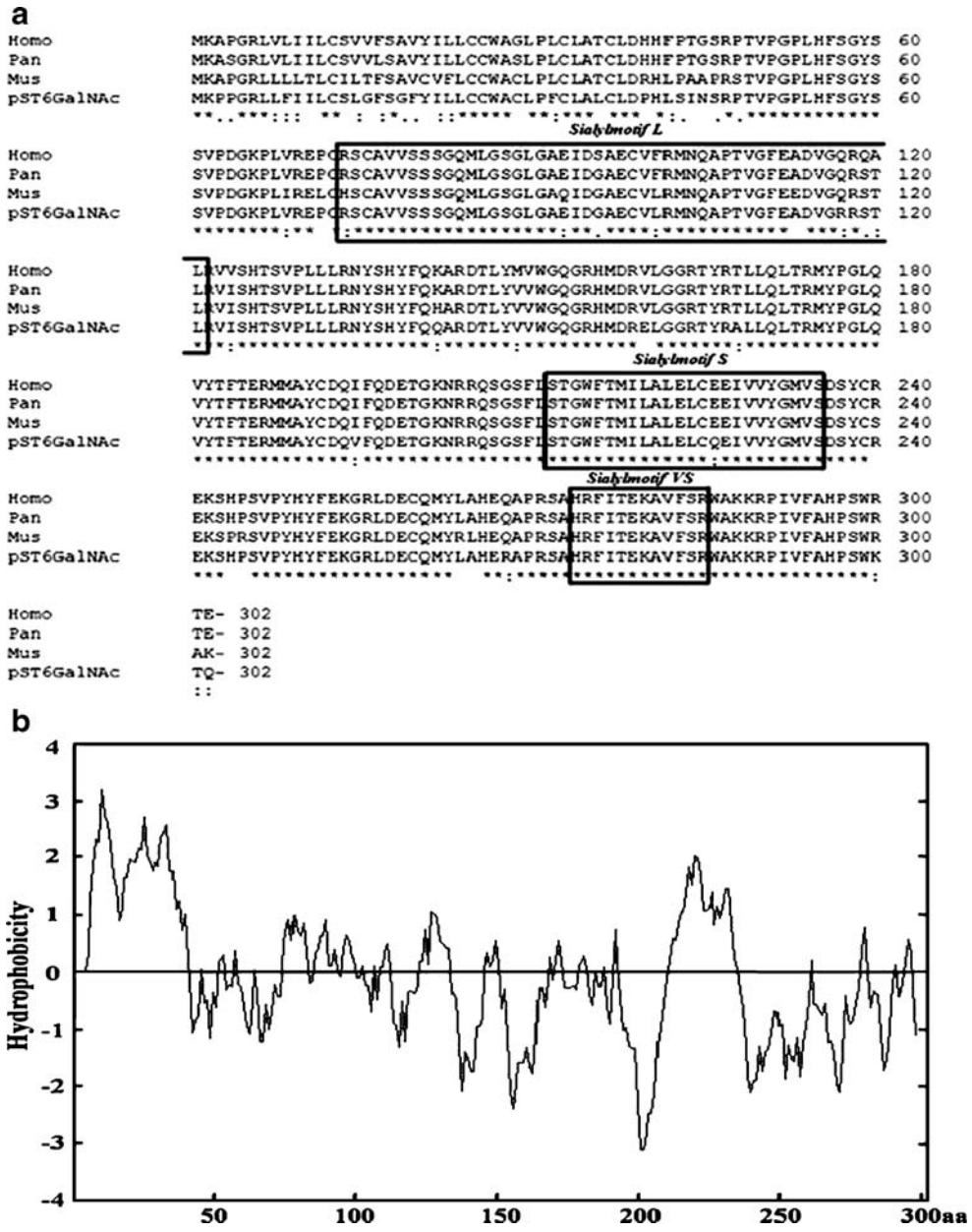

Fig. 1 The multiple alignment analysis and hydropathy plot of the deduced amino acid sequence of pST6GalNAc IV cDNA. **a** Alignment of various animal-expressed ST6GalNAc IV amino acid sequences using alignment program Clustal W2. The sialyl motifs including 'L', 'S' and 'VS' are boxed. '*asterisk*' indicates same residues, '*colon*' indicates strong conservation residue and '*full stop*' indicates weak conservation residue. GenBank accession numbers: *Homo sapiens* NM_175039, *Pan troglodytes* CAG26699 and *Mus musculus* NP_035503. **b** The hydropathy plot was calculated by an analysis program of Kyte and Doolittle (http://www.expasy.ch/cgi-bin/protscale.pl)

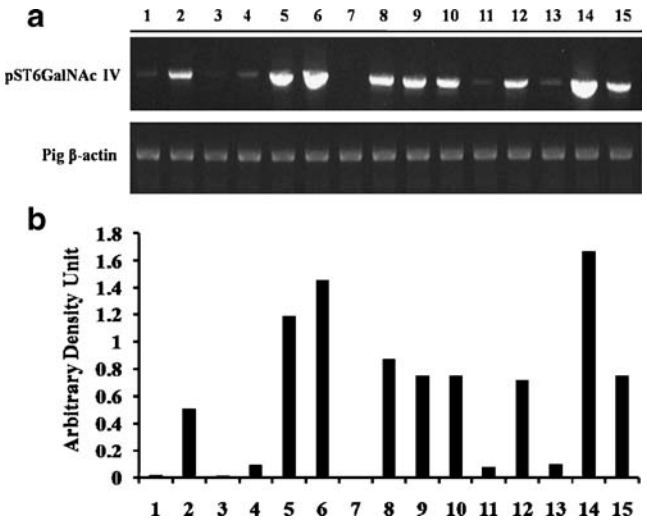

Fig. 2 Expression of pST6GalNAc IV mRNA in various pig tissues. **a** Expression level of pST6GalNAc IV mRNA was measured by RT-PCR. The β-actin was used as an internal control. 1, brain; 2, rectum; 3, bladder; 4, stomach; 5, tongue; 6, muscle; 7, pancreas; 8, spleen; 9, small intestine; 10, testicle; 11, kidney; 12, liver; 13, spinal cord; 14, heart; 15, colon. **b** Graphical representation of the densitometric analysis using Image J software from the RT-PCR data shows the expression level of the pST6GalNAc IV in various pig tissues as in panel **a**

pST6GalNAc IV gene was abundantly expressed in tongue, muscle and heart, whereas not expressed in pancreas at all.

Change in glycosylation pattern in the pST6GalNAc IV-transfected PK15 cells

In order to assess the functional characterization of pST6GalNAc IV gene, the two different pST6GalNAc IV-transfected PK15 cells of pST6GalNAc IV-1 and pST6GalNAc IV-2, which stably express the pST6GalNAc IV gene, have been established (Fig. 3a). Although we have attempted the pST6GalNAc IV gene transfection using the primary culture of pig ear cells, which were newly established, it failed to transfect with the cloned DNA constructs, probably due to their limited cell division. Then, the sialylated carbohydrate pattern of the pST6GalNAc IV-transfected PK15 cells was analyzed by flow cytometry using MAA, MAL II, PNA and SNA lectins and immunofluorescence microscopy using a MAL II lectin. As shown in Fig. 3b and c, the binding of MAL II was increased in the pST6GalNAc IV-transfected PK15 cells compared with mock control. In addition, binding levels of MAA and SNA were increased in the pST6GalNAc IV-transfected PK15 cells, compared with mock control. However, binding of PNA was decreased in the pST6GalNAc IV-transfected PK15 cells, compared with mock control. These results indicate that the level of Galβ1-3GalNAc was reduced in the pST6GalNAc IV-transfected PK15 cells, whereas the level of terminal disialylated tetrasaccharide Neu5Acα2-3Gal-β1-3(NeuAc-α2-6)GalNAc residues or monosialylated disaccharide NeuAc-α2,6-GalNAc residues was increased in the pST6GalNAc IV-transfected PK15 cells.

Discussion

At present, six sialyltransferase ST6GalNAc cDNAs have been reported to date. Among these sialyltransferases, the ST6GalNAc IV gene was cloned and characterized from the human and mouse, but not from pig [3, 11]. However, pig pST6GalNAc IV gene has only been reported from the NCBI Genebank database (GenBank accession number EU643700 and AJ620652). In addition, the functional characterization of the pig pST6GalNAc IV was not reported to date. In this study, we have cloned the pST6GalNAc IV cDNA from the pig liver tissues and examined expression of pST6GalNAc IV gene in pig various tissues. In addition, to investigate functional characterization of the pST6GalNAc IV gene, we have established pST6GalNAc IV stable cell lines using PK15 cell line, which is frequently used in pig cell research.

The pST6GalNAc IV gene encoded a protein of 302 amino acids, which contained highly conserved regions such as sialyl motif 'L', 'S' and 'VS'. The amino acid sequence of pST6GalNAc IV has high homology with the ST6GalNAc IV of *Homo sapiens* (90%), *Pan troglodytes* (91%) and *Mus musculus* (87%), respectively (Fig. 1a). The analysis of hydropathy plot of the pST6GalNAc IV unraveled a hydrophobic segment of 23 amino acids in the amino-terminal region, predicting that the pST6GalNAc IV protein has type II transmembrane topology characteristic of many other glycosyltransferases (Fig. 1b), as for the pig glycolylneuraminic acid hydroxylase [16].

It was reported that human ST6GalNAc IV was expressed at low level in most human tissues and human cancer cell lines [11]. Furthermore, the mouse ST6GalNAc IV was specifically expressed in brain, colon and generally expressed in lung, thymus and spleen [3]. When the expression level of the pST6GalNAc IV gene was examined using various pig tissues and RT-PCR, the expression of pST6GalNAc IV gene was observed in a wide range of pig tissues. As shown in Fig. 2, the pST6GalNAc IV mRNA was specifically expressed in tongue, muscle and heart, but not expressed in pancreas.

To investigate changes in carbohydrate structure in the pST6GalNAc IV-transfected PK15 cells, the flow cytometry and immunofluorescence microscopy analysis have been performed using four different lectins including MAA, MAL II, SNA and PNA. It was known that lectin MAA (*Maackia amurensis* agglutinin) binds to α2-3 linked sialic acid not only in *N*-glycans, but also *O*-glycans [17, 18] and lectin SNA (*Sambucus nigra* agglutinin) displays higher affinity

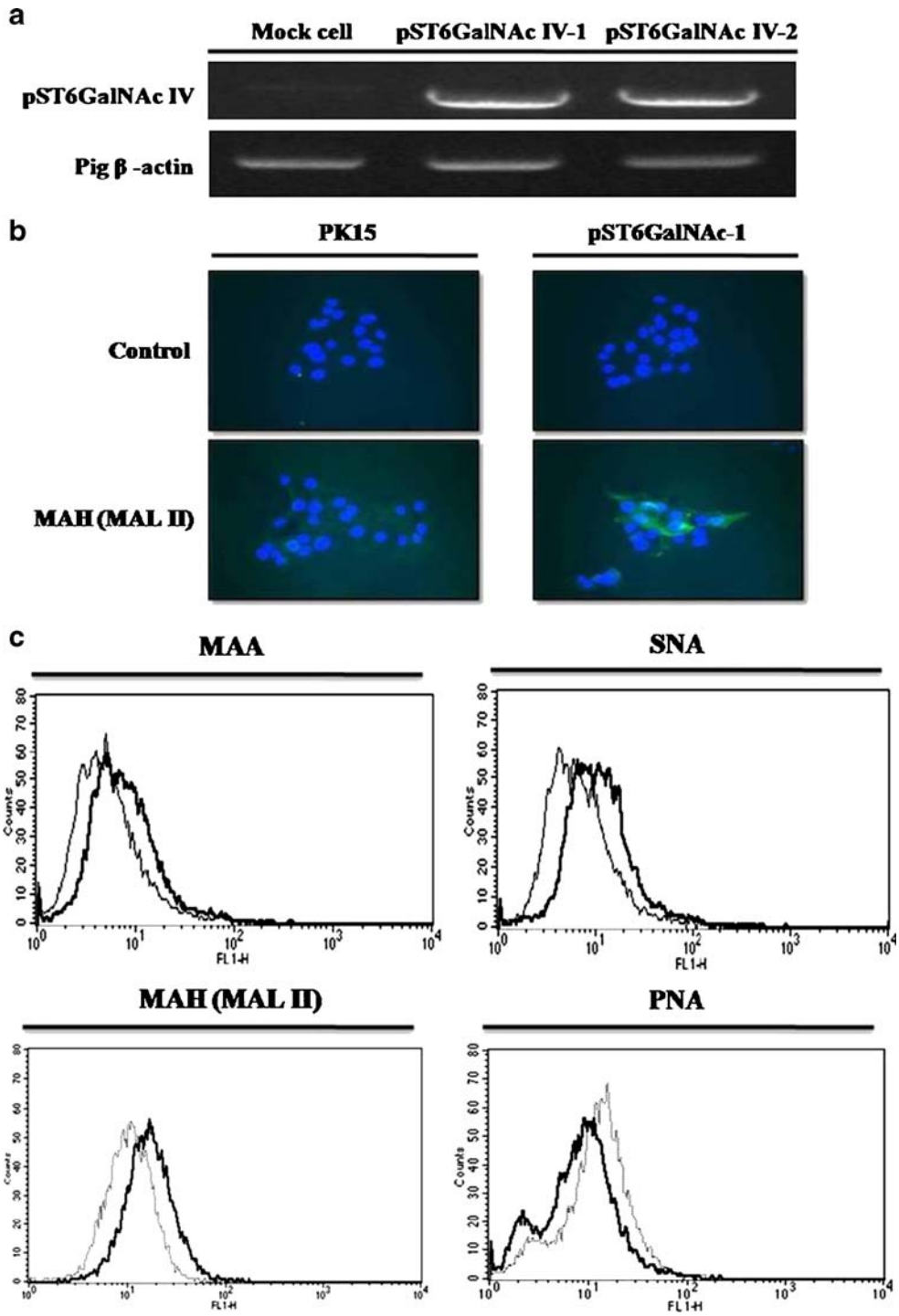

Fig. 3 Change in MAL II lectin binding ability in pST6GalNAc IV-transfected PK15 cells. **a** Construction of stable pST6GalNAc IV cell lines of pST6GalNAc IV-1 and pST6GalNAc IV-2 and confirmation using RT-PCR. **b** Effect of pST6GalNAc IV gene transfection on morphological changes in PK15 cells. **c** Analysis of glycosylation pattern in the pST6GalNAc IV-transfected PK15 cells using Flow cytometry with MAA, SNA, MAH (MAL II) and PNA lectins. The thin line: lectins binding to mock transfectants; thick line: lectins binding to pST6GalNAc IV-transfected PK15 cells

for sialic acids α2-6 linked to Gal or GalNAc residue [17, 19]. In the present study, SNA binding capacity was increased in the pST6GalNAc IV-transfected PK15 cells, compared with mock control. Although MAA binds to α2-3 linked sialic acid, the MAA binding capacity was unexpectedly increased in the pST6GalNAc IV-transfected PK 15 cells. Previously, it was reported that lectin MAA contains two types of the lectin, MAL (*Maackia amurensis* leukoagglutinin) and MAH (*Maackia amurensis* hemagglutinin) [20]. The MAL, called as MAL I, recognizes sialyllactosamine (Neu5Acα2-3Galβ1-4GlcNAc) found on *N*-linked glycan, whereas MAH, called as MAL II, binds to the disialylated tetrasaccharide (Neu5Acα2-3Galβ1-3(NeuAcα2,6)GalNAc) found on *O*-linked glycoprotein [21, 22]. Therefore, the present results suggested that the increased MAA lectin binding capacity was caused by lectin MAH included in MAA. We have further investigated the binding capacity of MAL II (MAH commercially produced) to the

pST6GalNAc IV-transfected PK 15 cells using flow cytometry and immunofluorescence microscopy (Fig. 3c). The results have clearly shown that the MAL II lectin binding capacity was increased in the pST6GalNAc IV-transfected PK15 cells (Fig. 3b and c).

On the other hand, the binding capacity of lectin PNA (*peanut* agglutinin), recognizing the terminal Galβ1-3 GalNAc residues found on *O*-linked glycoprotein [17, 23], were decreased in the pST6GalNAc IV-transfected PK15 cells (Fig. 3c). These result revealed that overexpression of pST6GalNAc IV in PK15 cells results in decrease of Galβ1-3GalNAc carbohydrate structure, whereas increase of Neu5Acα2-6GalNAc or NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc carbohydrate structure. Human and chicken ST6GalNAc II are reported to catalyze the transfer of sialic acid onto *O*-linked Galβ1-3GalNAc or Neu5Acα2-3Galβ1-3GalNAc carbohydrate structure [24, 25]. Our results suggested that pST6GalNAc IV can use Galβ1-3 GalNAc or Neu5Acα2-3Galβ1-3GalNAc carbohydrate structure as substrates, like human or chicken ST6GalNAc II. Further studies are needed to investigate the acceptor substrate specificity of pST6GalNAc IV enzyme.

In this study, we firstly cloned the pig pST6GalNAc IV gene from pig liver tissues and investigated expression level of pST6GalNAc IV gene in various pig tissues. In addition, we established pST6GalNAc IV-overexpressing PK15 cells and investigated changes in lectin binding specificities in the pST6GalNAc IV overexpression PK15 cells using four different lectins of MAA, MAL II, SNA and PNA. Consequently, results on the change in lectin binding pattern in the pST6GalNAc IV-transfected PK15 cells suggested that the pST6GalNAc IV gene plays an important role in transfer of sialic acids to Neu5Acα2-3Galβ1-3GalNAc carbohydrate structure.

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