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Expression of Sialyl-Tn antigen in breast cancer cells transfected with the human CMP-Neu5Ac: GalNAc α2,6-sialyltransferase (ST6GalNAc I) cDNA

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Sialyl-Tn antigen (STn) is a cancer associated carbohydrate antigen over-expressed in several cancers including breast cancer, and currently associated with more aggressive diseases and poor prognosis. However, the commonly used breast cancer cell lines (MDA-MB-231, T47-D and MCF7) do not express STn antigen. The key step in the biosynthesis of STn is the transfer of a sialic acid residue in α2,6-linkage to GalNAcα-*O*-Ser/Thr. This reaction is mainly catalyzed by a CMP-Neu5Ac GalNAc α2,6-sialyltransferase: ST6GalNAc I. In order to generate STn-positive breast cancer cells, we have cloned a cDNA encoding the full-lenght human ST6GalNAc I from HT-29-MTX cells. The stable transfection of MDA-MB-231 with an expression vector encoding ST6GalNAc I induces the expression of STn antigen at the cell surface. The expression of STn short cuts the initial *O*-glycosylation pattern of these cell lines, by competing with the Core-1 β1,3-galactosyltransferase, the first enzyme involved in the elongation of *O*-glycan chains. Moreover, we show that STn expression is associated with morphological changes, decreased growth and increased migration of MDA-MB-231 cells.

Keywords: **breast cancer, Sialyl-Tn, ST6GalNAc I, *O*-glycosylation**

Abbreviations: **T antigen, Thomsen-Friedenreich antigen; Tn antigen, Thomsen-nouveau antigen; Neu5Ac, 5 N-Acetyl-neuraminic acid; ST6GalNAc I, CMP-Neu5Ac: R-GalNAcα1-*O*-Ser/Thr α2,6-sialyltransferase (EC 2.4.99.3); ST6GalNAc II, CMP-Neu5Ac: (Neu5Acα2-3)Galβ1-3GalNAcα1-*O*-Ser/Thr α2,6-sialyltransferase (EC 2.4.99.-); Core1β3GalT, UDP-Gal: GalNAc β1,3-galactosyltransferase (EC 2.4.1.122); GAPDH, Glyceraldehyde phosphate deshydrogenase; PNA, *Arachis hypogaea* agglutinin.**

Introduction

The modification of cellular glycosylation is a common phenotypic change observed in malignancy and the over-expression of antigenic sialylated structures at the surface of cancer cells have been widely reported [1,2].

Relevant to cancer is the abnormal expression of structures related to the Thomsen-Friedenreich antigens family such as Tn antigen (GalNAcα1-*O*-Ser/Thr) and STn (STn) antigen (Neu5Acα2-6GalNAcα1-*O*-Ser/Thr). STn antigen is usually carried by membrane-bounded mucins such as MUC1 [3] but has been also characterized on a CD44 splicing variant [4]. The premature sialylation of core carbohydrate structures stops further elongation of the oligosaccharide chains, leading to the appearance of short truncated and sialylated *O*-glycans. STn expression has been observed in normal secretory tissues such as salivary glands, gastric, intestinal and uterine mucosae, but STn is absent from most of other tissues such as normal colon, pancreas or breast tissues [5].

Over-expression of STn has been reported for cancers of pancreas, stomach and ovary with varying frequencies [6]. This expression is a strong, independent indicator of poor prognosis [7], and was associated with other poor prognosis markers such as high histologic grades, survival decrease and lymphoid

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node invasion [8–12]. Several studies have also reported STn expression in breast cancers, ranging from 16 to 85% of patients [6,13–15]. STn expression was significantly associated with high histologic grades and aneuploïdy [16], survival decrease [17], loss of estrogen receptors expression and c-erb-B2 oncogene activation [18]. Miles et al. have also shown that expression of STn appears to be a marker of resistance to adjuvant chemotherapy in node-positive breast cancer [19].

STn antigen is associated with cancer aggressiveness and poor prognosis, however the exact biological function of this structure remains unclear. By the way, STn antigen has been considered as a real interest for the elaboration of a cancer vaccine. Synthetic STn hapten, conjugated to Keyhole limpet hemocyanin (STn-KLH) and injected with Detox-B stable emulsion has been used to treat patients with ovarian or breast cancer after high-dose chemotherapy. Clinical responses indicate a decrease of the risk for relapse and death of treated patients [20].

A family of enzymes named sialyltransferases catalyzes the biosynthesis of sialylated structures. To date 18 distinct human sialyltransferase genes have been cloned [21]. These enzymes are expressed in a tissue and cell-type specific manner, and their expression is regulated at developmental stages [22]. They are expressed in the Golgi apparatus and the trans-Golgi-network. Sialyltransferases are type II transmembrane glycoproteins, with a short NH_2-terminal cytoplasmic tail, a 16–20 amino acid signal anchor domain and a large COOH-terminal catalytic domain in the Golgi apparatus lumen [22]. They all catalyze the transfer of a sialic acid residue from CMP-Neu5Ac to oligosaccharide chains of glycolipids and glycoproteins. The linkage formed can be α2-3 or α2-6 to galactose (Gal) residue, α2-6 to *N*-acetylgalactosamine (GalNAc) residue or α2-8 to another sialic acid residue. Each enzyme can catalyze only one linkage and has its own substrate specificity [21].

Six α2,6-sialyltransferases catalyze the transfer of Neu5Ac to GalNAc. Three of them (ST6GalNAc I, II, and IV) are active on glycoproteins, whereas the others (ST6GalNAc III, V, and VI) exhibit specificity towards glycolipids [21]. So far, the cloning and characterization of the first three enzymes from human sources has been reported [23–25]. It has also been shown that ST6GalNAc I and ST6GalNAc II share nearly the same substrate specificity: both are able to transfer sialic acid to T antigen (Galβ1-3GalNAcα1-*O*-Ser/Thr) or to Sialyl-3T antigen (Neu5Acα2-3Galβ1-3GalNAcα1-*O*-Ser/Thr) leading to Sialyl-6T antigen (Galβ1-3[Neu5Acα2-6]GalNAcα1-*O*-Ser/Thr) and disialyl-T antigen (Neu5Acα2-3Galβ1-3[Neu5Acα2-6]GalNAcα1-*O*-Ser/Thr), respectively [23,24]. The main difference in acceptor substrate specificity takes place in the α6 sialylation of Tn antigen [23,26]. Whereas the mouse ST6GalNAc II was shown to transfer sialic acid onto asialo-ovine submaxillary mucin *in vitro* [26], ST6GalNAc I was shown to be much more efficient towards this acceptor substrate [23,24]. Furthermore, STn antigen expression in malignant rat colonic epithelial cells depends upon ST6GalNAc I expression [27], which thus appears as the most probable candidate for human STn biosynthesis [23,27].

Brockhausen et al. have reported that breast cancer cell lines MDA-MB-231, BT-20, MCF-7 and T47-D do not exhibit ST6GalNAc I activity onto GalNAc-mucin used as substrate [28]. In order to elucidate the biological effect of the expression of STn antigen in breast cancer, it was necessary to obtain cell lines expressing STn. In this paper, we report on the molecular cloning of a human ST6GalNAc I cDNA from HT-29-MTX colon cancer cells used for stable transfection in breast cancer cell lines. The modifications of the *O*-glycosylation pattern of the transfected cells are detailed and we show that STn expression modify cell migration.

Materials and methods

Cell culture: Breast cancer cell lines: MDA-MB-231, BT-20, MCF-7 and T47-D were obtained from the American Type Cell Culture Collection. Cells were routinely grown in EMEM medium (Bio Whittaker) supplemented with 5% foetal calf serum (FCS) (Life Technologies), 100 $\mu g \cdot ml^{-1}$ penicillin, 100 $UI.ml^{-1}$ streptomycin, and 45 $\mu g \cdot ml^{-1}$ gentamycin. HT-29-differenciated subpopulations (mucus secreting cells) were derived from the original cell line [29] and adapted to 10^{-6} M Methotrexate (MTX) [30].

Molecular cloning of hST6GalNAc I: Nucleotide BLAST analysis (BLASTn) with the nucleotide sequence of chicken ST6GalNAc I (GenBank accession number X74946) was used to search the hST6GalNAc I sequence in the human genome survey sequence database at the National Center for Biotechnology Information (NCBI). An expression plasmid designed to encode the hST6GalNAc I full length was constructed by PCR using HT-29-MTX cDNA and the following primer pair: sense primer is $5'\text{-}_{30}$GCAGAAGCGGCCGCTAGAACCCGACCCACCACCATG-3′ and contains a NotI restriction site (underlined), antisense primer is $3'\text{-}_{1846}$GGTTTCGGTTCTTGACTGGCCCAGATCTCGACGGTA-5′ and contains a XbaI restriction site (underlined). Numbers on the left side of each sequence indicates the position of the first nucleotide in the sequence of the cDNA (Y11339). Forty cycles (1 min at 94°C, 1 min at 68°C and 1.5 min at 72°C) were run using 2 U of Advantage-HF Taq polymerase (Clontech). The resulting 1859 bp amplified fragment was subcloned into pCR2.1 vector (TOPO TA-cloning kit, Invitrogen). The inserted fragment was cut out by digestion with NotI and XbaI and inserted into the NotI and XbaI sites of pRc-CMV expression vector (Invitrogen). Restriction digestion and DNA sequencing confirmed the insert junctions and the total conservation between the inserted sequence and hST6GalNAc I sequence (accession # Y11339). The resulting plasmid (pRc-CMV-hST6GalNAc I) was further purified and used for transfection as described below.

RT-PCR: Total cellular RNA was isolated using RNeasy Mini Kit from Qiagen. Following the supplier's recommendations,

Table 1. Sequences of the three primer pairs used for PCR amplification

Target[a] *cDNA*	*Primer set*[b]	*GC content (%)*	*Tm (°C)*	*PCR product size (bp)*
GAPDH	5′-$_{4406}$GGTGGACCTGACCTGCCGTCTA-3′	63.0	73.1	
[J04038]	3′-$_{4765}$GAGGTCCACCACCCTGTTGCTG-5′	63.0	73.4	256
hST6GalNAc I	5′-$_{1407}$GGACTATGAGTGGCTGGAAGCA-3′	54.5	61.7	
[Y11339]	3′-$_{1801}$ TCCCTATTAGGCCGACATGGTC-5′	54.5	66.2	421
Core1β3GalT	5′-$_{376}$ AACACGTCAAAGCTACTTGGGC-3′	50.0	61.2	
[AF155582]	3′-$_{973}$ AGATCAGAGCAGCAACCAGGAC-5′	54.5	60.8	598

[a]Genbank accession number are shown in brackets.
[b]The number on the left side of each sequence indicates the position of the first nucleotide in the coding sequence of each cDNA, except for GAPDH, which indicates the first 5′ nucleotide in the gene sequence.

we treated the silica membrane retaining RNA with DNAse I (RNAse free DNase set protocol, Qiagen). The amount of RNA was quantified by measuring the absorbance at 260 nm. Total cellular RNA (5 μg) was used for RT-PCR as described previously [31] using hST6GalNAc I, Core1β3GalT, or GAPDH specific primers (Table 1). Briefly, the 25 μl of PCR mixture consisted of 1 unit of Hi-Taq DNA polymerase (Quantum Appligene), 10 mM Tris-HCl pH 8.8, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% triton X-100, 0.2 mM dNTP and 0.6 μM of each primer. Samples were overlaid with two drops of mineral oil (Sigma Chemical Co.). Reactions were run in a PTC-100 thermal cycler (M.J. Research, Inc.) using the following conditions: for specific GAPDH fragment amplification: 1 min at 94°C, 1 min at 69°C and 1.5 min at 72°C, 22 cycles; for specific hST6GalNAc I fragment amplification: 1 min at 94°C, 1 min at 65°C and 2 min at 72°C, 40 cycles (Figure 1) or 27cycles (Figure 4); for specific hCore1β3GalT fragment amplification: 1 min at 94°C, 1 min at 60°C and 1.5 min at 72°C, 27 cycles. In all experiments, negative control reactions were performed by replacing cDNA template with sterile water. Amplification products were seen on a 1.5% agarose gel containing ethidium bromide.

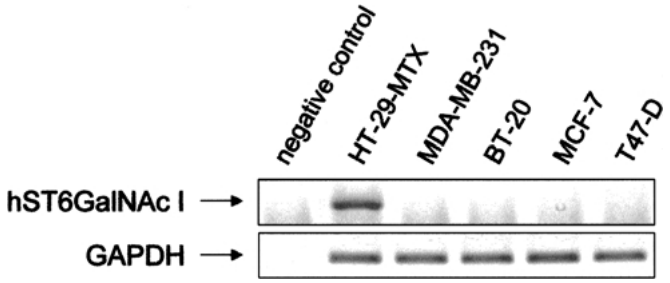

Figure 1. RT-PCR analysis of the expression of hST6GalNAc I in breast cancer cell lines. PCR was performed using cDNA prepared from total RNA of MDA-MB-231, BT-20, T47-D and MCF-7 breast cancer cells. A positive control was performed by using HT-29-MTX cDNA as template. The 420 bp length product of 40 cycles of amplification of hST6GalNAc I cDNA fragment is shown at the top of the figure. The 256 bp length product of 22 cycles of amplification of GAPDH cDNA fragment is shown at the bottom of the figure.

Transient transfections: Cell lines MDA-MB-231, MCF-7 and T47-D were transiently co-transfected with a pECFP-C1 plasmid containing Green Fluorescent Protein (GFP) cDNA (Clontech) and either of the pRc-CMV expression plasmid, containing or not the hST6GalNAc I cDNA sequence. Briefly, cells were seeded on eight chambers slides (LAB-TEK Nalge Nunc International) and cultured in standard conditions until mid-confluence. Then they were transfected with 0.5 μg pECF-C1 plasmid, 0.5 μg plasmid of interest, and 0.6% lipofectamine (Gibco) for MDA-MB-231 and T47-D or 0.8% lipofectine (Gibco) for MCF-7, in Opti-MEM (Life Technologies). After transfection, cells were cultured for 24 hours in FCS-containing fresh medium.

Stable transfections: Cells were cultured in 100 mm diameter dishes (Nalge Nunc International) and transfected with pRc-CMV or pRc-CMV-hST6GalNAc I as described above. Two days after transfection, the dishes were split 1:10 and cultured for two weeks in EMEM containing 5% FCS and geneticin 418 (G418) (1000 $\mu g \cdot ml^{-1}$). After 15 days of selection, the resistant clones were picked-up with a trypsin soaked 1 mm^2 piece of sterilized Whatmann paper and seeded in 35 mm diameter dishes. Resistant clones were then cultured in same conditions as original cells unless selection by G418 which was applied one week in two.

Confocal microscopy: Confocal microscopy was performed on cell monolayers grown on eight chambers-slides. Cells were fixed with 4% paraformaldehyde (30 min at 4°C) and quenched with 50 mM NH_4Cl in Phosphate buffered saline (PBS) (30 min). The saturation (30 min at 4°C) and the following incubations were performed in PBS containing either 1% bovine serum albumin (BSA) for mAbs, or 2% polyvinylpyrolidone for PNA. Laser confocal microscopy analysis was performed using a Leica-instrument (Model TCS-NT).

Desialylation of the cell monolayer was performed before the incubation with mAbs or PNA, using 5 mU of type V neuraminidase from *Clostridium perfringens* (Sigma) in 50 mM citrate buffer, 0.9% NaCl, 0.1% $CaCl_2$, 1% BSA and slides were incubated 3 hours at 37°C in water saturated atmosphere.

STn antigen detection was performed by using a mouse IgG1 mAb (HB-STn1, Dako) diluted 1/50 (overnight at 4°C) and revealed by a Texas-Red-conjugated goat anti-mouse IgG1 (Jackson) diluted 1/150. Tn antigen detection was performed by using a mouse IgM mAb (HB-Tn1) diluted 1/50 (overnight at 4°C) and revealed by a Texas-Red-conjugated goat anti-mouse IgM (Jackson) diluted 1/150 (4 hours at 4°C). T antigen detection was performed by using Texas-Red-conjugated PNA lectin at 50 $\mu g \cdot ml^{-1}$.

Flow cytometric analysis: Cells (6×10^6) were incubated with the HB-STn1 mAb (1/50 in PBS, 1% BSA) for 1 hour on ice, and then washed twice with PBS 1% BSA, followed by incubation with FITC-conjugated anti-mouse whole IgG (1/80 in PBS 1% BSA) for 30 min on ice. Cells were then subjected to flow cytometric analysis using a FACScalibur instrument from Becton Dickinson.

Sialyltransferase assays: Sialyltransferase assays were performed as described previously [32]. Briefly, enzyme activity was measured at 37°C for 4 hours in 50 μl reaction mixture comprising 0.1 M cacodylate buffer pH 6.2, 10 mM $MnCl_2$, 0.2% Triton CF-54, 50 μM CMP-[^{14}C]-Neu5Ac (1.85 kBq), with 20 μl of the enzyme source and 1 mM of the acceptor substrate. This acceptor is an hexadecaglycopeptide GTTP-SPVPT[GalNAc]TSTTSAP (Neosystème, Strasbourg), corresponding to the MUC5AC tandem repeat sequence substituted by a GalNAc residue on Thr-9. The reactions were stopped by addition of 450 μl H_2O and products were separated from the donor by hydrophobic chromatography on C_{18} SepPak cartridges (Waters). After washing with 7 ml H_2O, the peptide was eluted with 7 ml 70% CH_3OH. The radioactive material was counted by liquid scintillation. The rate of this reaction was linear with time at least for 8 hours.

Cells growth analysis: Cells (2×10^4) were seeded in 35 mm diameter dishes and grown in 5% FCS-containing medium. They were collected after 48, 72, 96 or 120 hours, using 500 μl of Trypsin-EDTA 0.2 mM (Bio Whittaker) and counted on Malassez slide.

Cells migration analysis: Cells (2×10^4) were seeded in Transwell twelve wells plates (Costar) and grown 18 hours in 5% FCS-containing medium. They were fixed with cold methanol (10 min) and stained with Hoescht 33528 (Sigma) (20 min in the dark). Then the up-side of the well's porous membrane was scraped and washed and the membrane was mounted on slide with Glycergel mounting medium (Dako). Remaining stained cells (on the membrane's down-side) were observed and counted by fluorescence microscopy.

Results

Screening for the expression of hST6GalNAc I in various breast cancer cell lines

The expression of the hST6GalNAc I gene was analysed by PCR using cDNAs prepared from breast cancer cells total RNA, using a primers pair designed to generate a 421 bp hST6GalNAc I DNA fragment (Table 1). cDNA from HT-29-MTX colon cancer cells were used as a positive control. As shown in Figure 1, after 40 PCR cycles, no amplification product was obtained with breast cancer cells cDNAs, indicating the absence of hST6GalNAc I transcript in these cell lines. In fact, within the twenty two cell lines of various cellular types we analysed, only two of them: Jurkat (a lymphoid cell line) and HT-29-MTX (as shown in Figure 1) showed an amplified fragment corresponding to the ST6GalNAc I cDNA (data not shown). This result confirms previous data showing the absence of Neu5Acα2-6 transfer to GalNAcα-*O*-Ser/Thr [28] and the absence of STn antigen in breast cancer cell lines [33].

Molecular cloning of the full-length hST6GalNAc I cDNA from HT-29-MTX cells

In order to express STn antigen in the breast cancer cell lines, the full-length cDNA encoding hST6GalNAc I was cloned by PCR from a cDNA library of HT-29-MTX cells as described in *Materials and Methods* section. The resulting 1859 bp cDNA was found to be 100% identical to the long-form (#Y11339) characterized by Ikehara et al. from gastric cancer cells [23]. The sequence has been submitted and registred in NCBI database (#AY096001). This cDNA contains an open reading frame of 1803 bp encoding a 600 amino acids protein. The cDNA was subcloned in pRc-CMV expression vector and used for transient transfections of MDA-MB-231, MCF-7 and T47-D cells. Cells were also co-transfected with pECFP-C1 plasmid encoding the GFP. Transfected cells were immunostained using an anti-STn mAb (HB-STn1). Figure 2 clearly shows the expression of STn in the three cell lines transfected with pRc-CMV-hST6GalNAc I vector.

STn expression in stably transfected breast cancer cells

In order to obtain breast cancer cells stably expressing STn antigen, MDA-MB-231 were transfected with the pRc-CMV-hST6GalNAc I plasmid and were selected on the basis of their resistance to G418 for two weeks. The resistant clones were isolated and dispatched in new dishes. Few of these clones were effectively expressing STn antigen, as detected by immunostaining, and only two STn-positive clones (named MDA-STn-a and MDA-STn-b) persisted after three months of culture.

The expression of ST6GalNAc I transcript was controlled in the selected clones by RT-PCR. As shown in Figure 3A, both selected clones expressed the corresponding hST6GalNAc I mRNA, whereas the mock transfected cells (MDA-neo) do not.

The enzymatic activity of hST6GalNAc I was also measured from STn-positive clones using the synthetic hexadecaglycopeptide GTTPSPVPT[GalNAc]TSTTSAP, which corresponds to a MUC5AC tandem repeat substituted by a unique GalNAc residue on Thr-9. This acceptor substrate allowed to discriminate between ST6GalNAc I activity and other ST6GalNAc activities, such as those of hST6GalNAc II and IV known to be expressed in MDA-MB-231 cells [24,25]. As

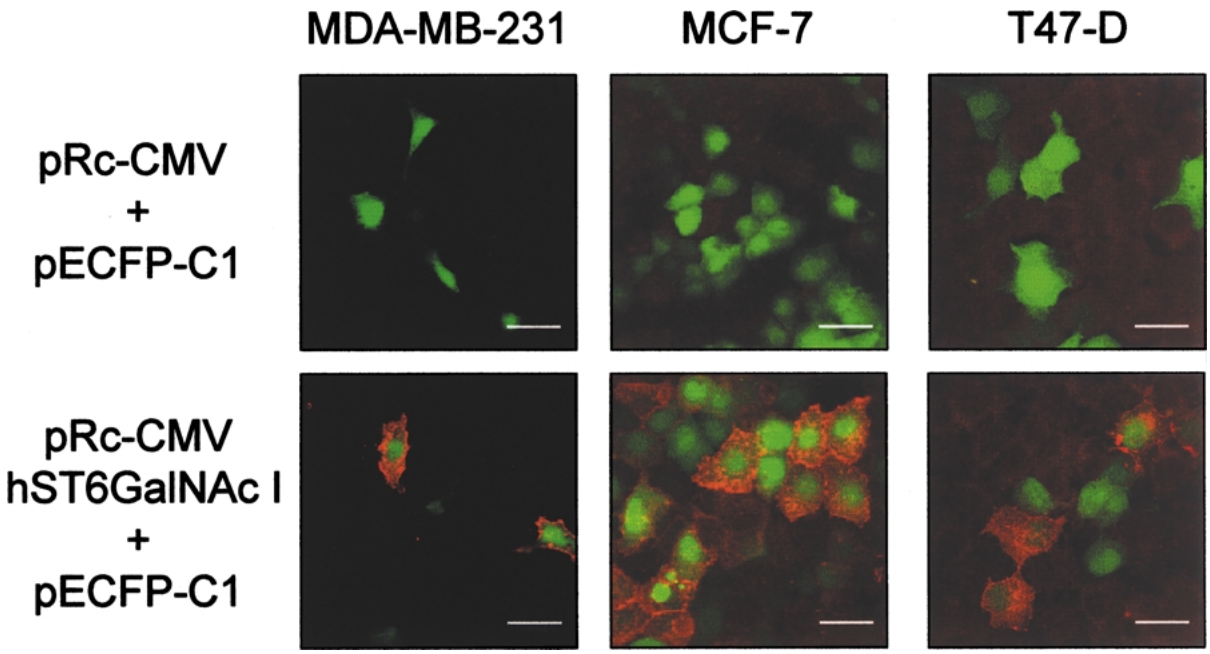

Figure 2. Detection of STn expression by confocal microscopy analysis of transiently transfected breast cancer cells. Cells were cultured in EMEM containing 5% FCS on eight chambers-slides (LAB-TEK) until mid-confluence. They were co-transfected with pECFP-C1 and either pRc-CMV or pRc-CMV-hST6GalNAc I, as described in Materials and Methods section. One day after transfection, cells were fixed, but not permeabilized, and labeled with the HB-STn1 mAb. A secondary anti-mouse revealed hB-STn1 labeling IgG1 Texas-Red conjugated antibody. Bars: 50 μm.

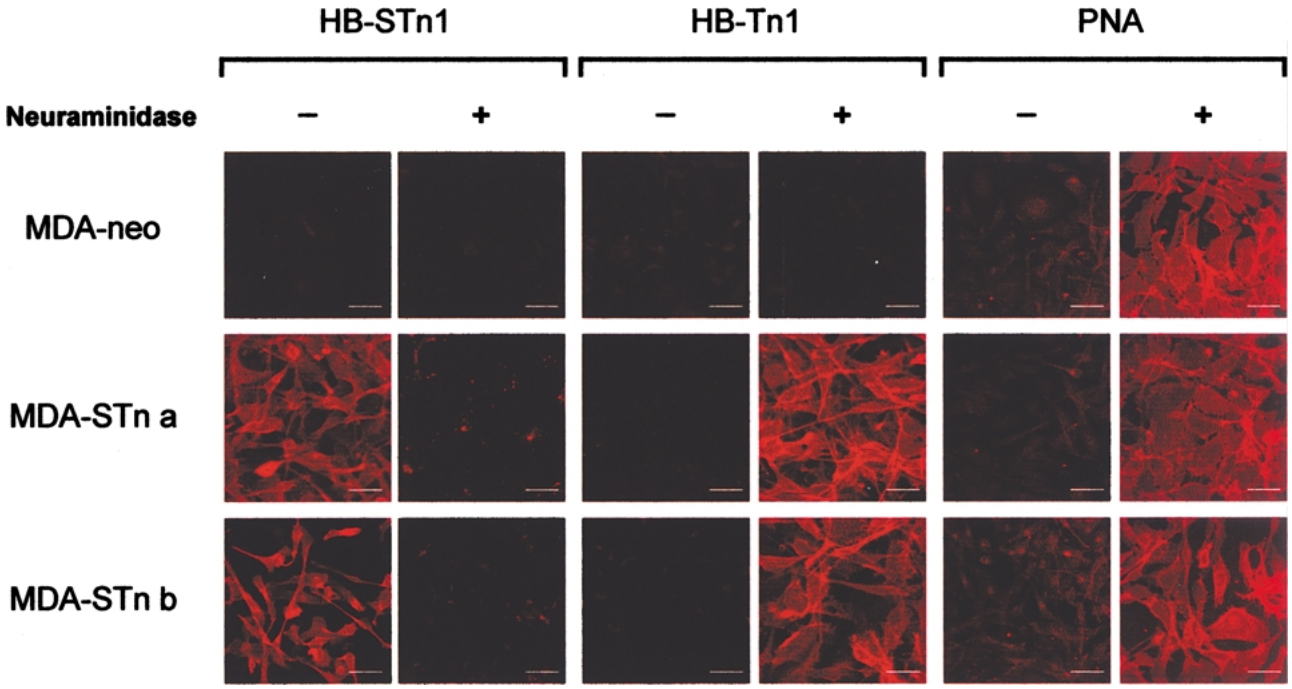

Figure 5. Confocal microscopy analysis of STn, Tn and T antigens expression in selected clones. Cells were treated or not with 5 mU of sialidase before the first immuno- or lectin labeling as described in Materials and Methods section. STn antigen was labeled by HB-STn1 mAb, Tn antigen by HB-Tn1 mAb, T antigen by Texas-Red conjugated PNA. Bars: 50 μm.

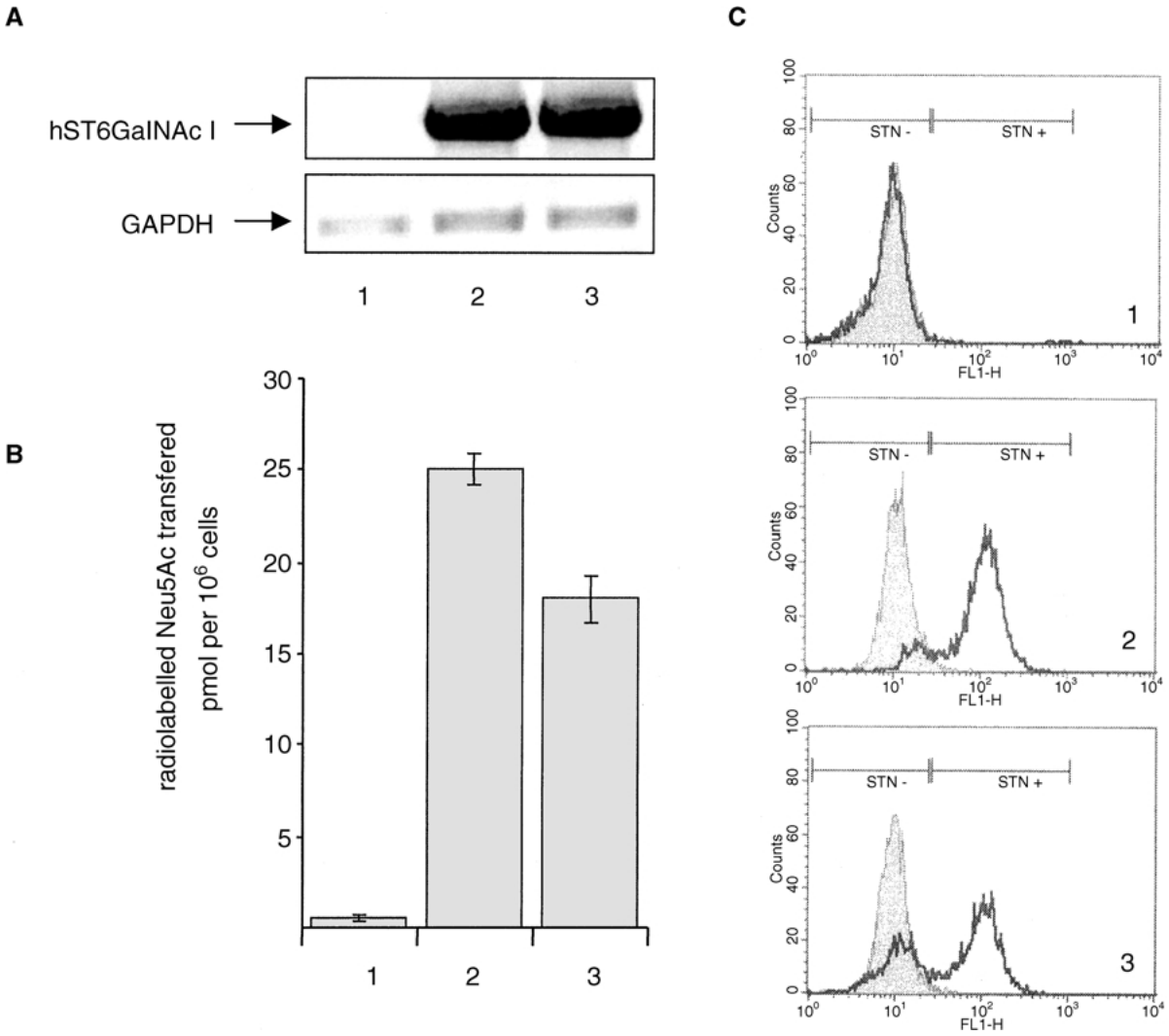

Figure 3. hST6GalNAc I induced STn expression at the surface of stably transfected breast cancer cells. For the four panels the selected clones are listed as below: 1, MDA-neo; 2, MDA-STn-a; 3, MDA-STn-b. A: RT-PCR analysis of the expression of hST6GalNAc I mRNA in selected clones. The 420 bp length product of 27 cycles of amplification of hST6GalNAc I cDNA fragment is shown at the top of the panel. The 256 bp length product of 22 cycles of amplification of GAPDH cDNA fragment is shown at the bottom of the panel. B: hST6GalNAc I enzymatic activity measured in selected clones. Enzymatic activity was measured by incubation with a mono-GalNAc substituted hexadecaglycopeptide chemically synthesized and corresponding to the MUC5AC tandem repeat sequence. Results are expressed in pmol of transferred Neu5Ac per 10^6 cells. C: Flow cytometric analysis of STn-positive cells pattern in selected clones. The filled peaks correspond to negative controls with a non-relevant mouse IgG1 kappa. Black lines correspond to positive signal observed with HB-STn1 mAb incubation. Based on the comparison of basal and specific signals obtained in this experiment, we determined the percentage of STn-positive cells for each clones.

shown in Figure 3B, both STn-positive clones were able to transfer a Neu5Ac residue in α2-6 linkage to GalNAc-*O*-Thr, whereas the mock-transfected cells did not.

The proportion of STn-positive cells in selected clones was determined by flow cytometric analysis (Figure 3C). The clones MDA-STn-a and MDA-STn-b presented 89% and 64% of STn-positive cells respectively. These percentages filled well with hST6GalNAc I activity determined *in vitro* (Figure 3B). Finally, confocal microscopy analysis confirmed that both hST6GalNAc I expressing clones expressed STn antigen at the cell surface (data not shown). All together, these results indicated that the expression STn antigen is directly determined by the hST6GalNAc I transfection.

RT-PCR analysis of Core1β3GalT expression in the MDA-STn cells

The hST6GalNAc I expressed in breast cancer cell lines might compete with other enzymes that use the Tn carbohydrate antigen (GalNAcα-*O*-Ser/Thr) as an acceptor substrate, especially

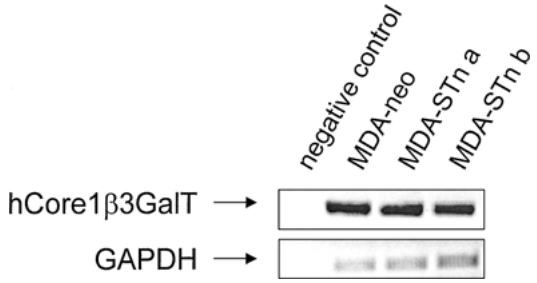

Figure 4. RT-PCR analysis of the expression of Core1β3GalT in selected clones. PCR were performed using cDNA prepared from total RNA of MDA-STn clones and mock transfected cells. The 598 bp length product of 27 cycles of amplification of *Core1β3GalT* cDNA fragment is shown at the top of the figure. The 256 bp length product of 22 cycles of amplification of GAPDH cDNA fragment is shown at the bottom of the figure.

the Core1β3GalT, which catalyzes the transfer of a Gal residue in β1,3-linkage to GalNAc, leading to the synthesis of the Core 1 structure (Galβ1-3GalNAcα1-*O*-Ser/Thr). We have analysed by RT-PCR, the level of expression of the Core1β3GalT in the selected clones. As shown in Figure 4, the expression of hST6GalNAc I did not modified the level of expression of the Core1β3GalT in MDA-STn cells.

Analysis of the *O*-linked carbohydrate structures expressed at the cell surface

We have analyzed the *O*-glycosylation pattern of both mock transfected cells and STn-positive selected cells, based on the detection of STn, Tn and T antigens by fluorescence microscopy. STn and Tn antigens were revealed by HB-STn1 and HB-Tn1 mAbs, respectively (Dako), and T antigen was revealed by PNA lectin (EY laboratories). All experiments were performed with or without desialylation before the primary staining.

As shown in Figure 5, mock transfected cells (MDA-neo) did not express neither STn nor Tn antigens. On the other hand, they expressed the T antigen, as revealed by PNA, and also the sialylated derivatives of T antigen, as the desialylation by neuraminidase treatment improved the PNA labelling (last column). So, the shorter *O*-linked carbohydrate structure detected in these cells is the T antigen (Galβ1-3GalNAcα-*O*-Ser/Thr), a core 1 based structure.

Contrary to mock transfected cells, selected clones expressed STn but not Tn antigen. For these clones, the desialylation completely abolished the STn staining. This indicates that the sialic acid removal prevents the mAb binding, confirming the specificity of the HB-STn1 mAb for sialylated structures. At the same time, a binding of HB-Tn1 mAb was observed when cells were desialylated before the primary staining. Thus, after sialic acid removal, the STn antigen expressed in ST6GalNAc I transfectants is converted in Tn antigen by unmasking the GalNAc residues. This further confirmed the expression of STn at the cell surface on transfected cells, and that the shorter *O*-linked carbohydrate structure detected in these cells is the STn antigen. As mock transfected cells do not express Tn antigen capable to be sialylated by ST6GalNAc I, we concluded that ST6GalNAc I compete with the Core1β3GalT to synthesize STn antigen. Finally, the staining of T-antigen, before or after desialylation, showed only a slight decrease of PNA binding on the transfected cells compared to mock transfectants, suggesting that only a part of the *O*-glycosylation sites expressed at the cell surface of transfected cells are substituted by STn antigen.

MDA-STn-a clone has a decreased cell growth and an increased mobility

The expression of STn antigen at the cell surface of MDA-STn-a cells induced morphological changes compared to STn-negative cells. The STn-positive cells appeared more stretched than the STn-negative cells (Figure 6A, panels a and c). Moreover, STn-positive cells seemed to be less sensitive to contact inhibition, they did not establish cell-cell interactions when confluency is reached but they were able to spread on each other and form local area of cells multilayer (Figure 6B, panels b and d).

Secondly, we observed that MDA-STn-a clone has a decreased growth compared to STn-negative cells (Figure 6B). Finally, in migration assay using a compartmented chambers system (Transwell, Costar), we observed that there was six-fold more STn-positive cells which moved through the porous membrane, than STn-negative cells (Figure 6C).

Discussion

As a first step towards understanding the implication of STn expression on the biology of breast cancer cells, we have generated STn-positive breast cancer cells. We have cloned a hST6GalNAc I cDNA from HT-29-MTX colon cancer cells and transfected MDA-MB-231, MCF-7 and T47-D cells with this cDNA. These three cell lines do not express neither the STn antigen nor the hST6GalNAc I transcript. They were chosen according to the fact that they exhibit different *O*-glycosylation patterns as determined by the structural analysis of the *O*-glycans carried by MUC1 [33]. T47-D cells proteins are highly *O*-glycosylated [34] and mainly express core 1-based *O*-glycans which are mono- and disialylated. In contrast, MDA-MB-231 cells express predominantly α3-sialylated core 2 based glycans, and MCF-7 cells shows also a completely different pattern of glycosylation expressing mainly neutral, more extended, fucosylated glycans (up to fucosylated octasaccharides). After transient transfections of the hST6GalNAc I cDNA, we have detected the expression of STn antigen at the cell surface of the three cell lines, regardless of their respective initial glycosylation pattern. The expression of hST6GalNAc I mRNA was confirmed by RT-PCR in both MDA-MB-321 stable transfectants, as well as the hST6GalNAc I activity, measured in cell lysates using a MUC5AC tandem-repeat derived glycopeptide as acceptor substrate. These observations clearly indicated that

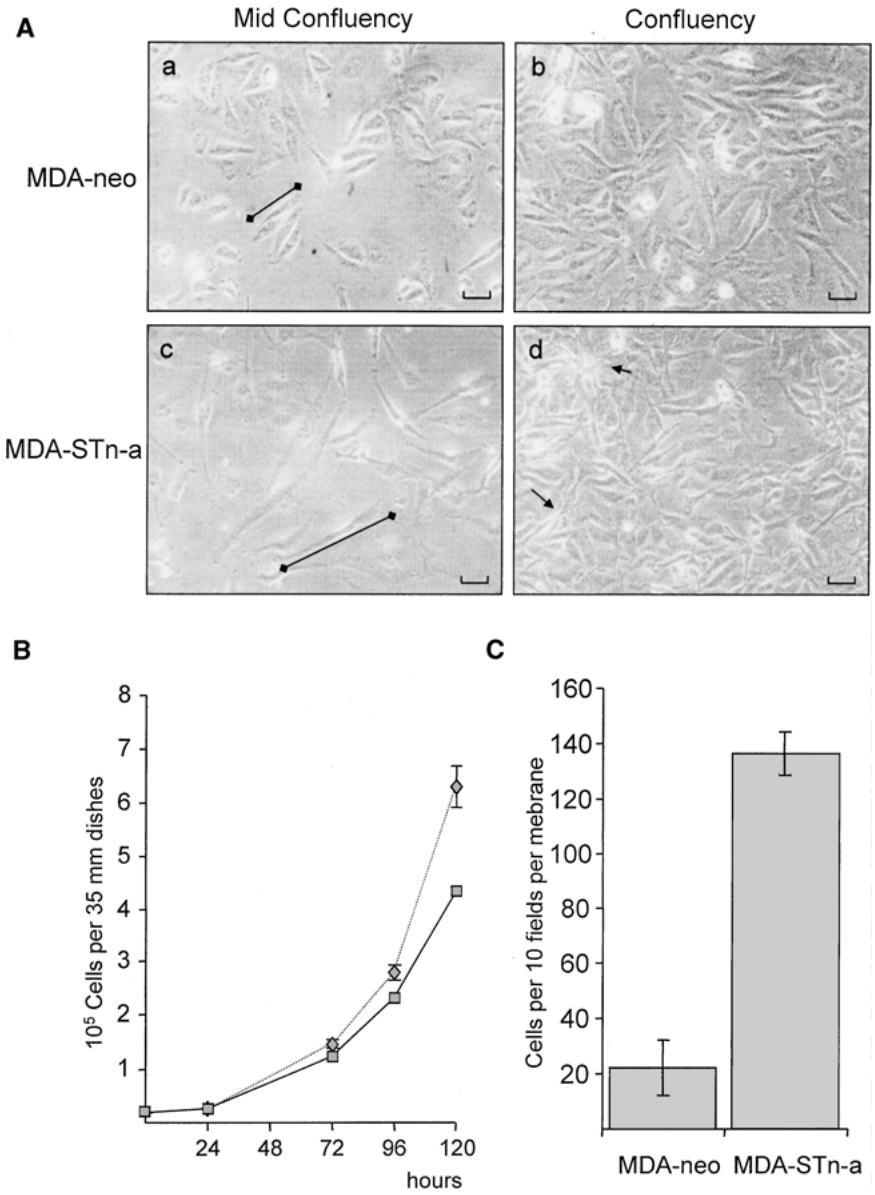

Figure 6. MDA-STn clone shows a decreased growth and an increased mobility in cell culture condition. A: Phase contrast microscopy of MDA-neo (a–b) and MDA-STn a (c–d). Black lines symbolize the cell size (a and c). Arrows indicate local multilayer areas (d). Bars: 25 μm. B: Growth rate of MDA-neo and MDA-STn a. C: Analysis of cells migration. Cells were counted in ten well-distributed fields per membrane.

the STn-negative status of breast cancer cell lines is mainly due to the absence of ST6GalNAc I activity rather than to the lack of acceptor substrates expressed at the cell surface.

The human ST6GalNAc I was previously cloned by Ikehara et al. from human pyloric mucosa and the authors have shown that the transfection of HCT15 human colon cancer cells with a plasmid encoding hST6GalNAc I induced STn expression [23]. Brockhausen et al. have also shown that the expression of STn in rat colonic cancer cells was dependent upon ST6GalNAc I expression [27]. On the other hand, ST6GalNAc II was shown to catalyze the biosynthesis of STn in *in vitro* assays [26], but there is no evidence for the implication of ST6GalNAc II in STn expression *in vivo*. As shown in this study, commonly used breast cancer cell lines (i.e. MDA-MB-231, MCF-7 and T47-D) are STn-negative whereas they all express ST6GalNAc II mRNA [24]. ST6GalNAc I seems to be therefore the main enzyme involved in the STn biosynthesis in breast cancer cells.

We have also demonstrated that the biosynthesis of STn short cuts the original glycosylation pathway of the cells, recruiting precursor structures that are originally involved in the biosynthesis of extended Core 1 based *O*-glycans. In other words, expression of STn antigen reflects a competition between ST6GalNAc I and Core1β3GalT for GalNAc-*O*-Ser/Thr structure as acceptor. Wang et al. have recently reported that

STn sialylation occurs in trans-Golgi in colorectal adenoma cells but that this activity is also detectable in the ER and the cis-Golgi lumen in adenocarcinoma cells [35]. Both enzymes might be therefore present in the same cellular subcompartment (i.e. early Golgi apparatus) and physically compete for the same acceptor. Alternatively, ST6GalNAc I might have the opportunity to act before the Core1β3GalT in an earlier compartment like cis-Golgi or ER. As we do not know exactly where the Core1β3GalT is expressed, both hypothesis are valid and further experiments are required to precisely define the first steps of biosynthesis of *O*-glycans in STn-positive cell lines. It has been widely reported that breast cancer cells over-express the MUC 1 protein, a membrane mucin. This protein has been well studied to define the *O*-glycosylation patterns of the breast cancer cells [4,28,33,34]. In parallel, it has been reported that CD44*v6*, a splicing variant of the *O*-glycosylated CD44 membrane protein, expressed by HT-29 human colonic cancer cells, carried STn antigen [5]. Breast cancer cells have been also shown to express CD44*v6* [36,37]. For these reasons MUC1 and CD44*v6* seem to be interesting candidates for the fine study of *O*-glycosylation patterns of the MDA-STn clones.

In this study, we have generated two clones from MDA-MB-231 cell line, which stably express STn antigen. Flow cytometric analysis has revealed that these two clones expressed STn-positive cells in various proportion. Nevertheless, no phenotypic reversion was observed during six months of continuous culture (data not shown). The study of MDA-STn-a clone (89% of STn-positive cells) showed a decreased growth and an increased mobility compared to STn-negative cells. Unless STn antigen has a specific impact on proliferation or survival, which remains to be determined, we assume that the reduced cell growth observed is mainly due to the enhanced mobility, because when the cell move the mitosis frequency is reduced. Furthermore, the cell morphology observed at confluency reflected a weak adherence of these cells. Similar observations were recently reported by Lin et al. [38] after Transfection of MDA-MB-435 with a ST6Gal I cDNA. Transfected clones carrying increased amounts of α2,6-linked sialic acid showed reduced cell-cell adhesion and enhanced invasion capacity. From these data, it seems that increased α2,6-sialylation reduces cell adhesion by increasing the cell membrane negative charges and/or by modifying some membranous proteins conformation. Moreover, because hST6GalNAc I can compete with Core1β3GalT, STn expression could reduce the expression of other peripheral carbohydrate structures (sialylated or not) as it was shown by Ikehara et al. using HCT15 transfected cells [23]. The decrease or the lack of these structures might play a role in the observed new phenotype.

Modifications of sialylation in cancer have been already reported to change cell behaviour. For example, sialyl Lewis^a and sialyl Lewis^x were found on glycoproteins and glycolipids of several cancers such as lung, colon, stomach and breast cancer [39]. The aberrant expression of these carbohydrate structures at the cancer cell surface are thought to enhance the metastasis ability of these cells. ST3Gal III (CMP-Neu5Ac: Galβ1-3(4)GlcNAc α2-3 sialyltransferase, EC 2.4.99.6), involved in the sialyl Lewis^a synthesis, has been shown to be highly expressed in breast cancers, in association with axillary node involvement and a decreased overall survival [31]. Furthermore, sialyl Lewis^x has been shown to serve as a ligand for selectins and adhesion of human breast cancer cells to vascular endothelium, mediated by sialyl Lewis^x/E-selectin interaction [40].

STn antigen is also specifically recognized by lectin-type proteins know as Siglecs (Sialic acid-binding Ig-like lectins) and especially by the Siglec 6 protein [41]. It was also recently shown that human interleukin-7 specifically binds STn antigen [42]. The new model of STn-positive breast cancer cells we generated will allow us to study the effect of Siglec 6 or interleukin-7 on cell behaviour, both *in vitro* and *in vivo*. This will help us to better understand the apparent aggressiveness of the STn-positive cancers. Furthermore, the STn-positive cells will also allow to identify the different *O*-glycosylated proteins substituted by STn antigen and potentially involved in the cell proliferation and migration and this is of interest in the development of immunotherapetic strategies using neoglycoproteins.

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