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Cultured Ehrlich Ascites Tumor Cells Show Increased N-linked α2,6-Sialyltransferase Activity[1]

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Ehrlich ascites tumor cells (EAT cells) are routinely grown in the peritoneal cavity of mice. These cells, EAT-wt, grow in suspension and exhibit a high level of α-2,3-O-linked sialyltransferase activity with benzyl-T-antigen (Galβ1,3GalNAc-α-O-$CH_2C_6H_5$) as acceptor. These cells also contain a very low level of α-2,6-O-linked and α-2,6-N-linked sialyltransferase activity. A variant of these cells, EAT-c, has been selected to grow in cell culture, attached to the surface of culture flasks. EAT-c cells exhibit a selective increase of two- to fivefold in the activity of α-2,6-N-linked sialyltransferase activity, using asialo-α_1-acid glycoprotein as acceptor. Since a similar selective increase has been previously observed in metastatic human colorectal cancer tissues, the EAT-wt/EAT-c cell system may serve as a good experimental model for the investigation of sialyltransferases and their cell surface sialylated products in relation to cancer, metastasis, and cell–cell interaction. © 1993 Academic Press, Inc.

Sialic acid generally occurs at the terminus of carbohydrate chains, usually in α-2,3- or α-2,6-linkage to galactose (Gal)[2] or to *N*-acetyl-D-galactosamine (GalNAc) residues of glycoproteins. The presence of sialic acid on carbohydrates has been associated with a variety of biological interactions such as in provoking or masking antigenicity (1–3). Changes in the quantity of sialic acid and/or its specific type of linkage to other cell surface glycoconjugates has been observed in cancer metastasis and invasion (4, 5). Increases in sialic acid levels on cell surfaces have been correlated with an increase in cancer metastasis (7, 8). It has been observed that the ability to colonize the lung appears to be reduced when cancer cells lose their surface sialic acids (9).

Incorporation of sialic acid into glycoconjugates is catalyzed by a number of highly specific sialyltransferases that recognize the terminal and subterminal sugar, their sequence, and specific linkage to each other as well as the linkage to the proteins, i.e., whether it is O-linked to Ser/Thr residues or N-linked to Asn residues. Thus the degree and type of sialylation depends on the level and relative activities of these enzymes.

Recent reports (7–9) comparing sialyltransferase activities of human colorectal cancer tissues to normal tissue showed an increase in the N-linked but not in the O-linked sialyltransferase activities. The increased activity, furthermore, was determined to be due to an increase in the α-2,6- but not in the α-2,3-sialyltransferase activity. Affinity chromatography, on elderberry bark and on wheat germ lectin columns, of cell surface glycoproteins of metastatic colon tumors showed a two- to threefold increase in N-linked sialyltransferase activity compared to that of the poorly metastatic tumor tissues. Also, the expression of α-2,6-sialyltransferase activity correlated with the presence of α-2,6-sialylated glycoproteins but not glycolipids (9).

The objective of the present study was to compare the changes in the relative activities of O- and N-linked α-2,3- and α-2,6-sialyltransferases in Ehrlich ascites tumor cells (EAT) that occur when these cells are changed from intraperitoneal to culture dish growth conditions. The findings from this model system could perhaps yield some useful information about the role of sialylation and its

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[2] Abbreviations used: EAT, Ehrlich ascites tumor cells; EAT-wt, Ehrlich ascites tumor cells grown in the peritoneal cavity of mice; EAT-c, Ehrlich ascites cells adapted to grow in cell culture; Gal, galactose; GalNAc, *N*-acetyl-D-galactosamine; LacNAc, *N*-acetyllactosamine; LNT, lacto-*N*-tetraose (Galβ1,3-GlcNAc-β1,3Gal-β1,4Glc); NCDV, New Castle Disease Virus; SNA, *Sambucus nigra* agglutinin (elderberry bark lectin); T-antigen, Galβ1,3-GalNAc; VC, *Vibrio cholerae;* BSA, bovine serum albumin; TCA, trichloroacetic acid; Mes, 4-morpholineethanesulfonic acid.

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possible involvement in cell–cell and cell–matrix interactions.

MATERIALS AND METHODS

Charles River CD-1 female mice, 20–30 g body weight, were used for growth of EAT-wt cells. Cells in culture were grown in the modified Eagle's medium fortified with fetal calf serum and epidermal growth factor. Tritiated CMP-sialic acid (26.2 Ci/mmol; 0.1 mCi/ml) was purchased from Dupont–New England Nuclear (Boston, MA). The *O*-benzyl glycoside of Gal-β1,3-GalNAc (T-antigen), α_1-acid glycoprotein and lacto-*N*-tetraose (LNT) were obtained from Sigma (St. Louis, MO). T-antigen–BSA and sialylated T-antigen–BSA, sialylated either α-2,3 to Gal or α-2,6 to GalNAc, were gifts from Chembiomed (Edmonton, Ontario). Elderberry bark lectin (SNA) was purified by Z. Song of this laboratory. Affi-Gel-15 and Bradford protein assay reagent were purchased from Bio-Rad. Other reagent-grade chemicals were available in this laboratory.

Enzyme Preparation—Buffers

Buffer A is a stock (4× strength) assay buffer consisting of 200 mM Mes, pH 6.5, 6% (v/v) Triton X-100, 4 mg/ml BSA, and 20 mM $MnCl_2$. Buffer B, the homogenization buffer, consists of 15 mM Tris–HCl, pH 7.5, 15 mM NaCl, 1 mM $MgCl_2$, and 25% glycerol. Buffer C, the membrane lysis buffer contains 20 mM Hepes buffer, pH 7.5, 4% lubrol PX, 1% Triton CF-54, 50% (v/v) glycerol, and 1 mM mercaptoethanol.

The EAT cells were grown either in the intraperitoneal cavity of the mouse (EAT-wt) or under culture conditions (EAT-c). Female CD-1 mice were injected intraperitoneally with 0.2–0.4 ml of fresh ascites fluid and the accumulated cells were aspirated after 8–10 days. Cells were sedimented and washed free of red blood cells as described by Elices *et al.* (10). Cultured EAT cells (EAT-c), selected from the ascitic EAT cells (EAT-wt) by D. MacCallum and J. Lillie (Department of Anatomy and Cell Biology, University of Michigan), were grown in 75-cc culture flasks. EAT-c cells were harvested using a buffer containing 5 mM EDTA, were washed several times with saline solution, and were either used immediately or kept at −20°C until use.

Cells were homogenized in 4× their wet volume of buffer B with the aid of a Polytron homogenizer. The homogenate was centrifuged at 1000*g* for 10 min to remove nuclei and unbroken cells. The supernatant solution was then centrifuged at 16,000*g* for 10 min to sediment mitochondria and the supernatant fraction was centrifuged at 100,000*g* for 60 min in an SW 60 rotor in a Beckman refrigerated ultracentrifuge to obtain the microsomal/Golgi pellet. Lysis buffer (buffer C) was added to one-tenth (EAT-wt) or one-half (EAT-c) of the original packed cell volume and the suspensions were sonicated with a Branson sonicator for three bursts of 5 s duration, each with intermittent cooling. The sonicates were recentrifuged at 100,000*g* for 60 min and the supernatant fractions were divided into small aliquots and kept at −20°C until assayed for enzymatic activity.

Enzyme Assays

The incubation medium was essentially that described by Elices *et al.* (10). Each 100 μl of the assay solution contained 1 μg of acceptor substrate, 1 pmol of CMP-[^{3}H]sialic acid (26.2 Ci/mmol), or approximately 60,000 dpm, and 10 μl of the enzyme preparation. Enzyme assays with added acceptors were accompanied by control experiments (no exogeneous acceptor added) to monitor and subtract any radioactivity incorporated into endogeneous acceptors. Using benzyl-T-antigen as acceptor, the approximate rate of incorporation was approximately 5000 dpm/30 min/10 ml of the enzyme preparation. An amount of enzyme that produced incorporation of 1 μmol/min of sialic acid under saturating substrate conditions is defined as 1 unit of enzyme activity.

Assays for Incorporated [^{3}H]Sialic Acid

Depending on the nature of the acceptor sugars, two types of assays were used to determine the sialyltransferase activities.

The Dowex 1 × 8 ion-exchange resin assay. This assay is suitable for relatively low molecular weight acceptors, e.g., water-soluble oligosaccharides. The procedure was essentially that of Paulson *et al.* (11). Briefly, the phosphate form of Dowex 1 × 8, equilibrated to pH 6.8, was packed to a height of 3–4 cm in disposable Pasteur pipets. At the end of the assay period, the reaction mixture was diluted to 1 ml with 5 mM phosphate buffer, pH 6.8, passed through the column 3× and the final flow-through fraction which contained only the sialic acid in covalent linkage to the oligosaccharide was subjected to scintillation counting.

The glass-fiber disk assay. This assay was performed with natural or synthetic glycoproteins such as asialo-α_1-acid glycoprotein or T-antigen linked to BSA. At the end of the enzyme assay period, the reaction mixture was diluted to 1 ml with cold 10% TCA solution, kept at 4°C for 10 min. The precipitated material was collected on 2.4-cm glass fiber filters, washed with cold 5% TCA, dried at 110°C for 2 h, cut into small pieces, and added to 5 ml of Ready-Solve (Beckman) for scintillation counting.

Affinity Chromatography

SNA was covalently linked to Affi-Gel-15 (Bio-Rad) according to the procedure of the supplier. The product contained 2.6 mg protein per milliliter of packed gel as determined by the Bradford protein assay performed on aliquots of the protein solution before and after linking the protein to Affi-Gel. The gel material was packed into small columns (Pasteur pipets) to a height of 2 cm. At the end of an enzyme assay period the contents of the assay tube were diluted to 1 ml with 0.05 M Mes buffer (pH 7.0) and passed 5× over the column. The column was then washed with the same buffer until no radioactivity was detected in the flow-through. Finally, the affinity-absorbed material was eluted three times with 0.2 ml of 0.2 M lactose and radioactivity counted.

RESULTS

Measurements of the Enzyme Activities Using Benzyl-T-Antigen as Acceptor

The postmitochondrial supernatant fraction from 30 g EAT-wt contained a total of 4200 mg protein and 1.43 units of total enzyme activity with benzyl-T-antigen (Galβ1,3-GalNAc-α-O-$CH_2C_6H_5$), as an O-linked substrate. The microsomal/Golgi fraction prepared at 100,000*g* from the above homogenate yielded 110 mg protein with a total of 1.3 units of activity or a specific activity of 0.012 units/mg microsomal/Golgi protein. One gram of culture cells treated in similar fashion yielded 280 mg protein and 0.36 units of activity in the postmitochondrial homogenate and 19 mg protein with 0.30 units of activity in the final microsomal/Golgi preparation for a specific activity of 0.015 units/mg microsomal/Golgi protein. Thus, both cell types showed a similar level of overall specific activity with the T-antigen as the acceptor substrate. The O-linked sialyltransferase activity, assayed using T-antigen as acceptor substrate, is by far the highest sialyltransferase activity in both EAT-wt and EAT-c cells.

Comparison of O-Linked vs N-Linked Sialyltransferase Activities

Using Galβ1,3-GalNAc and LacNAc, covalently linked to BSA (T-BSA and LacNAc-BSA, respectively), the rel-

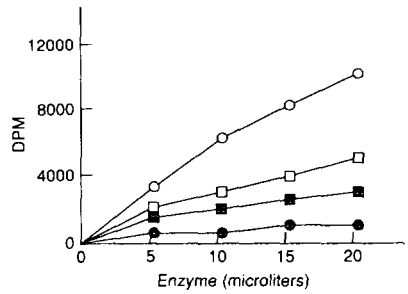

FIG. 1. Relative N- to O-linked sialyltransferase activities in EAT-wt and EAT-c cells. Enzyme preparations of EAT cells were incubated with 1 mg/ml BSA–LacNAc or BSA–T-antigen in fixed-time (30 min) assays and the sialylated product in each case was measured by the filter assay (see Materials and Methods). ○, O-linked sialyltransferase activity; ●, N-linked sialyltransferase activities present in EAT-wt cells (N/O ratio = 0.01). □ and ■ show the corresponding activities for EAT-c cells (N/O ratio = 0.5).

ative activities of transferases present in the microsomal/Golgi enzyme preparations of EAT-wt and EAT-c cells were measured in a fixed-time assay. The results shown in Fig. 1. indicate that under the assay conditions described, the EAT-wt cells have very little N-linked activity whereas this activity is fivefold higher in EAT-c cells. The rate of radioactive sialic acid incorporated/μl enzyme solution/30 min for each type of activity was determined from the slopes in Fig. 1 and the ratios of N- to O-linked sialyltransferase activities were calculated for the wild and the culture type enzyme preparations. Since such N- to O-ratios are internally fixed for each type of cell and are independent of absolute amount (activities) in each preparation, they represent a useful comparison of sialyltransferase activities in day-to-day preparations.

Determination of α-2,3- and α-2,6-Sialyltransferase Activities Using O-Linked and N-Linked Acceptor Carbohydrates

(A) Determination of the O-linked activities. Since T-antigen can be a sialyl acceptor for both the O-linked α-2,3- (at terminal Gal) or the O-linked α-2,6- (at GalNAc moiety) sialyltransferases, it cannot be used to distinguish between these two types of enzymatic activity. To determine the relative levels of α-2,3- and α-2,6-activities in these cells, T-antigen, covalently linked to BSA and sialylated either α-2,3 at Gal or α-2,6 at GalNAc, was used to distinguish the two types of O-linked activities in the wild and culture enzyme preparations. Rates of both sialylations were linear during a 1-h assay period. Comparison of the rates of [^{3}H]sialic acid incorporation catalyzed by each of these enzymes showed that the *relative activity* of the α-2,6 compared to the activity of the α-2,3 enzyme was not significantly different in the two types of cells (data not shown).

(B) Determination of the N-linked sialyltransferase activities. To determine the relative N-linked α-2,3- and α-2,6-sialyltransferase activities, asialo-α_1-acid glycoprotein was used as the acceptor substrate. Inasmuch as asialo-α_1-acid glycoprotein contains tetraantenary N-linked oligosaccharides with terminal galactosyl residues linked β1,4 to penultimate GlcNAc, it is a potential substrate for both N-linked α-2,6- as well as N-linked α-2,3-sialyltransferases (12). It was therefore necessary to distinguish between these two activities. This was accomplished by taking advantage of the carbohydrate binding specificity of SNA lectin toward α-2,6-sialylated oligosaccharides. This lectin specifically binds to α-2,6- but not to α-2,3-sialyl-LacNAc (13). Both EAT-wt and EAT-c enzyme preparations were incubated with asialo-α_1-acid glycoprotein as well as benzyl-T-antigen and LNT, and the incubation mixtures were passed through SNA–Affi-Gel affinity columns. The radioactivity retained on the columns after extensive washing was eluted with 0.2 M lactose and counted. Column flow-through fractions were also assayed by appropriate Dowex or filter-disk assays (see Materials and Methods). As can be seen from the results shown in Table 1, the highest amount of radioactivity retained by the SNA column resulted from the incubation of enzyme from EAT-c cells with asialo-α_1-acid glycoprotein. As expected, neither the α-2,3-sialylated benzyl-T-antigen nor the α-2,3-sialylated LNT, bound to the SNA affinity column, were found to be present in the flow-through fractions as determined by the Dowex assay. In the case of the EAT-wt enzyme preparation, the asialo-α_1-acid glycoprotein is not a very effective substrate, indicating that EAT-wt cells appear to contain a low level of α-2,6-sialyl transferase enzyme.

Neuraminidase Digestions

The material from the asialo-α_1-acid glycoprotein, retained by the SNA affinity columns may contain both α-

TABLE 1

Results of SNA–Lectin Affinity Chromatography of the EAT-c and EAT-wt Cells Enzyme Incubations with Various Acceptors

	EAT-c		EAT-wt	
Acceptor	dpm retained	dpm excluded	dpm retained	dpm excluded
Benzyl-T-antigen	27	2330	14	6546
Asialo-α_1-acid GP	829	185	232	336
Lacto-*N*-tetraose	20	1123	15	929

Note. All acceptors were used at a concentration of 1 mg/ml. At the end of incubation periods, the assay mixtures were passed through an SNA affinity column, the column was washed with buffer and eluted with 0.2 M lactose. The flow-through fractions and the eluted materials were processed as described under Materials and Methods followed by scintillation counting. Values given are averages of at least three experiments.

2,3-linked and α-2,6-linked sialic acid residues on some of their antennae. Therefore, to determine the contribution of each to the total incorporated sialic acid, the sialylated α_1-acid glycoprotein eluted from the SNA column with 0.2 M lactose was subjected to digestion with neuraminidase from New Castle Disease Virus (NCDV), which hydrolyzes only α-2,3-*N*-acetylneuraminic acid and, separately, with *Vibrio cholerae* (VC) neuraminidase, which hydrolyzes both α-2,3- and α-2,6-linked sialic acid residues from glycoproteins. The results, shown in Table II, indicate that about 90% of the sialylation occurring in the EAT-c enzyme preparation is not susceptible to NCDV and hence is presumably in α-2,6-N-linkage. In contrast, nearly 70% of the radioactivity incorporated by EAT-wt enzymes is prone to removal by NCDV sialidase and is presumed to be sialic acid in α-2,3-linkage. These values are consistent with the data shown in Table I. The last column of Table II shows that the VC neuramidase, which removes sialic acid irrespective of the nature of linkage, does in fact remove most of the sialic acid residues from all sialylated products.

In a further experiment, we conducted an overnight incubation with an assay mixture containing LacNAc and 50% (v/v) glycerol for enzyme stabilization. Aliquots of LacNAc sialylated by EAT-wt or by EAT-c cell enzyme preparations were processed in the following manner: Aliquots were passed over SNA–Affi-Gel columns, washed, and eluted with 0.2 M lactose as described above. The flow-through material, presumably containing α-2,3-sialo-LacNAc was assayed by the Dowex procedure. The lactose-eluted material was divided into three equal aliquots which were incubated overnight, respectively, with NCDV-neuraminidase, VC-neuraminidase or in the absence of neuraminidase (control) followed by assay by the Dowex procedure. Results of this experiment, shown in Fig. 2, are in accord with the data already presented. As can be seen from a comparison of the hashed bars A and B, about 75% of the counts incorporated into LacNAc by EAT-c enzymes and retained by the SNA column are resistant to NCDV neuraminidase and therefore are assumed to be α-2,6-N-linked. In comparison, as shown by the open bars A and B, less than 20% of the corresponding counts from EAT-wt enzymes remain after the same treatment, indicating that less than 20% of the sialic acid residues in this case are α-2,6-N-linked.

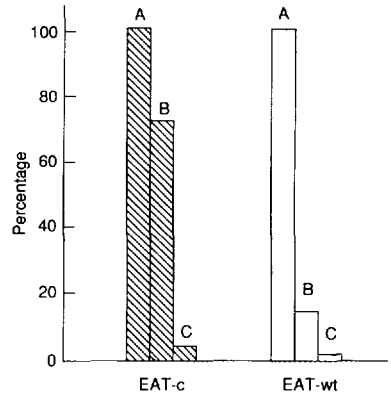

FIG. 2. Relative α-2,6N-linked enzyme activities of EAT-wt and EAT-c cells. Enzyme preparations were incubated overnight with soluble acceptors. Total O-linked and N-linked activities were determined using T-antigen (not shown) and LacNAc as specific acceptors. The LacNAc incubation mixtures were then divided into aliquots and used to determine total incorporation taken as 100% counts bound to elderberry bark lectin, (A), and the percentage counts remaining after overnight digestion with NCDV (B), and VC neuraminidases (C). Hashed columns, EAT-c enzyme activity; Open columns, EAT-wt enzyme activity.

TABLE II

Effect of NCDV and VC Neuraminidase on the ^{3}H-Sialylated α_1-Acid Glycoprotein Eluted from SNA Column by 0.2 M Lactose

	dpm		
	Total (no treatment)	Resistance to NCDV	Resistance to VC
EAT-c	986 (100%)	882 (89%)	116 (12%)
EAT-wt	402 (100%)	136 (33%)	67 (16%)

Note. Numbers in parentheses are percentages relative to nontreated material (i.e., an aliquot of the lactose-eluted counts incubated under identical conditions but without neuraminidase). Sialylated α_1-acid glycoprotein, eluted from the SNA–Affi-Gel column, was incubated separately, overnight with the two neuraminidase enzymes.

DISCUSSION

Ehrlich ascites tumor cells, which grow in suspension in the mouse peritoneal fluid (EAT-wt), can be selected to grow in culture dishes (EAT-c), attached to a plastic substratum. The EAT-c cells exhibit increased agglutination with SNA lectin which is specific for N-linked α-2,6-sialylated carbohydrates whereas there is very little of this activity in the original EAT-wt cells (18). Recent reports (7–9) also indicate that there is an increase of about two- to threefold in the N-linked α-2,6- but not α-2,3-sialyltransferase in human colorectal cancer compared to poorly metastatic tumor tissues. Additionally, there was no significant change in the activity of O-linked enzymes (9). A recent communication reports on "the expression of α2,6-linked sialic acid residues of neoplastic but not in normal colonic mucosa" (19).

The EAT-c and EAT-wt cells provide a model system for comparison of sialyltransferase activities and also for the *in vivo* expression of the enzyme activity which occurs in the form of sialylated oligosaccharides on cell surfaces. To selectively measure and compare the relative activity of the different sialyltransferases in these cells we made use of the substrate specificity of these enzymes, using specific acceptor substrates. An alternate approach to this

type of study would have been to purify each of these enzymes to homogeneity in order to establish their presence without ambiguity and measure enzymatic parameters more quantitatively. Indeed all of the enzymes dealt with in this paper have been previously purified from various sources and characterized in the laboratories of R. Hill and J. Paulson (14–17), each purification requiring kilogram quantities of the source materials. The drawback of such extensive purification schemes, aside from the scarcity of starting materials and difficulties of purifications (e.g., *uneven recoveries*), is that a good comparisons of enzyme activities close to the *in vivo* conditions would not be possible because of partial or complete loss or inactivation of one or more of the enzymes during the multistep purification procedures. Therefore, we measured the enzyme activities present in the 100,000*g* microsomal/Golgi preparations. These preparations were lysed in a detergent-containing buffer and used with the assumption that they contained their *in vivo* complements of the four sialyltransferases studied.

Measurements of the relative O-linked and N-linked activities of EAT-wt and EAT-c cells using the T-antigen (Galβ1,3-GalNAc) as acceptor for the O-linked, and LacNAc as the acceptor for the N-linked activity, showed that the level of N-linked relative to O-linked sialyltransferase activity increases several fold in the EAT-c cells. For reasons that are not yet clear but perhaps are related to cell-culturing conditions, the increase can vary from two- to fivefold. It should perhaps be noted that inasmuch as the N- and O-linked activities are compared relative to each other, their absolute amounts have no bearing on these results because all values compared are relative values within each preparation. The one caution that must be noted is that despite minimal manipulations to extract the enzymatic activities under identical conditions, there could be an uneven extraction of one type over the other type of cells. This possibility, however, appears remote and according to our observations, uniform variations in substrate concentrations, enzyme amounts, or incubation times, do not change the relative ratio of the N- to O-linked activities.

Both the α-2,3- and the α-2,6-O-linked as well as N-linked sialyltransferase activities were present in both types of cells but in different ratios. In the case of O-linked sialyltransferase activities, there is no significant change in either the α-2,6- or the α-2,3-enzyme activity under peritoneal or culture growth conditions. Also, the N-linked α-2,3-enzyme activity, measured using LNT as acceptor, remains relatively constant (see Table I). The only increase occurs in the N-linked α-2,6-enzyme activity of the EAT-c cells. The combined SNA–lectin affinity chromatography and NCDV sialidase experiments with sialylated α_1-acid glycoprotein (Tables I and II) indicate that approximately 80% of the sialic acid residues incorporated into asialo-α_1-acid glycoprotein by the EAT-c cell enzymes are in α-2,6-linkage, whereas less than 20% are α-2,6-linked by the EAT-wt enzyme preparations. The data using LacNAc (Fig. 2) confirm these values. Again it must be emphasized that these percentages are for relative activities found in microsomal/Golgi preparations from cells and do not indicate absolute amounts of enzymes.

In summary, the data presented in this paper indicate that EAT cells selected to grow in culture, exhibit a two- to fivefold increase in their α-2,6-N-linked sialyltransferase activity. Whether this change in the enzyme activity has any functional significance with regard to the interaction of cells with each other or their ability to attach to surfaces remains to be elucidated. Since a similar selective increase has been previously observed in metastic human colorectal cancer tissues, the EAT-wt/EAT-c cells may serve as a good experimental model for the investigation of sialyltransferases and their cell surface sialylated products in relation to cancer, metastasis, and cell–cell interactions.

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