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Short Communication

Transgene Expression of α(1,2)-Fucosyltransferase-I (FUT1) in Tumor Cells Selectively Inhibits Sialyl-Lewis x Expression and Binding to E-Selectin without Affecting Synthesis of Sialyl-Lewis a or Binding to P-Selectin

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During inflammation, E- and P-selectins appear on activated endothelial cells to interact with leukocytes through sialyl-Lewis x and sialyl-Lewis a antigens (sLe$^{x/a}$). These selectins can also interact with tumor cells in a sialyl-Lewis-dependent manner and for this reason, they are thought to play a key role in metastasis. Diverting the biosynthesis of sialyl-Lewis antigens toward nonadhesive structures is an attractive gene therapy for preventing the hematogenous metastatic spread of cancers. We have previously shown that transfection of α(1,2)-fucosyltransferase-I (FUT1) in Chinese hamster ovary (CHO) cells had a slight effect on the overall sialylation while the synthesis of sLE^{x} was dramatically prevented. We herein delivered the gene of FUT1 by a human immunodeficiency virus-derived lentiviral vector to three human cancer cell lines including pancreatic (BxPC3), hepatic (HepG2), and colonic (HT-29) cancer cells. We found that on FUT1 transduction, all cells exhibited a dramatic decrease in sLe^{x} synthesis with a concomitant increase in Le^{y} and Le^{b} expression, without any detectable effect on the level of cell surface sLe^{a} antigens. In parallel, FUT1-transduced HT-29 and HepG2 cells, but not BxPC3 cells, failed to interact with E-selectin as assessed by E-selectin-binding assay or dynamic adhesion to activated endothelial cells. We show also that transduced FUT1 efficiently fucosylates the P-selectin ligand PSGL-1 without altering P-selectin binding. These results have important implications for understanding cell-specific reactions underlying the synthesis of selectin ligands in cancer cells and may provide a basis for the development of anti-metastatic gene therapy. *(Am J Pathol 2004, 164:371–383)*

E- and P-selectins appear on the surface of activated endothelial cells to bind to leukocytes harboring sialyl-Lewis x (sLe^{x}, NeuAcα2,3Galβ1,4[Fucα1,3]GlcNAc) and sialyl-Lewis a (sLe^{a}, NeuAcα2,3Galβ1,3[Fucα1,4]GlcNAc) antigens and their sulfated variants.[1] In addition to their natural function in leukocyte trafficking, E- and P-selectins can also interact with tumor cells bearing sialyl-Lewis antigens, thus initiating adhesion to the endothelium and subsequent migration into the underlying connective tissue.[2] It has been repeatedly shown that the amount of sLe^{x} and sLe^{a} structures are increased in carcinoma cells[3–6] and a correlation has been established between increased expression of sialyl-Lewis antigens and metastatic potential.[7–10]

Inhibiting the synthesis of E- and P-selectin ligands would provide a way to prevent the hematogenous metastatic spread of cancers. However, unlike P- or L-selectin that have a limited number of sialyl-Lewis protein carriers,[11] there is no such well-defined counterreceptors for E-selectin and many tumor-associated glycoproteins and glycolipids have been reported as potential ligands for E-selectin. In the lack of a protein or a lipid target, we

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herein investigated whether inhibiting glycosyltransferases that participate in forming sialyl-Lewis structures would result in blocking tumor cell attachment to endothelia. Sialyl-Lewis antigens are synthesized on glycoproteins and glycolipids by an ordered series of sugar transfer reactions catalyzed by several glycosyltransferases; the final reactions being controlled by one or more α(1,3/1,4)-fucosyltransferases and α(2,3)-sialyltransferases. So far, six human fucosyltransferases (ranging from FucT-1 to FucT-7 and FucT-9)[12] and six α(2,3)-sialyltransferases (ranging from ST3Gal-I to ST3Gal-VI)[13] can use either type I (Galβ1,3GlcNAcβ1-R) or type II (Galβ1,4GlcNAcβ1-R) oligosaccharides to synthesize sialyl-Lewis antigens. Regarding sialylation for example, not all type I and type II precursors end with sialylated-Lewis ligands[13] and the synthesis of such structures may also depend on the expression level of a given sialyltransferase *versus* other competing glycosyltransferases, as well as on how these enzymes segregate into distinct compartments of the Golgi complex.[14]

It has been reported that the α(1,2)-fucosyltransferase (FUT1, E.C. number 2.4.1.69) reduced terminal α(2,3)-sialylation in transfected cells and transgenic animals.[15,16] Likewise, we have recently shown that transfection of a sLe^x-expressing CHO cell model with FUT1, led to a discrete decrease in the level of cell surface sialic acid while the synthesis of sLe^x structures was strongly inhibited.[17] We have furthered this line of investigation by studying the intracellular distribution of FUT1 and found that, unexpectedly, this terminally-acting enzyme is in fact a medial-Golgi protein because it co-localized with the medial-Golgi marker α-mannosidase-II.[18] This finding provided a possible explanation for the FUT1-mediated inhibition of sLe^x expression. Thus, because of its compartmentalization in the Golgi, FUT1 may intercept the type II oligosaccharide precursors before they enter the compartment of the α(2,3)-sialyltransferases. These data being acquired, we further wanted to extend this approach to cancer cells. In this regard we have recently described an inverted E-selectin binding assay that exploit the green and the red fluorescence of EGFP and DsRed2, respectively, to monitor and easily quantificate tumor cell adhesion to E-selectin.[19] We herein used this assay together with a human immunodeficiency virus type 1-based lentiviral vector to deliver the FUT1 gene into three different tumor cell lines and evaluate the effect of α(1,2)-fucosylation on sialyl-Lewis antigen expression and interaction with E- and P-selectin.

Materials and Methods

The anti-sLe^x antibody SNH-3 was purchased from Wako Chemicals (Neuss, Germany) and Cslex-1 was prepared from the hybridoma HB8580 (American Type Culture Collection, Rockville, MD) as described.[20] The antibodies KM93 (anti-sLe^x), 2D3, and IH4 (anti-sLe^a) and 2-25LE and H18A (anti-Le^b and anti-Le^y, respectively) were from Seikagaku (Tokyo, Japan). The anti-PSGL-1 mAb PL-1 and rhodamine isothiocyanate (RITC)-conjugated anti-human IgM and IgG were from Beckman Coulter (ImmunoTech, Marseille, France). Rhodamine-conjugated anti-mouse IgM and IgG were from Sigma-Aldrich and cyanin-5 (Cy5)-conjugated antibodies were from Jackson Immunoresearch (West Grove, PA). The *Ulex europaeus* agglutinin-1 (UEA-1) lectin conjugated to RITC was from EY laboratories (Biovalley, Marne-La-Vallée, France) and its agarose-immobilized form was from Calbiochem (San Diego, CA). PNGase-F and ethylenediaminetetraacetic acid-free protease inhibitor cocktail was from Roche Diagnostics (Meylan, France).

Plasmid DNAs and Lentiviral Constructs

The construct (FUT1) fused to EGFP (FUT1-EGFP) in pcDNA3 (pcDNA3/FUT1-EGFP) has been described in a previous work.[18] The FUT1-EGFP DNA was excised from pcDNA3/FUT1-EGFP with *Bam*HI and *Bsr*GI, gel purified, and ligated into *Bam*HI/*Bsr*GI-digested pRRLpgK-GFP-.sin18 lentiviral vector (referred to as pL1-gfp; kindly provided by Dr. Trono, University of Geneva, Geneva, Switzerland). The resulting plasmid (referred to as pL1FUT1-gfp) contains an in-frame C-terminal fusion of FUT1 to EGFP. Vesicular stomatitis virus G protein-pseudotyped human immunodeficiency virus type-1 vector particles were prepared by transiently co-transfecting subconfluent 293T cells with 20 μg of either pL1-gfp or pL1FucT1-gfp, 10 μg of the packaging plasmid pCMVΔR8.91, and 5 μg of the vesicular stomatitis virus G protein envelop plasmid pMD.G. The polyethylenimine-mediated transfection procedure was performed according to Naldini and co-workers.[21] The vector-conditioned medium was collected 48 and 72 hours after transfection, cleared of debris by low-speed centrifugation, and filtered through a 0.22-μm pore-size filter before being aliquoted and frozen at −80°C. High-titer vector stocks were prepared by ultrafiltration using Vivaspin 20 concentrator (100,000 MWCO PES membrane; Vivascience, Palaiseau, France), centrifuged at 3000 × *g* for 30 minutes. To determine the vector titer, HeLa cells in 12-well plates (10^5cells/well) were transduced in the presence of a serial dilution of DNA and 4 μg/ml of polybrene. The transduction media were removed 16 hours later and replaced with fresh media. Three days after transduction, the relative end-point vector titer (transducing units/ml; TU/ml) was determined by a fluorescence-activated cell sorter (FACscan; Becton Dickinson, Mountain View, CA).

Cell Lines, Transfection, and Lentiviral Transduction

The colon adenocarcinoma HT29, the hepatocarcinoma HepG2, and the human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium and the pancreatic cancer cells BxPC3 were cultured in RPMI 1640. The generation of CHO cells expressing both E-selectin (Es) and the red fluorescent protein DsRed2 (red) (referred to as CHOred/Es) has been described previously.[19] The CHO-K1 cells, stably co-expressing the enzymes Core2-β1,6*N*-acetylglucosaminyltransferase-I (C2GnT-I), the α(1,3)-fucosyltransferase-VII (FucT-VII),

and the P-selectin glycoprotein ligand-I (PSGL-1) have been described elsewhere.[18] In the present study these cells were named CHO271; the numerals 2, 7, and 1 correspond to C2GnT, FucT7, and PSGL-1, respectively. Recombinant E-selectin IgM and P-selectin IgM chimeras were produced by transiently transfecting subconfluent 293T cells with pcDNA1/E-selectin IgM or pcDNA1/P-selectin IgM plasmids[22] kindly provided by Pr. M. Fukuda (The Burnham Institute, La Jolla, CA). Culture media (OptiMEM) were collected between the 4th and the 7th day after transfection, centrifuged at 1000 rpm for 5 minutes to remove detached cells and debris, and used undiluted in E- and P-selectin-binding experiments. All transfections were performed using Lipofectamine Plus reagent, according to the manufacturer's instructions and all culture media (Life Technologies, Inc., Gaithersburg, MD) contained 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs; Clonetics Corp., San Diego CA) were cultured in complete endothelial cell growth medium (EGM, Clonetics Corp.) according to the manufacturer's instructions.

For transduction experiments, 10^5 cells per well were seeded in a 12-well plate and incubated for 16 hours in 0.5 ml of fresh medium containing virus supernatant supplemented with 4 μg/ml of polybrene. The cells were then cultured in complete medium for 6 days before analyses. The percentage of EGFP-positive cells and the mean fluorescence intensity (MFI) were determined by flow cytometry.

Flow Cytometry and Fluorescence Microscopy

We used three different antibodies, Cslex-1, KM93, and SNH3 (IgM) to assay for the expression of sLe^x antigens and two anti-sLe^a antibodies 2D3 (IgM) and IH4 (IgG). The expression of the di-fucosylated glycotopes Le^b and Le^y was determined using 2-25LE and H18A antibodies (both IgG), respectively. All these monoclonal antibodies are murine IgM or IgG as indicated. Fluorescence was either determined by flow cytometry (fluorescence-activated cell sorting) or fluorescence microscopy essentially as described.[18] For fluorescence-activated cell sorting analyses, immunoreactivities were assessed by incubating cells with appropriate antibodies and counterstaining with Cy5-conjugated goat anti-mouse IgM (for Cslex-1, KM93, SNH3, and 2D3) or Cy5-conjugated goat anti-mouse IgG (for IH4, 2-25LE, and H18A). All incubations were performed at 4°C in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (FL-buffer). Cells were then fixed in 1% paraformaldehyde and samples were analyzed by measuring the fluorescence of 10,000 cells and displayed on a 4-decade log scale and data were plotted using FlowJo online software (Tree Star Inc., San Carlos, CA).

For fluorescence microscopy, cell monolayers were fixed in 1% paraformaldehyde in PBS and incubated for 30 minutes at 4°C with appropriate antibodies at 0.2 to 1 μg/ml in FL-buffer. They were then counterstained with RITC-conjugated anti-mouse IgM or with RITC-conjugated anti-mouse IgG, depending on the first antibody, and visualized on a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, Göttingen, Germany). In double-staining experiments with the anti-sLe^x KM93 and the anti-sLe^a IH4, cells were incubated stepwise with KM93 followed by RITC-conjugated anti-mouse IgM and then with IH4 followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. Fluorescence was determined as above using a dual-color (FITC/RITC) ×40 objective with no bleed through between the two channels.

E- and P-selectin binding was performed by incubating cell monolayers at 4°C for 30 minutes with media from 293T cells producing either E-selectin IgM or P-selectin IgM chimeras. Cells were then rinsed three times with $PBS^{+}/^{+}$ and incubated at 4°C for another 30-minute period with RITC-conjugated anti-human IgM antibodies in OptiMEM containing 1 mg/ml of bovine serum albumin. Cells were then fixed in 3.5% paraformaldehyde in $PBS^{+}/^{+}$ for 5 minutes at room temperature and fluorescence was visualized as above. In some P-selectin binding experiments, cells were pretreated with increasing concentrations of anti-PSGL-1 mAb PL1.

Lectin Chromatography, Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis, and Immunoblotting

Cells were solubilized for 20 minutes at 4°C in 50 mmol/L of Hepes, pH 7.5, containing 0.15 mol/L NaCl, 1% octylglucoside, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and ethylenediaminetetraacetic acid-free protease inhibitor cocktail (lectin buffer). After 30 minutes of centrifugation at 9000 × *g*, the supernatant (250 μl, 0.5 mg/ml) was incubated batchwise with 100 μl of slurry UEA-I-agarose for 2 hours at 4°C. Chromatography was monitored spectrophotometrically at 280 nm. After removing the unbound fraction, the gel was rinsed by several centrifugations until no absorbance at 280 nm was detected. Proteins were then extracted by adding sodium dodecyl sulfate sample buffer to the lectin gel and boiling for 3 minutes at 100°C and resolved on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel according to Laemmli.[23] After electrophoresis, proteins were transferred to nitrocellulose membranes, stained with Ponceau red, and blocked overnight at 4°C in Tris-buffered saline containing 0.05% Tween (TBST; 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.5) and 5% nonfat milk. Membranes were probed with KPL-1 mAb followed by an alkaline phosphatase-conjugated anti-mouse secondary antibody and protein bands were developed according to Burnette.[24]

Low-Shear Force Adhesion Assays

Control tumor cells and FUT1-EGFP-expressing cells were assayed for adhesion to CHOred/Es cells as described earlier.[19] Briefly, CHOred/Es cells were harvested, resuspended at 2×10^5 cells/ml in RPMI containing 1 mg/ml bovine serum albumin and loaded at 2 ×

10^4 cells/well onto tumor cells that have been seeded in 24-well plates a day before the experiment. CHOred/Es cells were allowed to adhere to tumor cells on a constantly rocking platform for 30 minutes at 4°C. After three rinses with cold RPMI 1640, cells were fixed in 3.5% paraformaldehyde $PBS^{+}/^{+}$ for 5 minutes at room temperature and bound red fluorescent cells were counted using the computer-assisted method developed by the NIH Image program. Six randomly chosen areas were visualized and photographed using the AxioVision system (Carl Zeiss, Göttingen, Germany). The digitized images were gray scale-converted using Adobe Photoshop software (Adobe Systems, Mountain View, CA) and spots were counted using the public domain NIH Image program developed at the National Institutes of Health and available on the internet at *http://rsb.info.nih.gov/nih-image/*).

Measurement of Rolling on Activated Endothelial Cells

Endothelial cells were seeded on glass coverslips 1 day before the experiments and activated for 4 hours at 37°C in the presence or the absence of 10 ng/ml of tumor necrosis factor (TNF)-α. Coverslips of control and TNF-α-activated HUVECs were used as the bottom plates of a parallel flow chamber as described[25] with minor modifications. Briefly, control or FUT1-expressing cancer cells were assembled in a parallel plate laminar flow chamber (Satim, Evenos, France) and mounted on the stage of an inverted microscope (Olympus CK40, Japan) equipped with a charge-coupled device camera (Sony SPT-M 108CE) connected to a videotimer (VTG33 Musetta, Marseille, France) and a videotape recorder for delayed analysis. In a typical experiment 1 ml of cell suspension (5×10^5 to 10^6) were driven through the chamber with a wall shear rate set at $4s^{-1}$. The flow was generated with a 5-ml plastic syringe mounted on an electric syringe holder (Bioblock, Illkirch, France).

The video signal was subjected to real-time digitization with a PCVision+ card (Imaging Technology, Bedford, MA). A custom-made software allows cell tracking by manual superimposition of a cursor driven by the computer mouse on live images. The motion of individual cells was recorded with 1 pixel (0.7 μ) and 0.02 second accuracy. Cells interacting with HUVEC monolayers were detected by discriminating their velocity from the hydrodynamic velocity distribution of cells freely flowing near the monolayer. Cells were found to display numerous arrests with durations ranging between a fraction of a second and several seconds. Arrests were defined as durable when they lasted more than 10 seconds and transient when their duration was lower than 10 seconds. The binding frequency P was calculated as the ratio between the number N of (transient or durable) arrests and the total displacement length measured on a given cell sample: $P = N/L$. This parameter was expressed as μm^{-1}.

Results

It has been reported that the FUT1 reduced terminal α(2,3)-sialylation in transfected cells and transgenic animals.[15,16] Furthermore, using a sLe^x-expressing CHO cell model, we have previously shown that FUT1 transfection led to a dramatic decrease in the level of sLe^x antigens while a relatively small portion (~17%) of glycoconjugates have been modified by α(1,2)-fucosylation, as assessed by flow cytometry and lectin-column chromatography.[17] This apparent selectivity encouraged us to extend our investigations to three tumor cells known for their high-level expression of sialyl-Lewis antigens and their metastatic behavior, namely the pancreatic BxPC3 cells,[4,26] the hepatocarcinoma HepG2 cells,[27] and the colonic HT-29 cells.[28] To this end, we initially attempted to stably transfect these three tumor cell lines with FUT1 cDNA in a pcDNA3 vector[17] using a number of transfection kits and protocols with only a very limited success. One major difficulty raised from the strong clone-to-clone variations within mock-transfected cells essentially because of the heterogeneity of sLe^x and sLe^a expression by BxPC3 and HT-29 cells.

Expression Pattern of Sialyl-Lewis Antigens by Tumor Cells

As shown in Figure 1A, the pancreatic BxPC3 and the colonic HT-29 cells heterogeneously express the KM93 and IH4 epitopes whereas HepG2 cells exhibit a homogenous and exclusive KM93 immunoreactivity. It is noteworthy that in the case of BxPC3 and HT-29 cells, some cells exclusively express sLe^x (green) or sLe^a (red), or both (yellow and orange) or none (Figure 1, A and a). However, although both HT-29 and BxPC3 cells express sLe^x and sLe^a structures, there is a striking difference between the two cell lines in terms of the staining pattern. In fact, as illustrated in Figure 1B, the antibody KM93 does not label individual BxPC3 cells, but rather stains large areas that do not fit exactly with the cell shapes. Current efforts are devoted to understand this phenomenon.

To overcome the difficulties because of the heterogeneity within cell lines, we herein used an EGFP-tagged version of FUT1 to monitor the rate and the level of expression of the enzyme in living cells as well as a lentivirus-mediated gene delivery system. One of the attractive characteristics of this system over others is because of the human immunodeficiency virus type 1-based lentiviral vector that can infect even nondividing cells. Using this system, we obtained ~100% infection efficiency for HepG2 and HT-29 cells and 70 to 80% FUT1-EGFP-positive cells for BxPC3, as determined by fluorescence microscopy and flow cytometry (data not shown). Furthermore, we did not notice any significant difference in the level or the homogeneity of FUT1-EGFP expression in any cell line for up to 8 months, nor did we detect differences in parameters tested in the present study, between parental and mock-transduced cells.

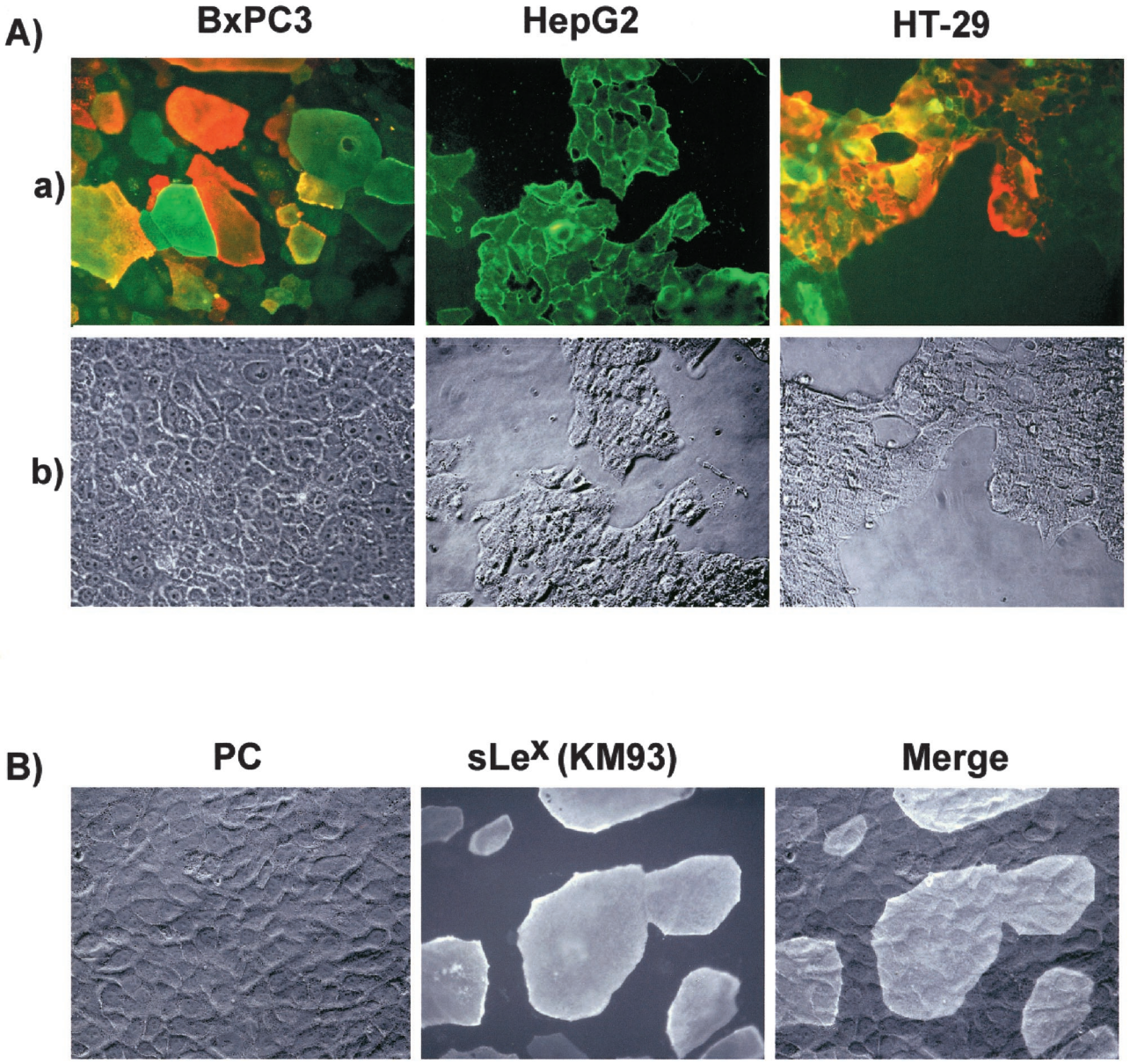

Figure 1. The expression pattern of sLe^x/sLe^a antigens on tumor cell lines. **A:** Cells were incubated with the anti-sLe^a mAb IH4 (IgG), followed by a RITC-conjugated anti-mouse IgG antibody (red), and again incubated with the anti-sLe^x mAb KM93 (IgM) and counterstained with a FITC-conjugated anti-mouse IgM antibody (green). The double staining (**a**) of cell monolayers (**b**) was observed by fluorescence microscopy using a dual-color (FITC/RITC) ×40 objective. **B:** The staining pattern of a BxPC3 cell monolayer with the anti-sLe^x mAb KM93. The merged image (merge) clearly shows that the anti-sLe^x KM93 stains areas that do not exactly fit with the underlying cell bodies. Such a pattern was repeatedly observed with BxPC3 cells (in more than 10 different experiments) regardless of the passage or the cell culture duration. It is, therefore, not an artifact. Note that these cells show similar kinds of staining with the anti-sLe^a mAb IH4 (**A**) and with E-selectin IgM (see Figure 5). PC, phase contrast.

Transduced FUT1 Reduces Cell Surface sLe^x but Not sLe^a

The α(1,2)-linked fucose transferred by FUT1, can be detected by the lectin UEA-1. Cells were stained with an RITC-conjugated UEA-1 and the results are presented in Figure 2. Control (untransduced) HepG2 cells do not express α(1,2)-linked fucose whereas BxPC3 and HT-29 express a low level of α(1,2)-fucosylated antigens. On infection with the *FUT1*-EGFP construct, all cells exhibit a typical Golgi staining (EGFP) together with a strong cell surface UEA-1 reactivity, indicating that the transduced FUT1-EGFP is fully active *in vivo*.

We then investigated whether *FUT1*-EGFP gene transfer into tumor cells would result in an alteration of sialyl-Lewis antigen expression. To this end, parental and FUT1-transduced cells were stained with one of the following antibodies to Lewis-related carbohydrates, three anti-sLe^x (Cslex-1, KM93, and SNH3), two anti-sLe^a (2D3 and 1H4), as well as the anti-Le^b 2-25LE and the anti-Le^y H18A and fluorescence was measured by flow cytometry. We used Cy5-labeled secondary antibodies be-

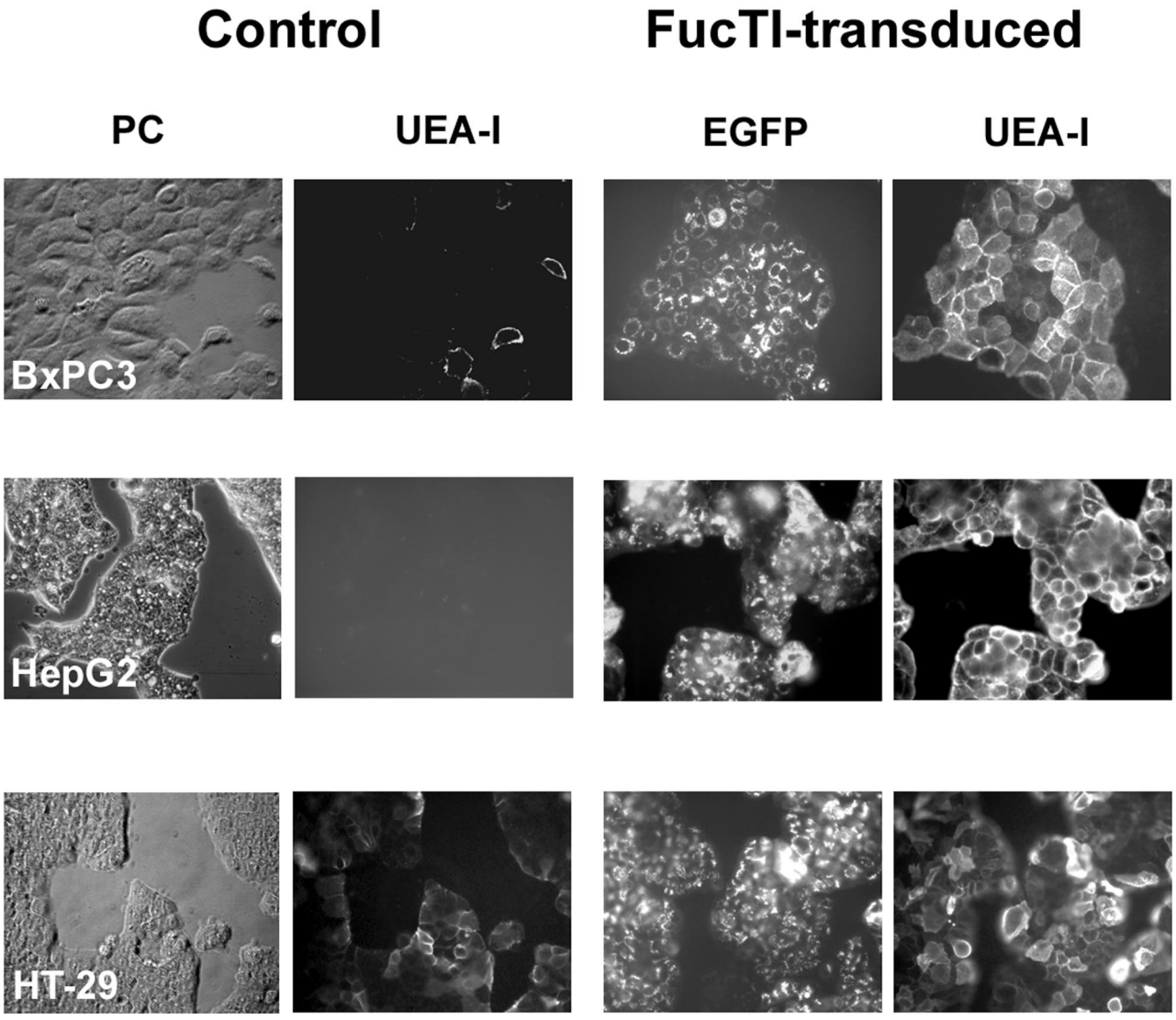

Figure 2. Transgene expression of FUT1-EGFP in tumor cells triggers cell surface appearance of α(1,2)-linked fucose. Untransduced (control) and FUT1-transduced cells (EGFP) were stained with UEA-I-RITC (UEA-I) and examined by fluorescence microscopy. PC, phase contrast.

cause the emission wavelength of Cy5 (670 nm) is far enough from that of EGFP (507 nm) to avoid overlapping between intracellular (EGFP) and cell surface fluorescence. Figure 3, left, shows that the sLe^x epitopes Cslex-1, KM93, and SNH3 are present on the three cell lines, although Cslex-1 is only faintly expressed on HT-29 cells (16% positive cells). However, all FUT1-transduced cells exhibit a dramatic decrease in both Cslex-1 and KM93 immunoreactivities while SNH3 expression was only slightly affected. This result indicates that FUT1 targets the precursors of Cslex-1 and KM93 antigens but not those of SNH3.

If the synthesis of sLe^x antigens is altered in the presence of FUT1, then one should expect the appearance on the cell surface of the di-fucosylated Le^y epitope [Fucα1,2Galβ1,4(Fucα1,3)GlcNAc-R] (Figure 4). In fact, as shown in Figure 3, the Le^y epitope on untransduced cells was either absent (HepG2) or low (HT-29), indicating that the latter may express an endogenous FUT1 activity, consistent with data from Figure 2 and from previous findings on these cells.[29] After FUT1-transduction the Le^y expression either appeared (HepG2, Figure 3B) or was markedly increased in HT-29 cells (from 4.79 to 92.1% positive cells, Figure 3C). In the case of BxPC3 cells, the untransduced cells express already a high level of Le^y (53.5% positive cells, Figure 3A) that was not significantly enhanced after FUT1 gene transfer (63.1% positive cells), indicating that BxPC3 cells naturally express an elevated endogenous FUT1 activity. Given that BxPC3 cells highly express both Le^y and sLe^x structures, this result suggests that the sLe^x precursors may escape the endogenous FUT1 but not the transduced FUT1, because the latter dramatically inhibits KM93 and Cslex-1 immunoreactivities in these cells.

Regarding the expression of sLe^a antigens defined by either the 2D3 or IH4 mAbs (Figure 3, right), we found that the hepatocarcinoma cells HepG2 express a negligible amount of these epitopes, whereas BxPC3 and HT-29 cells show a strong immunoreactivity with the antibodies, consistent with data from Figure 1. Transgene expression

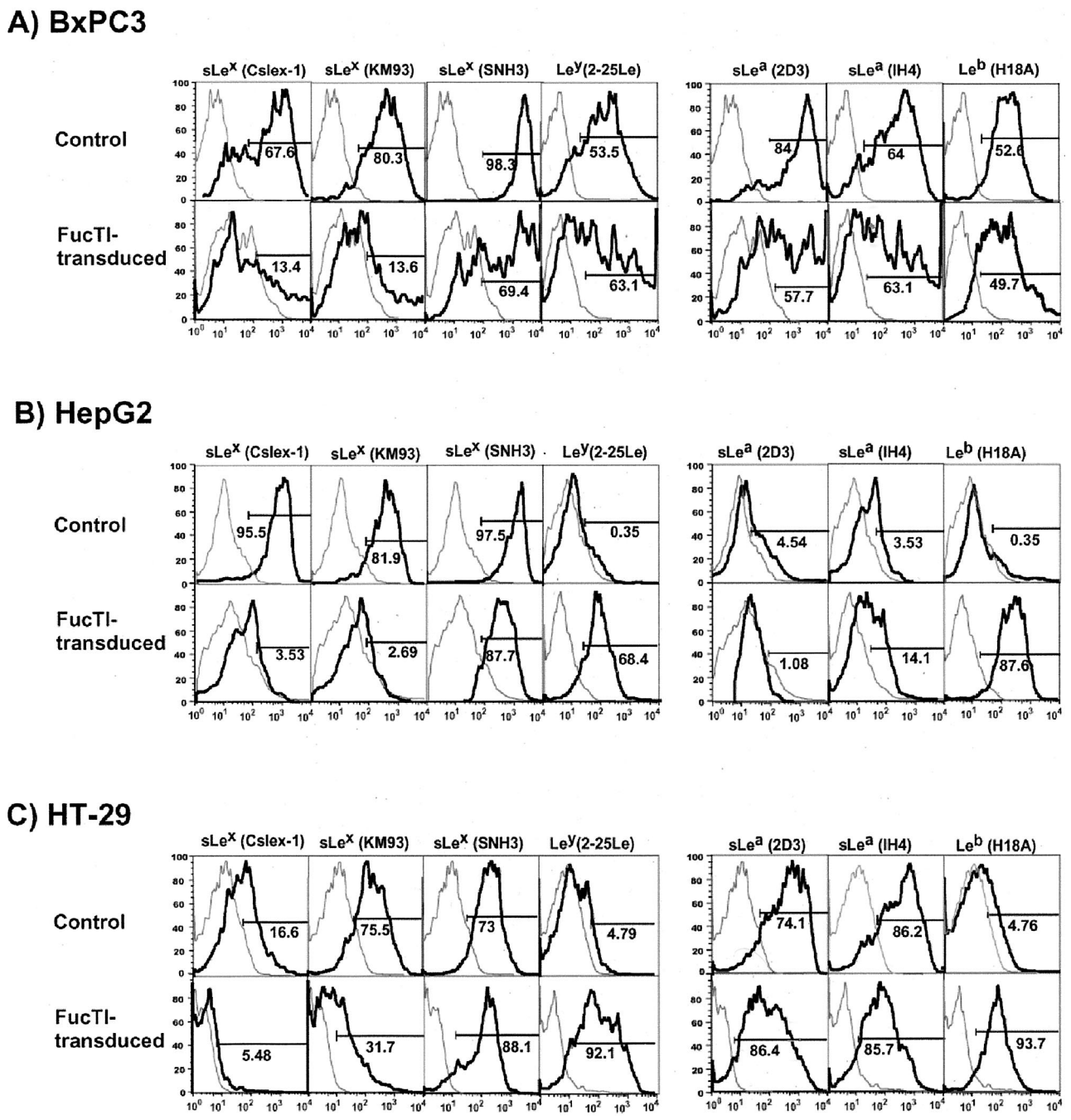

Figure 3. Flow cytometric analysis of Lewis antigens on tumor cells before and after infection with FUT1-EGFP-coding lentiviral vector. Control and FUT1-transduced cells were incubated with anti-carbohydrate antibodies, counterstained with Cy5-conjugated secondary antibodies, and analyzed by flow cytometry. **Dotted lines**, the results of staining with the secondary antibodies Cy5-conjugated anti-mouse IgM for Cslex-1, KM93, SNH3, and 2D3, or Cy5-conjugated anti-mouse IgG for IH4, 2-25Le, and H18A. **Bold lines**, the expression pattern of Lewis determinants on control and FUT1-transduced cells. This experiment was repeated three times.

of FUT1 in either BxPC3 or HT-29 cells does not affect sLe^a expression (Figure 3, A and C). However, the expression of Le^b epitope, which is naturally present on parental BxPC3 cells (Figure 3A), is triggered by the transduced FUT1-EGFP in both HT-29 and HepG2 cells (Figure 3, B and C). Given that the Le^b structures come from the α(1,2)-fucosylation of Le^a precursors (Figure 4) and the fact that in HT-29 cells, the transduced FUT1 does not affect the expression of sLe^a antigens while generating the Le^b epitope, the above data suggest that in HT-29 cells FUT1 may α(1,2)-fucosylate a population of Le^a oligosaccharides that is not intended to be further sialylated. Surprisingly, HepG2 cells that faintly express sLe^a antigens (Figure 3B) and by inference a low level of Le^a oligosaccharides, exhibit a strong Le^b immunoreactivity after FUT1 gene transfer (Figure 3B). This result indicates that, like HT-29 cells, the hepatocarcinoma cells may synthesize a large amount of Le^a oligosaccha-

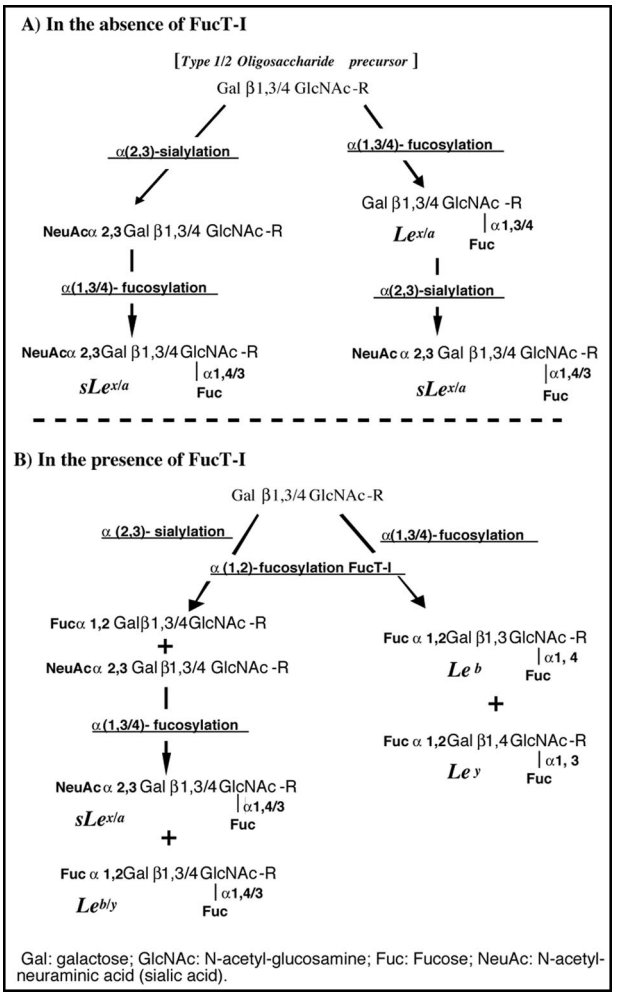

Figure 4. Biosynthesis of Lewis-related antigens in the absence (**A**) and the presence (**B**) of FUT1.

rides but unlike HT-29 cells, HepG2 cells are not able to produce sLe^a because they might be deficient in sLe^a-specific α(2,3)sialyltransferases. More work is needed to address this issue.

Transduced FUT1 Alters E-Selectin Binding to HepG2 and HT-29 but Not to BxPC3 Cells

The above data show that *de novo* expression of FUT1 in the hepatocarcinoma cells HepG2 or its overexpression in the colonic HT-29 cells or the pancreatic cells results in a dramatic decrease in the level cell-surface sLe^x antigens; without any apparent alteration in sLe^a expression.

We next examined how the decreased sLe^x expression affects the E-selectin binding to tumor cells. To this end, parental and FUT1-transduced cells were incubated with recombinant E-selectin IgM chimera and, because of the presence of the EGFP tag, bound proteins were revealed by an anti-human RITC-conjugated secondary antibody and concomitant EGFP/RITC staining was analyzed by fluorescence microscopy as above. Figure 5 shows that all parental cells bind E-selectin IgM. However, when FUT1-EGFP is present in these cells, the E-selectin binding is dramatically inhibited on both HepG2 and HT-29, but not at all on BxPC3 cells. Given that the synthesis of sLe^x, but not that of sLe^a antigens, is impaired in the presence of FUT1 regardless of the tumor cell considered, this result demonstrates that the transduced FUT1 selectively inhibits sLe^x-dependent E-selectin binding.

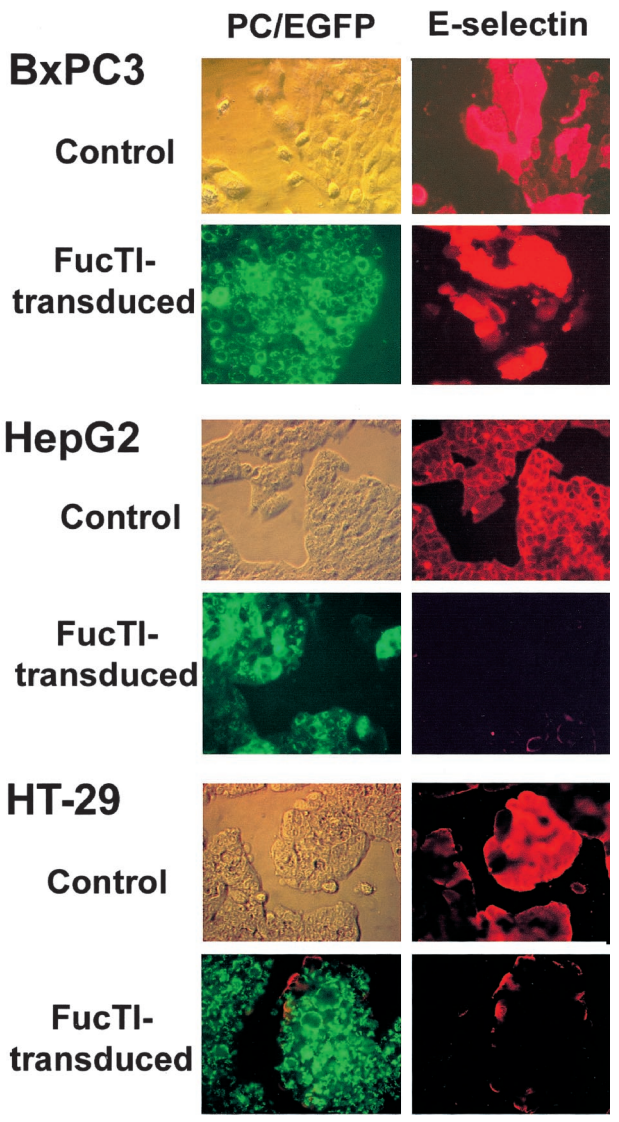

Figure 5. Altered E-selectin binding to FUT1-transduced tumor cells. Shown are phase contrasts (PCs) of control cells and fluorescence of FucT-transduced tumor cells (EGFP) together with their corresponding E-selectin-IgM-binding patterns (E-selectin) as revealed by RITC-conjugated secondary antibody. A double-stained EGFP/RITC FUT1-transduced HT-29 cell monolayer is presented to show that some cells still bind E-selectin-IgM chimera, although expressing FUT1-EGFP, probably through sLe^a oligosaccharides.

Adhesion to E-Selectin-Expressing Cells and Rolling on Activated Endothelial Cells Are Altered in FUT1-Transduced HepG2 and HT-29 Cells but Not in BxPC3 Cells

The above data suggest that among the three cell lines tested, the hepatocarcinoma cells HepG2 and the colon carcinoma cells HT-29, but not the pancreatic cancer

cells BxPC3, bind to E-selectin through their sLe^x oligosaccharides. To further evaluate the biological impact of sLe^x alteration on E-selectin-mediated cell-cell interactions, we tested adhesion of FUT1-transduced tumor cells on E-selectin-expressing cells, under low-shear and dynamic conditions. Adhesions under low-shear conditions were performed using the CHOred/Es system as described before.[19] We have previously shown that E-selectin-binding criteria of CHOred/Es cells are in the same range as those of TNF-α-activated HUVECs (ie, calcium and sialic acid dependence and inhibition by blocking antibodies), while greatly simplifying the quantification of E-selectin-mediated tumor cell interaction.[19] Figure 6A shows that more than 85% of CHOred/Es cells adhere to FUT1-transduced BxPC3 (provided that adhesion of CHOred/Es to untransduced cell is taken as 100%) whereas the adhesion of HepG2 and HT-29 cells expressing FUT1-EGFP, respectively, dropped to 10% and 22% of the adhesion of their untransduced counterparts. Except for BxPC3 cells, the extent of FUT1-induced inhibition is comparable with that achieved by treating cells with sialidase,[19] suggesting that the decreased adhesion may be the consequence of reduced cell surface sialylated glycoconjugates including sLe^x antigens.

Before their arrest in the vasculature, the circulating tumor cells have to slow down their velocity by interacting with the endothelial cells through E- and P-selectin.[11] This phenomenon called rolling, can be reproduced *in vitro* under hydrodynamic conditions close to those encountered in the postcapillary venules. We have previously shown that our three tumor cell lines adhere to TNF-α-stimulated HUVECs in an E-selectin-dependent manner.[19] Regardless of their origin and states (untransduced or FUT1-transduced), all cells were found to display numerous arrests with durations ranging between a fraction of a second and several seconds. As shown in Figure 6B, except for HT-29 cells, combining transient and durable arrest frequencies did not allow distinguishing between the flow behaviors of parental (open bars) and FUT1-transduced cells (filled bars). However, durable arrest frequencies decreased dramatically for all FUT1-tranduced cells, except for BxPC3 cells (Figure 6C). In fact, we noticed that while untransduced cells rolled and became firmly adherent, the FUT1-infected HepG2 and HT-29 cells continuously rolled on the endothelial monolayer. This result confirms the data obtained above and again, demonstrates the central role of sLe^x antigens as high-affinity counterreceptors to endothelial E-selectin.

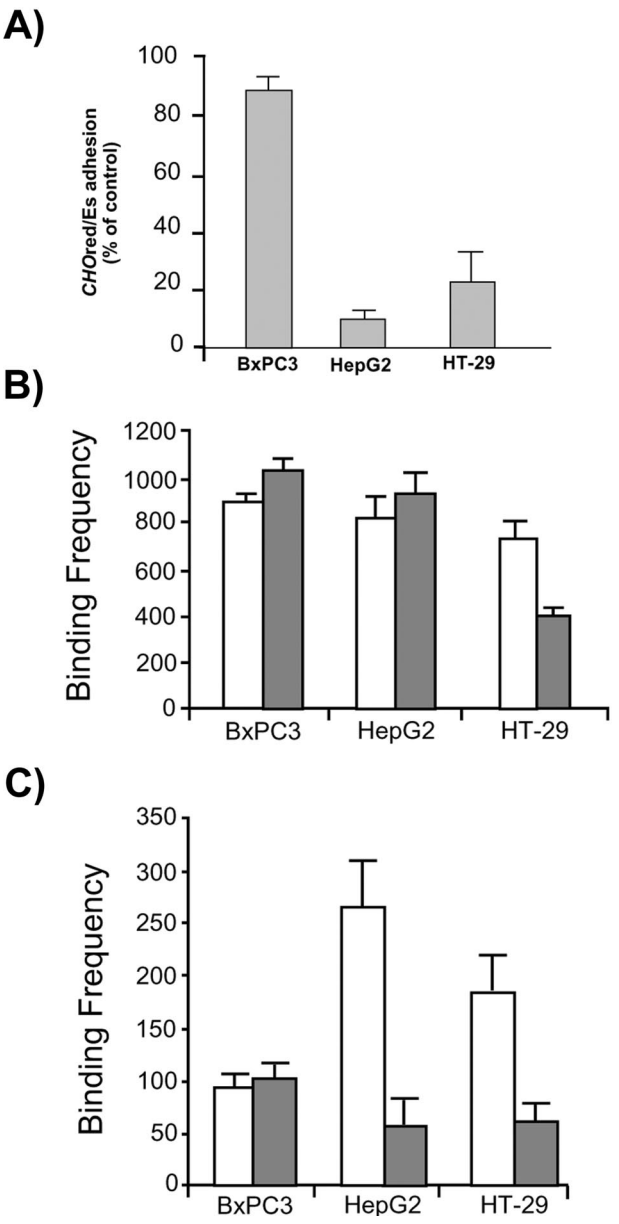

Figure 6. Altered adhesion and rolling of FUT1-EGFP-transduced tumor cells. **A:** Tumor cell monolayers were overlaid with CHOred/Es and bound fluorescent cells were counted as described in Materials and Methods. The adhesion was normalized to the values obtained for parental cells (untransduced). **B:** Rolling experiments with control and FUT1-transduced tumor cells on TNF-α-activated HUVECs quantified by combining transient binding frequencies (less than 10 seconds) and durable arrests (more than 10 seconds). **C:** Binding frequencies for arrest durations of more than 10 seconds. Data are presented as mean ± SD of total rolling events. These experiments were repeated at least three times.

The α(1,2)-Fucosylation Does Not Alter P-Selectin Interaction with Its Ligand PSGL-1

It is suggested that P-selectin also can contribute to the metastatic spread of cancers. For example it has been reported that the deletion of P-selectin in mice impairs the hematogenic dissemination of the colon carcinoma cells LS180 in these animals.[30] It is therefore interesting to investigate whether FUT1 could affect the interaction of tumor cells with P-selectin. However, preliminary data showed that none of our tumor cells bound P-selectin (data not shown). In the lack of a tumor model, we reproduced a P-selectin-binding system by stably transfecting CHO cells with the required molecules according to previous reports.[31,32]

As shown in Figure 7, the binding of P-selectin-IgM chimera to CHO271 cells was not significantly modified by transduced FUT1-EGFP (Figure 7B). In parallel, the binding of E-selectin-IgM protein to the same FUT1-transduced CHO271 cells was strongly affected (Figure

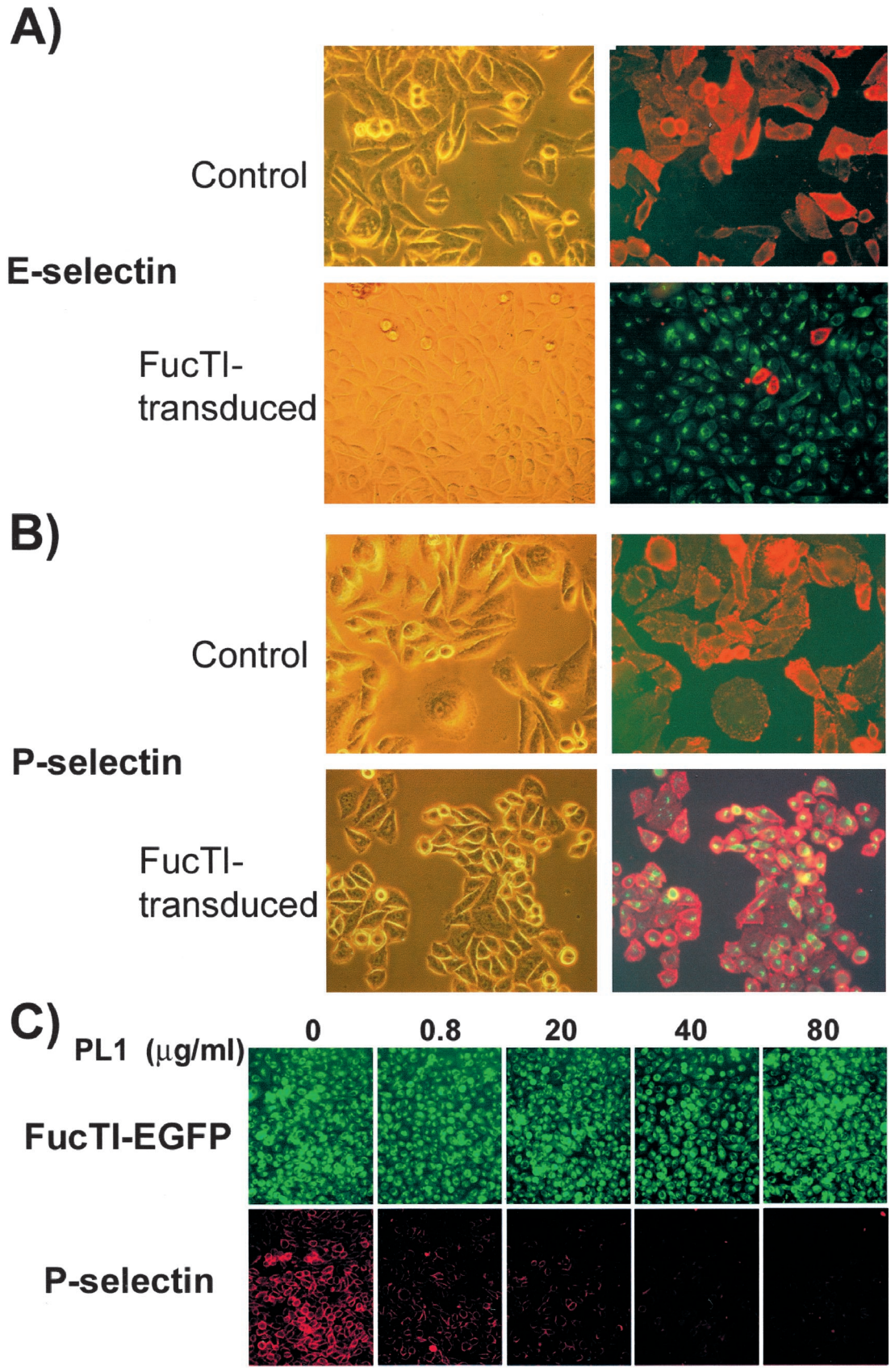

Figure 7. E-selectin binding to FUT1-transduced CHO271 is altered, whereas binding of P-selectin is not. Simultaneous imaging of intracellular distribution of FUT1-EGFP and cell surface-bound E-selectin-IgM (**A**) and P-selectin IgM (**B**). The staining conditions are as in Figure 5. **C:** Dose-dependent inhibition of P-selectin-IgM binding to FUT1-transduced CHO271 cells by PL-1 mAb.

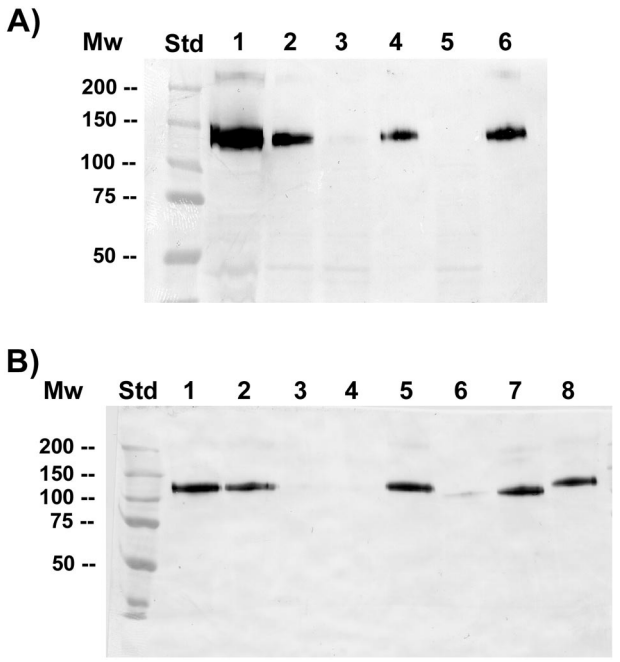

Figure 8. PSGL-1 oligosaccharides are both α(1,2)-fucosylated and α(2,3)-sialylated. Cell extracts were prepared from parental or FUT1-transduced CHO271 cells and mixed with UEA-I-agarose or wheat germ agglutinin-agarose gels and PSGL-1 molecules were detected by immunoblotting using PL-1 mAb. **A:** Crude extract from parental CHO271 cells (**lane 1**); crude extract from FUT1-transduced CHO271 cells diluted (1/2) with the lectin buffer (**lane 2**); chromatography of FUT1-transduced CHO271 cell extracts on UEA-I-agarose, flow-through fraction (**lane 3**) and bound material (**lane 4**); chromatography of FUT1-transduced CHO271 cell extracts on wheat germ agglutinin-agarose, flow-through fraction (**lane 5**) and bound material (**lane 6**). **B:** UEA-I chromatography of parental CHO271 cell crude extract (**lanes 1** and **8**), flow-through fraction (**lane 2**), and bound material (**lane 3**). UEA-I chromatography of FUT1-transduced CHO271 cell extract with or without PNGase-F treatment (from **lanes 4** to **7**), without any treatment unbound (**lane 4**) and bound material (**lane 5**) and after PNGase-F treatment unbound (**lane 6**) and bound fraction (**lane 7**).

7A). In addition, we show that P-selectin binding to FUT1-transduced CHO271 cells is abolished by PL-1 mAb in a dose-dependent manner (Figure 7C), indicating that P-selectin binding to these cells is solely mediated by PSGL-1.[33] These results suggest that if FUT1 can efficiently α(1,2)-fucosylate the sLe^x precursors on E-selectin ligands it does not affect in the same way, those carried by PSGL-1.

The presence of α(1,2)-linked fucose on PSGL-1 was then investigated using the property of UEA-1 to specifically bind to α(1,2)-fucosylated glycoconjugates. Figure 8A shows that PL-1 mAb recognizes a 120- to 130-kd protein in FUT1-transduced CHO271 cells (lanes 1 and 2) that is quantitatively retained on the UEA-I-column (lane 4) because almost no immunoreactivity could be seen in the flow-through fraction (lane 3). This result demonstrates that PSGL-1 produced in the presence of FUT1-EGFP carries α(1,2)-linked fucose. Yet, if PSGL-1 binds so avidly to the UEA-1, one would expect that a large part of its glycans would be α(1,2)-fucosylated rather than α(2,3)-sialylated. To test this hypothesis we used another plant lectin that binds to α(2,3)-sialylated glycoconjugates in general and to the leukocyte PSGL-1 in particular, that is the wheat-germ agglutinin.[34] Surprisingly, although heavily α(1,2)-fucosylated, the PSGL-1 molecules still avidly bind to wheat germ agglutinin (Figure 8A, lane 6) because no detectable immunoreactivity could be detected in the flow-through fraction (lane 5). These results indicate that PSGL-1 glycans produced in the presence of FUT1, carry both α(1,2)-linked fucose and α(2,3)-linked sialic acid on their penultimate galactose residues.

According to Moore and co-workers,[34] there are at least two *N*-linked oligosaccharides and numerous *O*-linked sugar chains attached to PSGL-1. To determine whether α(1,2)-fucosylation occurs on *N*- or *O*-linked sugars, cell extracts from FUT1-transduced cells were treated with the peptide:*N*-glycosidase F (PNGase-F), which cleaves asparagine-linked glycans,[35] and applied to UEA-1 chromatography as above. Figure 8B shows that after PNGase-F treatment, a significant increase in the electrophoretic mobility of PSGL-1 could be observed [Figure 8B, compare lanes 6 and 7 (treated) to lane 5 (control)], indicative of the cleavage of *N*-linked glycans and consistent with previously reported data.[34] However, despite the cleavage of *N*-glycans, the majority of PSGL-1 molecules remains bound to the UEA-I column [Figure 8B, compare lane 6 (flow-through) and lane 7 (retained)]. This result indicates that PSGL-1 expressed in FUT1-transduced cells carries α(1,2)-linked fucose mainly on its *O*-linked oligosaccharides. Taken as a whole, these data suggest that FUT1 is able to efficiently α(1,2)-fucosylate the *O*-linked glycans of PSGL-1 but not the sLe^x structures that bind to P-selectin.

Discussion

Our earlier studies have shown that transfection of a sLe^x-expressing CHO cell line with FUT1 led to a discrete decrease in the level of cell surface sialic acid with a concomitant strong inhibition of sLe^x synthesis.[17] We proposed that FUT1 may intercept the sLe^x precursors in the Golgi apparatus before they enter the compartment of α(2,3)-sialyltransferases. Indeed, we have found that FUT1 is not localized to the late Golgi compartments (ie, trans-Golgi and trans-Golgi network) like many other terminally acting enzymes including α(2,3)-sialyltransferases, but instead overlaps very well with the medial-Golgi marker α-mannosidase-II.[18] Because the sialyl-Lewis antigens are generally overexpressed on tumor cells and therefore, may mediate binding to endothelial selectins and promote the hematogenic spread of cancer cells, we herein extended our investigations to three tumor cell lines of the gastrointestinal tract. These include the hepatic HepG2, the colonic HT-29, and the pancreatic BxPC3 cells. We found that 1) there are differences in the level and the pattern of expression of sialyl-Lewis antigens and E-selectin binding from a cell line to another; 2) the transduced FUT1 abrogates sLe^x but not sLe^a expression, in all cell lines; and 3) for the hepatic HepG2 and the colonic HT-29 cells but not for the pancreatic BxPC3 cells, the loss of sLe^x expression results in reduced interactions with E-selectin and altered attachment to activated endothelial cells.

To our knowledge, the heterogeneity in carbohydrate expression and E-selectin binding within BxPC3 cells and

within HT-29 cells (Figure 1) has never been reported before, probably because immunoreactivities were often assessed by single labeling and/or flow cytometric analyses.[26,28,36] Despite this common feature, HT-29 cells, unlike BxPC3 cells, exhibited an important FUT1-induced decrease in E-selectin binding (Figure 5) and adhesion to E-selectin-expressing cells (Figure 6). One likely explanation for this is that on HT-29 cells, and probably on other colon carcinoma cells, sLe^x oligosaccharides may serve as high-affinity binding sites for E-selectin whereas sLe^a may represent the low-affinity binding sites. Consistent with the present work, the importance of sLe^x-mediated adhesion in invasiveness of colon carcinoma cells is supported by several reports including two recent ones concerning the colon carcinoma cells LS180[37] and LS174T[38] in which a central role for sLe^x antigens in metastasis has been clearly demonstrated.

Regarding BxPC3 cells, our data are consistent with studies from Aubert and co-workers[26] who showed that overexpression of FUT1 in these cells, resulted in a reduction of sLe^x antigen expression. However, our results are in striking contrast with their data concerning the inhibition of sLe^a expression and adhesion to E-selectin of FUT1-transfected BxPC3 cells. One likely explanation for this discrepancy could be provided by the posttransfection cloning approach used by the authors to isolate their transfectants. Indeed, given the heterogeneity of BxPC3 cells (Figure 1), one could isolate some clones that do not naturally express neither sLe^x (or sLe^a) nor bind to E-selectin. In addition, because the authors used an untagged enzyme, one could hardly distinguish between clones expressing the transfected and those expressing an endogenous α(1,2)-fucsyltransferase which synthesize the Le^y glycoantigen in these cells[39] (see Figure 3A, lane Le^y). Interestingly, the finding that neither sLe^a expression nor E-selectin binding were altered in FUT1-transduced BxPC3 cells suggests that the pancreatic adenocarcinoma cells may interact with E-selectin exclusively through sLe^a antigens. Other pancreatic cancer cell lines including SOJ-6, Panc-1, MiaPaca-2, and Capan-1 are currently studied in our laboratory to address this issue.

Having the cell heterogeneity in mind, we herein chose a lentiviral-mediated approach to transfer the gene of FUT1 fused to EGFP to avoid the cloning-related problems mentioned above and to allow a continuous monitoring of FUT1-EGFP expression as well as the homogeneity of cell populations. By doing so, we highlighted several parameters regarding the selectivity of FUT1 toward acceptor oligosaccharides. For example, we found that even among sLe^x structures, FUT1 is able to discriminate between the precursors of Cslex-1, KM93, and SNH3. In fact, as shown in Figure 3, regardless of the cell line considered, FUT1 prevents the synthesis of Cslex-1 and KM93 but not SNH3 antigens. According to Numahata and co-workers,[40] the SNH3 epitope might be carried by gangliosides whereas the epitopes defined by Cslex-1 and KM93 antibodies have been shown to be associated with mucin-like glycoproteins.[20,41] Thus, it seems likely that FUT1 may preferentially act on glycoproteins rather than glycosphingolipids, which is consistent with studies from Prieto and co-workers[42] who found that transfected FUT1 does not fucosylate glycolipids in CHO cells.

P-selectin is another adhesion molecule that is expressed on platelets and activated endothelia to interact with cells carrying sLe^x oligosaccharides on their surface.[43] However, unlike E-selectin, the interaction of P-selectin with PSGL-1 was not altered after infection with the FUT1-coding lentiviral vector. Here also we found that FUT1 selectively α(1,2)-fucosylates PSGL-1 on its PNGase-F-resistant oligosaccharides (presumably *O*-linked) but only marginally, if not at all, on its *N*-linked sugar chains. The binding characteristics of PSGL-1 to P-selectin are well documented and require sLe^x-structures carried by C2GnT-branched *O*-linked oligosaccharides located at the N-terminal portion of the protein in the close proximity of three sulfated tyrosines.[32] It is therefore possible that this particular environment may in turn, protect sLe^x precursors from α(1,2)-fucosylation. At least six α(2,3)-sialyltransferases (ST3Gal, ranged from ST3Gal-I to ST3Gal-VI) have been identified that may contribute to selectin ligand formation.[13] However, it is not accurately known which of these sialyltransferases are involved in sialylation of PSGL-1 sLe^x precursors *in vivo*, nor is known the Golgi localization of this (these) enzyme (s). In this regard, we have recently reported on the feasibility of perturbing glycosylation by mislocalizing glycosyltransferases from a Golgi compartment to another.[18] Therefore, once the Golgi compartmentalization of the sLe^x-specific α(2,3)-sialyltransferase is known, it would be conceivable to target FUT1 to an early compartment to favor the α(1,2)-fucosylation over the α(2,3)-sialylation of the P-selectin-binding domain on PSGL-1. Such a possibility is currently under investigation in our laboratory.

Altogether, our findings imply that FUT1 can efficiently prevent the synthesis of sLe^x antigens in hepatocarcinoma and colon carcinoma cells and their interaction with activated endothelia. Therefore, the FUT1-EGFP gene transfer approach would be expected to decrease or even suppress the initial vascular arrest of tumor cells and may provide a basis for the development of antimetastatic gene therapy.

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