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# Highly sensitive cell-based assay system to monitor the sialyl Lewis X biosynthesis mediated by α1-3 fucosyltransferase-VII☆

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**Abstract**

The sialyl Lewis X ($sLe^x$) determinant on leukocytes serves as a ligand for selectin family cell adhesion molecules, and selectin–carbohydrate interaction is considered to play an important role in the process of leukocyte extravasation during inflammation. Among several α1-3 fucosyltransferases (FucTs), FucT-VII plays a critical role in the biosynthesis of $sLe^x$-epitopes. Therefore, small molecules specifically designed to inhibit the FucT-VII enzyme may have potential as anti-inflammatory agents. Here, we have developed a versatile cell-based assay system to monitor $sLe^x$ biosynthesis using the GeneSwitch System. This system is a mifepristone (MFP)-inducible mammalian expression system, and human transfectant T lymphoblasts expressed the mRNA of FucT-VII and the $sLe^x$-epitopes on the cell surface in a time-dependent manner in the presence of MFP, with very low background transcription. Furthermore, when the transfectants were treated with the FucT-VII inhibitor panosialin, $sLe^x$ expression on the induced cells was inhibited dose dependently without alteration at the mRNA level of FucT-VII. These results suggest that the FucT-VII may be a major regulator of the biosynthesis of the $sLe^x$-epitopes on T lymphoblasts, and this cell-based assay may be utilized for a screening system of FucT-VII inhibitors.


*Keywords:* Fucosyltransferase; Sialyl Lewis X; Selectin; Adhesion; FACS; Glycobiology; Cell-based assay

Cell-surface antigen sialyl Lewis X ($sLe^x$) [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-R] plays an important role during the early steps of the inflammatory response as a ligand for selectin family cell adhesion molecules, and this interaction mediates the attachment and rolling of leukocytes to blood endothelial cells [1,2]. Furthermore, its related structures are also associated with cancer malignancy [3–6], and a number of mouse monoclonal antibodies (mAbs) against these antigens have been generated for cancer diagnosis [5,6]. The biosynthesis of $sLe^x$-epitopes requires the enzymatic activity of several glycosyltransferases. The final step in the biosynthesis of the $sLe^x$ determinant is regulated by α1-3 fucosyltransferase (FucT), which catalyzes the transfer of fucose from GDP fucose to *N*-acetyllactosamine via an α1-3-linkage [7–13]. Six enzymes, termed FucT-III, FucT-IV, FucT-V, FucT-VI, FucT-VII, and FucT-IX, have now been identified through molecular cloning procedures [8,12]. FucT-VII can clearly mediate $sLe^x$ biosynthesis in vitro, as well as in cultured cell lines transfected with expression vectors encoding this enzyme, and can also induce direct expression of E- and/or P-selectin ligands in transfected cells [9–12]. Furthermore, FucT-VII-deficient mice were shown to exhibit a leukocyte adhesion deficiency that was characterized by both the absence of leukocyte E- and P-selectin ligand activity, and a deficiency of L-selectin ligand activity in the high endothelial venules [2]. Therefore,

☆ *Abbreviations:* FucT, fucosyltransferase; $sLe^x$, sialyl Lewis X; MFP, mifepriston; mAb, monoclonal antibody; RT, reverse transcriptase; PCR, polymerase chain reaction; UAS, upstream activating sequences; DBD, DNA-binding domain; GFP, green fluorescent protein.

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inhibition of FucT-VII activity may suppress the expression of selectin ligands, thereby reducing selectin-dependent leukocyte adhesion to endothelial cells. Selective inhibitors of FucT-VII are expected to be potential therapeutics for the treatment of inflammatory disease.

Previous studies indicated that panosialin A and B inhibited human FucT-VII and the biosynthesis of $sLe^x$-epitopes in U937 cells, in which FucT-VII was constitutively expressed [14]. However, an adequate suppressive effect on the cell surface $sLe^x$-epitopes was observed after treatment with the panosialin for 48–144 h, suggesting that the turnover of $sLe^x$-epitopes on the cells may take over 48 h [14]. FucT-VII mRNA and selectin ligands on human T lymphocytes were also induced in response to mitogenic stimulation, but the expression of the $sLe^x$-epitopes was low and only detected after 144–192 h [15]. Although monitoring of the $sLe^x$-epitopes provided an indirect measurement of FucT-VII activity, these $sLe^x$-epitope detection methods were generally time-consuming, less sensitive, and had limited throughput. Thus, these conventional methods were not suitable for the screening of a large number of chemicals. To overcome these limitations, we intended to establish a high-throughput cell-based assay system, which can regulate the expression of the target protein.

Recently, the GeneSwitch System has been developed by Valentis [16]. In principle, this regulatory system is "OFF" in the absence of mifepristone, but upon addition of mifepristone, this system is turned "ON," resulting in the expression of a GAL4 regulatory fusion protein known as the GeneSwitch protein. Subsequently, the GeneSwitch protein binds to GAL4 upstream activating sequences in the promoter controlling the gene of interest, resulting in the activation of transgene expression. This system has been used successfully to regulate transgene expression in culture cells in vitro and ex vivo, in transgenic mice, and in mice injected with viral vectors [16–20].

Here, we investigated the ability of the GeneSwitch regulatory system to establish a cell-based assay for FucT-VII. The established assay system permitted the detection of $sLe^x$-biosynthesis accurately, and could be easily adapted to the high-throughput screening of FucT-VII inhibitors. We have also conducted a pharmacological study of a known FucT-VII inhibitor, and demonstrated the cascade scheme of the FucT-VII activity, $sLe^x$-biosynthesis, and cell adhesion to selectins.

## Materials and methods

### *Materials*

The GeneSwitch complete system was purchased from Invitrogen. Cells were obtained from American Type Culture Collection (Rockville, MD). Panosialin K was isolated from the fermentation broth of *Streptomyces* sp. The mouse anti-$sLe^x$ mAb CSLEX-1 (IgM) was purified from the supernatants of hybridoma (ATCC No. HB8580). FITC-conjugated anti-mouse IgM mAb and control mouse IgM mAb were obtained from Jakson Immunotech. P-/E-selectins/$IgG_1$ were obtained from Pharmingen. All radio-labeled nucleotide sugars were purchased from Amersham–Pharmacia. α2-3-sialyl *N*-acetyllactosamine was obtained from Dextra Laboratories. *N*-acetyllactosamine, $sLe^x$, $Le^x$, and sialidase were purchased from Oxford Glycobiosystems. All other chemicals were from Sigma, unless otherwise stated.

### *Cell culture*

Molt-4 Cells were cultured in RPMI 1640 medium (Gibco-RBL) supplemented with 10% fetal calf serum (FCS) at 37 °C in a 95% $O_2$/5% $CO_2$ humidified atmosphere. The transfected Molt-4 8H12G cells were also grown in RPMI 1640 medium with 10% FCS and 200 μg/ml hygromycin (Invitrogen) to maintain the transfected phenotype. Sf21 cells were cultured in serum-free medium EX-CELL400 (JRH Biosciences).

### *Isolation of cDNAs encoding FucT-VII*

A full-length cDNA of FucT-VII was amplified from single stranded cDNA of THP-1 by the polymerase chain reaction (PCR) using primers FT7-NE1 (5′-ATTGAATTCTCTCGGGTCTCTTGG CTGACTG-3′) and FT7-CX1 (5′-AGGGGAATTCGCCTGGTGG TTTGATTTCG-3′), digested with *Eco*RI, and inserted into the *Eco*RI site of pBluescript II SK+. The sequences of the constructs were determined by the dideoxy-chain-termination method, using an automated DNA sequencer (model 373A, Applied Biosystems) and the *Taq* Dye Primer Cycle Sequencing Kit (Perkin–Elmer Cetus). The resulting plasmid was designated pBluFucT-VII-L.

### *Transfection and identification of stable cell lines*

A 1.1-kb fragment, which encoded from amino acid 1 to 342 of FucT-VII, was obtained by digestion of the pBluFucT-VII-L with *Eco*RI and inserted in-frame into the *Eco*RI sites of the pGene/V5-His/A vector (Invitrogen) to generate pGene/FUT7. EGFP (*Hin*dIII/*Bam*HI fragment) was also inserted into the pGene/V5-His/A vector to generate pGene/EGFP. The pSwitch (hygromycin-resistance gene expression plasmid) was linearized with *Fsp*I, transfected into the Molt-4 cells ($8 \times 10^4$), and harvested in mid-log phase growth by electroporation at 270 V, 975 μF in phosphate-buffered saline (PBS) using a Gene Pulser (Bio-Rad). The cells were allowed to grow for 2 days before being subjected to selection based on their ability to grow in a medium containing 200 μg/ml of hygromycin. In order to select stable transfectants of pSwitch, $1.6 \times 10^6$ hygromycin-resistant cells were seeded in 6-well plates and transiently transfected with pGene/EGFP using a transient transfection kit PEI (TaKaRa). The GFP-expressing clones, in a MFP-dependent manner, were identified by a Laser Scanning Confocal Imaging System (Bio-Rad) and used in the experiments as follows.

pGene/FUT7 or pGene/V5-His/A (zeocin-resistance gene expression plasmid) were linearized with *Fsp*I and transfected into the stable transfectants of pSwitch (Molt-4 8H cells) using electroporation, as described above. The transfected cells were allowed to grow for 2 days before selection based on their ability to grow in a medium containing 50 μg/ml zeocin (Invitrogen) and 200 μg/ml hygromycin. The stable cell lines were cloned, expanded, and cultured for 3–72 h at 37 °C in 0.1% BSA-RPMI 1640 medium with or without MFP, and harvested to monitor the mRNA levels of the FucT-VII or $sLe^x$-epitopes on the cell surface by flow cytometry analysis, as described below.

### *Monoclonal antibodies and flow cytometry analysis*

Flow cytometry was performed using a FACStar analyzer (Becton–Dickinson), and the analysis was effected using the Cell Quest soft-

ware. The assays were conducted in V-bottomed microtiter plates for 30 min at 4 °C, as follows. The Molt-4 8H12G cells ($5 \times 10^5$) were incubated with CSLEX-1, washed once, and stained with fluorescein isothiocyanate-conjugated anti-mouse IgM mAb. Isotype-matched controls were incubated in all experiments and used to set the gates. A total of 10,000 cells were analyzed in each experiment, and viable cells were estimated by 1.0 μg/ml propidium iodide (Sigma) staining method.

In some experiments, the Molt-4 8H12G cells were preincubated with 25 mU *Salmonella typhimurium* sialidase (Oxford Glycosystems) in PBS(+) for 1 h at 37 °C before the cells were monitored by flow cytometry analysis, as described above. Untreated cells were also pre-incubated with PBS(+) for 1 h at 37 °C before staining to serve as the control.

Data were expressed as percentages of positive cells (compared with an isotype control).

### *RT-PCR*

Total cellular RNA was isolated from equal numbers ($5 \times 10^6$) of transfected Molt-4 cells using a RNeasy kit (Qiagen). The amount of isolated RNA was calculated by spectrophotometric absorbance at 260 nm, and 1.0 μg RNA was taken and used as a template for kinetic RT-PCR with superscript II RT (Life Technologies), *Taq* DNA polymerase (Life Technologies), and the primer sets (forward: 5′-CTC GGACATCTTTGTGCCCTATG-3′; reverse: 5′-CGCCAGAATTT CTCCGTAATGTAG-3′ (PCR product length is 291 bp) for FucT-VII, forward: 5′-CTTCATTGACCTCAACTACAT-3′; reverse: 5′-CTCAGTGTAGCCCAGGATGCC-3′ (PCR product length is 727 bp) for GAPDH). The reactions were carried out at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, allowed to continue for 30 (FucT-VII) or 25 cycles (GAPDH) in a thermal cycler GeneAmp PCR system 9700 (Perkin–Elmer). Ten microliters of PCR product was subjected to electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide under UV light.

### *Preparation of the FucT-VII protein*

Recombinant protein A fused human FucT-VII was produced by infecting Sf21 cells with recombinant baculovirus. The baculovirus transfer vector pAcProFucT-VII, carrying the secretary FucT-VII DNA encoding gp67 signal peptide, IgG-binding domain of *Staphylococcus aureus* protein A, and putative catalytic domain (amino acids 39–342) of FucT-VII, was prepared as described previously (manuscript submitted). The pAcProFucT-VII was co-transfected with linearized AcNPV (*Autographa californica* nuclear polyhedrosis virus) DNA (PharMingen) into Sf21 cells to obtain recombinant baculovirus BVProAFucT-VII. Sf21 cells ($1 \times 10^6$ cells/ml), cultured in EX-CELL 400 medium, were infected with the BVProAFucT-VII (MOI = 10). After a 72 h culture period, the cell supernatant was harvested and stored at −80 °C until use.

### *Assay of glycosyltransferase using SPA*

Truncated human FucT-VII activity was measured in a volume of 100 μl containing 1.0 μM GDP-β-fucose, 300,000 dpm GDP-[$^3$H]fucose, 1.25 mM $MnCl_2$, 25 mM Hepes/NaOH, pH 7.0, a fetuin-SPA bead suspension (1 mg beads/assay), and 1 μg enzyme for 120 min at 37 °C (manuscript submitted).

Truncated human FucT-VI (Calbiochem) was assayed in a volume of 100 μl containing 2.5 μM GDP-β-fucose, 120,000 dpm GDP-[$^3$H]fucose, 10 mM $MnCl_2$, 2.5 mM ATP, 0.0025% BSA, 5.0 mM L-fucose, 25 mM sodium cacodylate, pH 6.1, fetuin coated SPA beads (1 mg beads/assay), and 40 ng enzyme for 60 min at 37 °C.

Porcine α1-3 galactosyltransferase (Calbiochem) activity was assayed in a volume of 100 μl containing 2.0 μM UDP-α-D-galactose, 120,000 dpm UDP-[$^3$H]galactose, 0.005% BSA, 10 mM $MnCl_2$, 50 mM Hepes/NaOH, pH 7.0, asialofetuin-SPA bead suspension (1 mg beads/assay), and 0.1 mU enzyme for 45 min at 37 °C.

All assays were performed in 96-well flat white OptiPlates (Packard). GDP-[$^3$H]fucose incorporation was measured using a TopCount microplate scintillation counter (Packard).

### *Molt-4 cell adhesion assays*

Cell adhesion assays, using immobilized P-/E-selectin-IgG chimera (0.1 μg/well), were performed as follows. The microtiter plates (Sumitomo Bakelite) were coated with 100 μl PBS(+) containing P-/E-selectin-$IgG_1$ (0.1 μg/ml) overnight at 4 °C. The P-/E-selectin-coated plates were washed with PBS(+) and then blocked with PBS(+) containing 1% BSA. Molt-4 8H12G cells ($5 \times 10^5$) were pre-incubated with or without test compounds and stimulated with 1.0 nM MFP for the induction of FucT-VII for 16 h at 37 °C in RPMI 1640 medium supplemented with 10% FCS. The treated cells were suspended in PBS(+) containing 1% BSA, added to the P-/E-selectin-coated plates, and incubated for 60 min at 4 °C. After washing to remove unbound cells, the adhesion cells were counted with a cell counting kit (Promega). Data were expressed as percentages of the input cell number.

### *Inhibitor studies*

*Cell-based assay.* The cells were pre-incubated with the inhibitors for 60 min at 37 °C before being stimulated by MFP for 16 h, and the $sLe^x$-positive cells were monitored by flow cytometry analysis, as described above. The inhibitors were prepared as 100-fold concentration stocks in DMSO, and DMSO was added to a final concentration of 0.1% (v/v) in all samples assayed.

*Enzyme assay.* The enzyme inhibitors were pre-incubated with the enzyme for 30 min at 37 °C before initiating the SPA-based assay as described above. The inhibitors were prepared as 100-fold concentration stocks in DMSO, and DMSO was added to a final concentration of 1.0% (v/v) in all samples assayed.

### *Statistical analysis*

Data were represented as means ± SEM where applicable. Significance was determined using Student's *t* test.

## Results

### *Functional studies of the GeneSwitch system*

A schematic diagram of the principle of the GeneSwitch system is shown in Fig. 1. The mifepriston (MFP)-inducible regulator protein consists of yeast GAL4 DNA-binding domain (GAL4-DBD), the truncated human progesterone receptor ligand-binding domain (hPR-LBD), and the activation domain of the p65 subunit of human NF-κB (p65-AD). This protein (GeneSwitch protein) is initially produced as an inactive monomer. Binding of the MFP (antiprogestin) triggers conformational change of the GeneSwitch protein, and causes the formation of an active homodimer. The active homodimers bind to GAL4 sites in the inducible promoter (GAL4 UAS), then stimulate transcription of the transgene, and lead to the increased production of the protein of interest. Two plasmids

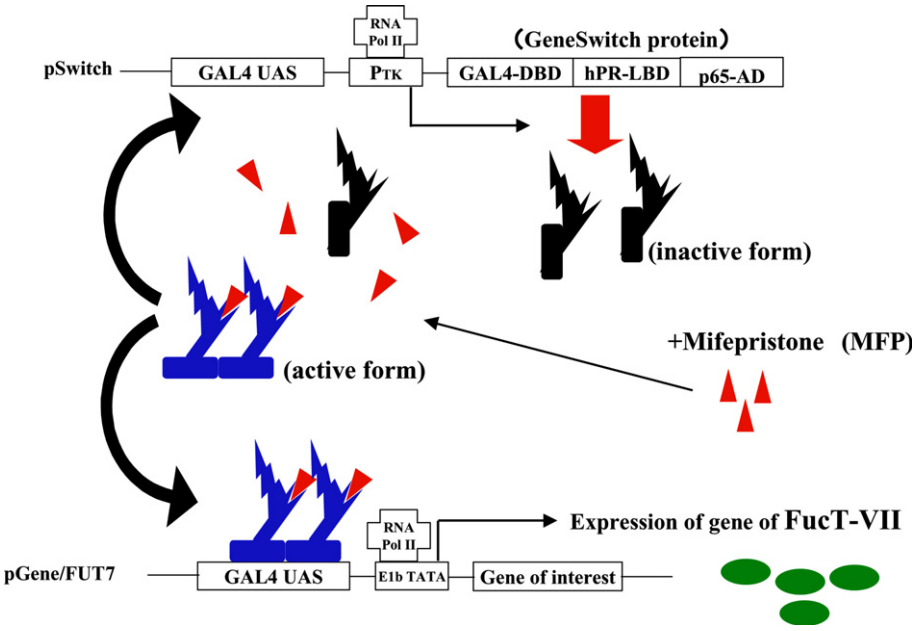


Fig. 1. The GeneSwitch system for FucT-VII expression in human T lymphoblastoma. An inducible expression plasmid, pGene/V5-His, for expression of the FucT-VII gene under the control of a hybrid promoter containing GAL4 upstream activating sequences (GAL4 UAS) and the adenovirus E1b TATA box. A regulatory plasmid, pSwitch, which encodes a fusion protein (GeneSwitch protein) consisting of the yeast GAL4 DNA-binding domain (GAL4-DBD), a truncated human progesterone receptor ligand-binding domain (hPR-LBD), and the human p65 activation domain from NF-κB (p65-AD). In the absence of the activator MFP as a progesterone antagonist, the GeneSwitch protein is expressed in the cells but remains transcriptionally silent (black). Therefore, the FucT-VII gene is not expressed. On the contrary, in the presence of MFP (red), the GeneSwitch protein forms a homodimer and becomes transcriptionally active (blue), which leads to the expression of the FucT-VII gene (green) and its own gene via autoregulatory feedback.

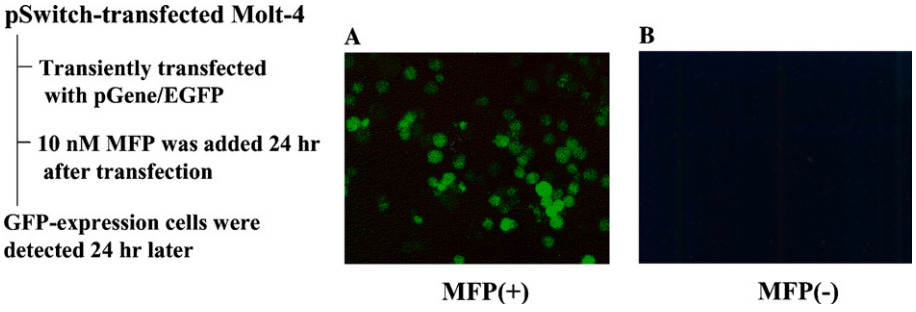


Fig. 2. MFP-mediated induction of transgene expression in the T lymphoblastoma. The stable transfectants of pSwitch (Molt-4 8H) were transfected with pGene/EGFP as the reporter gene by electroporation. The expression of GFP in the transfectants was monitored by confocal laser scanning microscopy after 24 h of culture with (A) or without (B) 1.0 nM MFP.

were used in this system. The first plasmid contained a gene for the GeneSwitch protein (pSwitch), and the second plasmid contained a gene encoding the protein of interest (pGene).

In order to develop a cell-based assay system for FucT-VII, the GFP/GeneSwitch system was tested for its regulatory potential of MFP-dependence in human T cells. At first, we introduced the pSwitch linearized with *Fsp*I into Molt-4 cells ($8 \times 10^4$) in mid-log phase growth by the electroporation method, and hygromycin-resistant cells were cloned and expanded. Next, in order to determine the inducing ability of the GeneSwitch protein, these hygromycin-resistant cells were transiently transfected with the reporter gene plasmid pGene/EGFP, and the GFP-expressing clones in the presence of MFP were identified by laser scanning confocal imaging system (Fig. 2). Three of 24 clones (#5A, #8H, and #11G) displayed inducible GFP expression in the presence of 1.0 nM MFP (Fig. 2A). In contrast, these positive clones did not express GFP in the absence of MFP (Fig. 2B). Furthermore, similar results were observed in the Jurkat cell line, and the GeneSwitch-working cells were obtained at a similar rate (data not shown). Thus, the GeneSwitch system is functional and MFP-mediated transgene expression can be achieved in these human T cell lines. Although Jurkat cells synthesized the E-selectin

ligand under the overexpression of FucT-VII, P-selectin ligands could not be detected in the cells, presumably due to their low level of UDP-Gal:GalNAc β1-3 galactosyltransferase [21]. Therefore, the Molt-4 8H clone, which showed preferential growth, was chosen and used for characterization of the FucT-VII/GeneSwitch.

*Selection of the sLe$^x$-epitope-expressing cells*

To establish the FucT-VII-expressing cell line under the control of the GeneSwitch system, we introduced the pGene/FUT7 into the Molt-4 8H stable cell line, and zeocin-resistant cells were cloned and expanded. The FucT-VII-inducible Molt-4 8H cells were selected by monitoring the sLe$^x$-epitopes stained with CSLEX-1. Six of 24 clones (#3H, #5E, #5F, #12G, #21F, and #30G) displayed inducible sLe$^x$-epitope expression, showing 80–95% positive cells among the transfected cells in the presence of 1.0 nM MFP for 48 h with very low background in the absence of MFP (Fig. 3). Thus, we selected Molt-4 8H12G for the subsequent studies because of its preferential growth (data not shown).

*FucT-VII expression following GeneSwitch activation*

In order to investigate the kinetics of FucT-VII expression, we quantified mRNA levels of FucT-VII after MFP application to Molt-4 8H12G using kinetic RT-PCR (Fig. 4). The mRNA was detectable within 6 h and reached a maximum by 24 h in the presence of 1.0 nM MFP. Furthermore, the mRNA of FucT-VII was also able to be detected until 48 h, whereas its expression was not able to be detected in the absence of MFP or the mock-transfected Molt-4 8H with 1.0 nM MFP (Fig. 4). In the GeneSwitch system, long-term inducible FucT-VII gene expression was able to be achieved in the T lymphoblastoma. The sLe$^x$-epitopes on the surface of FucT-VII-induced Molt-4 8H12G were also monitored by flow cytometry. As shown in Fig. 5A, expression of the sLe$^x$-epitopes was positive in 62.4% of cells within 15 h. The expression of the sLe$^x$-epitopes was linearly increased up to 24 h and reached a maximum (90.0% sLe$^x$ positive cells) at 72 h following induction with 1.0 nM MFP (Figs. 5A and B). Furthermore, the FucT-VII-induced Molt-4 8H12G, pretreated with

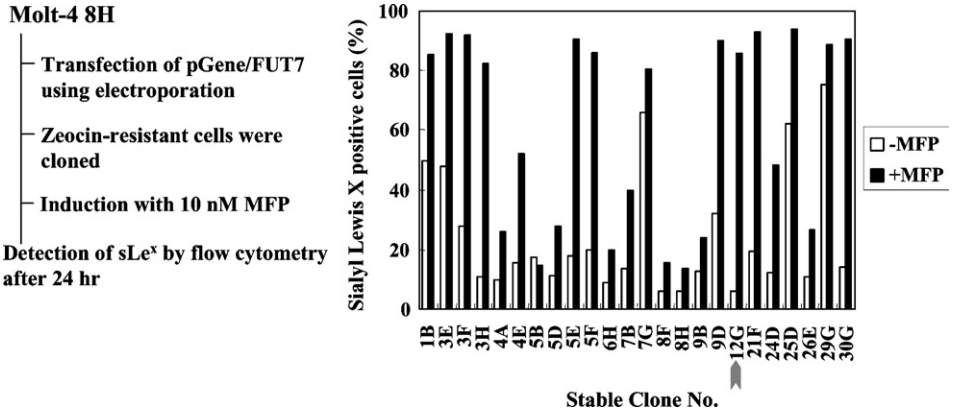


Fig. 3. Selection of sLe$^x$-inducible clones. The transfectants of pSwitch and pGene/FUT7 (Molt-4 8H12H) were cloned, expanded, and divided. One-half was stimulated in the presence (■) of 1.0 nM MFP and the remainder was uninduced (□) for 48 h. The sLe$^x$-epitopes were monitored by indirect immunofluorescence and flow cytometry, as described in Materials and methods. Data are expressed as percentages of positive cells (compared with an isotype control).

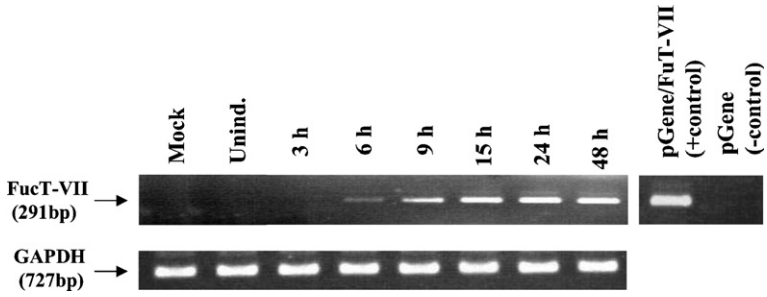


Fig. 4. Time-course of FucT-VII and GAPDH mRNA expression in Molt-4 8H12G cells stimulated with MFP. Molt-4 8H12G cells were induced or uninduced (unind.) with 1.0 nM MFP and collected 3, 6, 9, 24, 48, and 72 h after the induction. Kinetic RT-PCR was then performed, using a specific primer for the *FucT-VII* and the "housekeeping gene," *GAPDH*, described in Materials and methods.

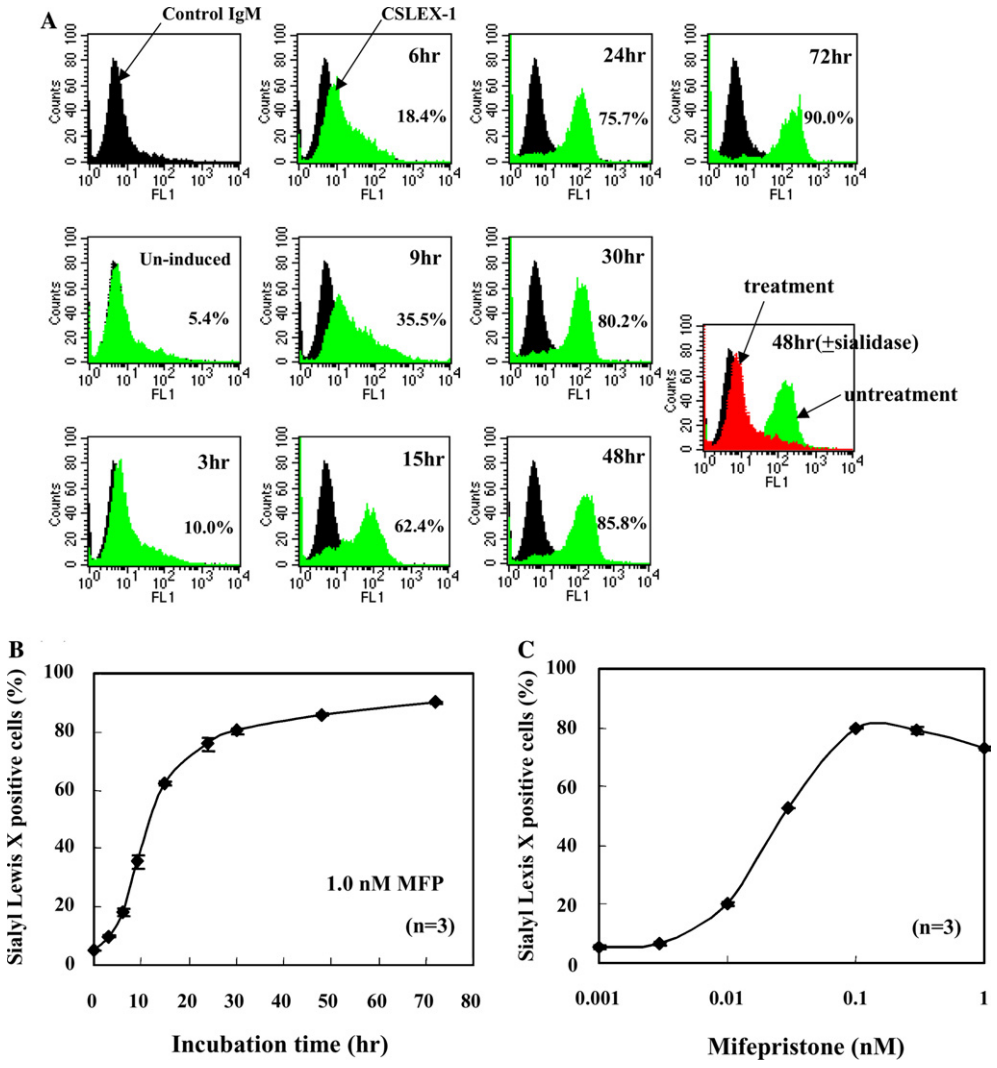


Fig. 5. Flow cytometry analysis of the sLe$^x$-epitope on the Molt-4 8H12G cells stimulated with MFP. (A) Time-course of sLe$^x$-epitope expression was analyzed in the presence of 1.0 nM MFP. The black histograms show the fluorescence of the cells stained with control IgM and the green histograms show the fluorescence of the cells stained with anti-sLe$^x$ antibody (CSLEX-1) after induction with 1.0 nM MFP. The red histograms show the Molt-4 8H12G cells treated with 25 mU of *Salmonella typhimurium* sialidase before being stained with CSLEX-1. (B) Quantification of sLe$^x$-epitopes was calculated from the histogram (A) and was shown as percentages of positive cells. (C) The Molt-4 8H12G cells were stimulated with the indicated concentration of MFP and were collected after 24 h, and the sLe$^x$-epitopes were analyzed by flow cytometry.

sialidase, was unresponsive to CSLEX-1, suggesting that CSLEX-1 binds sLe$^x$-epitopes selectively (Fig. 5A). Therefore, we fixed the incubation time at 16 h with 1.0 nM MFP to obtain sufficient expression of sLe$^x$-epitopes.

Furthermore, the sLe$^x$-epitope expression of the Molt-4 8H12G clone was induced by MFP concentrations ranging from 0.01 to 0.1 nM, with half-maximal expression at approximately 0.02 nM and peak expression at 0.1 nM (Fig. 5C). We carried out subsequent experiments with a MFP concentration of 1.0 nM, which was not toxic for the cells (data not shown).

In our study, FucT-VII activity in the T lymphoblasts correlated with the mean levels of sLe$^x$-epitopes. Therefore, expression of these epitopes provides an indirect measurement of FucT-VII activity in individual cells.

*Effects of panosialin K on the sLe$^x$-epitope expression*

To investigate the relationship between the enzyme assay and GeneSwitch cell-based assay, we performed the FucT-VII enzyme assay using the SPA-based method. In our experiments, the FucT-VII inhibitor panosialin K (A/B = 50/50) and GDP inhibited the FucT-VII activity in a dose-dependent manner with $IC_{50}$ values of 8.7 μg/ml and 2.3 μM, respectively. In contrast, weak inhibition of panosialin K and GDP was observed against α1-3 galactosyltransferase with $IC_{50}$ values of 48 μg/ml and 3.4 mM, respectively. These are consistent with the report by Shinoda et al. [13,14].

Panosialin A was also reported to reduce the cell surface expression of selectin ligands on U937 by 30% at a concentration of 25 μg/ml after a 72 h incubation time,

and did not alter the expression of other cell surface molecules [14]. Our results of the cell-based assay using the GeneSwitch system were almost equivalent to the report, the $IC_{50}$ value for panosialin K was 14.5 μg/ml (Fig. 6B), and GDP was not effective at 10 mM due to poor permeability to the cell membrane. Moreover, this method was more sensitive and rapid, because panosialin K completely inhibited the expression of $sLe^x$-epitopes at 20–40 μg/ml for a 16 h incubation time. Panosialin K did not influence cell viability and transcription activity up to 50 μg/ml (Fig. 6A).

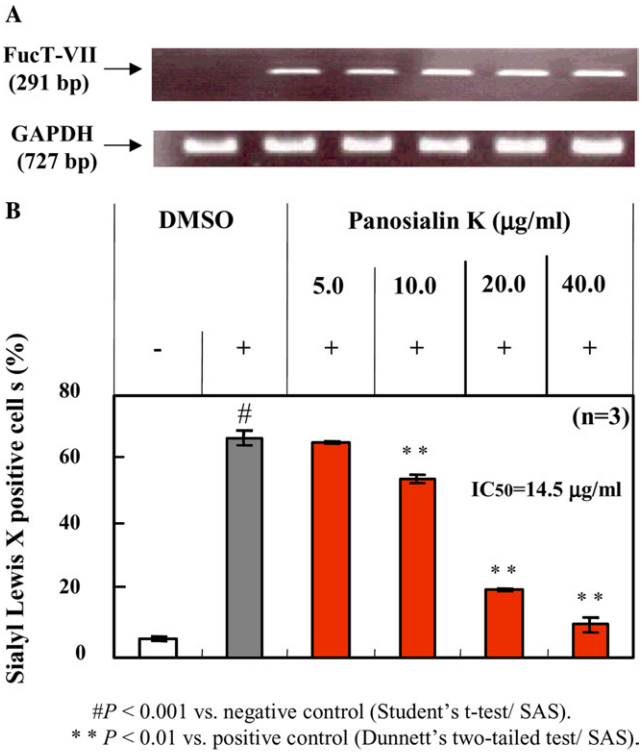


Fig. 6. Effects of panosialin K on the $sLe^x$-expression by Molt-4 8H12G cells. The Molt-4 8H12G cells were pre-treated with panosialin K (5–40 μg/ml) for 60 min and incubated in the presence (+) or absence (−) of 1.0 nM MFP for 16 h. Effect of panosialin K on mRNA expression of FucT-VII and GAPDH is shown in (A), and the expression of the $sLe^x$-epitope was monitored by flow cytometry (B). Quantification of the $sLe^x$-epitope was analyzed from the histogram (C) and shown as percentages of positive cells.

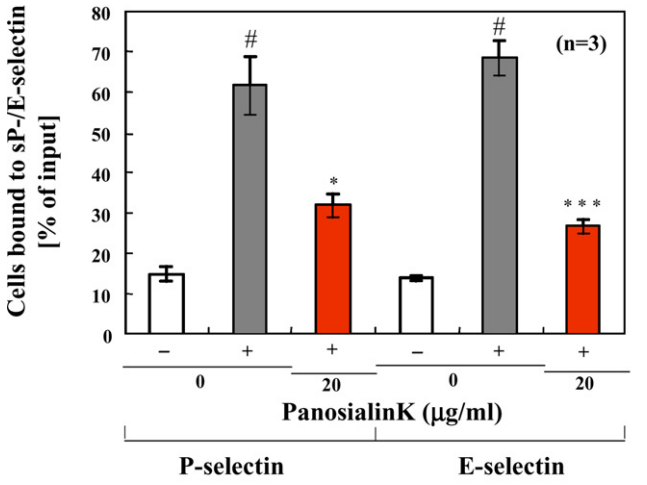


Fig. 7. Adhesion ability of panosialin K treated Molt-4 8H12 G cells to human P-/E-selectin chimera. Molt-4 8H12G ($5 \times 10^5$) cells were pre-incubated with 20 μg/ml (20) or without (0) panosialin K for 60 min, and incubated in the presence (+) or absence (−) of 1.0 nM MFP at 37 °C for 16 h. The treated cells were suspended in PBS(+) containing 1% BSA, added to the P-/E-selectin coated 96-well plates (0.1 μg/well), and incubated for 60 min at 4 °C. After washing to remove unbound cells, the adhered cells were counted. Data are expressed as percentages of the input cell number.

*Inhibition of panosialin K in the cell adhesion assay of FucT-VII-induced Molt-4 8H12G and P-/E-selectin*

Effects of the known FucT-VII inhibitor panosialin K on P-/E-selectin-dependent cell adhesion were studied. FucT-VII-induced Molt-4 8H12G preferentially adhered to immobilized P-/E-selectin (Fig. 7). By the treatment of FucT-VII-induced Molt-4 8H12G with 20 μg/ml panosialin K for 16 h, the adhesion to plates coated with recombinant P- and E-selectin-IgG was dramatically inhibited. These results suggested that the FucT-VII enzyme was necessary for the synthesis of P-selectin and E-selectin ligands in T lymphoblasts, and quantitative analysis of the cell adhesion number to the selectin may provide an indirect measurement of FucT-VII activity.

## Discussion

Several types of selectin inhibitors block the inflammatory reaction in vivo. The use of antibodies against

selectin resulted in the blockage of the development of skin and lung inflammatory reactions [22], and $sLe^x$ has been shown to block the development of lung inflammatory reactions [23]. Bimosiamose (TBC-1269) is reported to reduce the airway recruitment of eosinophils in asthma patients [24]. The data of TBC-1269 represent an important proof-of-concept and suggest that selectin inhibitors will be a potential treatment option for asthma. However, some low molecular weight compounds such as $sLe^x$, glycyrrhizin, inositol polyphosphates, and TBC-1269, in general, require near millimolar concentrations to achieve in vitro efficacy. Current efforts are now focused on developing small molecules with nanomolar activity or incorporating active ingredients into polyvalent displays. Recent studies on fucosyl- and sialyltransferases clarified the biosynthetic pathways of $sLe^x$. Among the different fucosyltransferases, FucT-VII appears to be important in the biosynthesis of the selectin ligand. Therefore, small molecules designed to inhibit this enzyme specifically will be of pharmacological interest.

Here, we developed a quantitative method to monitor $sLe^x$-epitopes and selectin ligands on the FucT-VII-induced cells using the Gene Switch system. This provided a rapid and sensitive assay, principally based on the MFP-regulated gene expression system with the ability to control the levels of the protein product quantitatively, which allowed for the screening of inhibitors. The MFP-regulated system has several benefits when investigating the gene regulator. First, it generates low background expression in the absence of the inducer. Second, the induction by MFP appeared to occur within a relatively wide range of inducer concentrations with no effect on the cell viability. Third, the induction response was relatively rapid upon addition of the inducer.

Current ligand-dependent gene regulation systems are classified into three groups based on the components of the chimeric regulator protein: bacteria repressor proteins such as tetracycline, β-galactoside, and streptogramin-regulated systems [25–27], heterodimeric proteins resulting from chemical-induced dimerization such as rapamycin-regulated systems [28], or nuclear hormone receptor ligand-binding domains such as antiprogestin, antiestrogen, ecdysteroid, or glucocorticoid-regulated systems [17,29–32]. Some of these systems have been tested to establish the cell-based assay of FucT-VII using human T lymphoblastoma. The bacteria repressor protein-induced gene was expressed by a highly active, non-specific promoter such as the CMV promoter, whereas the gene for the MFP-inducible regulator protein was expressed from one much less active than the bacteria repressor protein. Excessive protein expressions, more than those under physiological conditions, desensitize the chemical compounds towards the target protein. In order to optimize the sensitive cell assay for FucT-VII, a reasonable and tightly controllable promoter activity in the system is preferable. Of note is that MFP has recently been approved by the FDA for use in humans, and the side effects or cytotoxicity associated with its administration is generally considered minimal. All of the well-characterized inducible systems that have been developed over the past decade are complicated to some extent, i.e., the inducible agent causes unwanted side effects upon administration to the animal, or the activation of an immune response against the regulator protein in vivo/vitro. The tetracycline-based system has been shown to elicit immune responses against the transactivator protein [33], while rapamycin has been shown to be a potent suppressor of the immune system at the concentrations needed for transcriptional activation [34].

In our study, the GeneSwitch system was found to be dose-dependent with maximal activation occurring at a dose of 0.1–10 nM MFP, in contrast, there was an extremely low background cell activation in the absence of MFP in the human T lymphoblast Molt-4 cell line. The FucT-VII enzyme is required for the synthesis of the most active P- and E-selectin ligands on cultured T lymphoblasts [35]. Introduction of the FucT-VII gene into the human Molt-4 generated ligands that mediated slow rolling on immobilized selectins under shear stress. In contrast, neither wild-type Molt-4 cells nor T lymphoblasts derived from FucT-VII-deficient mice reacted to E-/P-selectin chimeras or initiated rolling on the immobilized selectins [35]. In our GeneSwitch system, we also showed a high level of expression of $sLe^x$-epitopes in the presence of MFP, which resulted in the adhesion of these cells to E-/P-selectin chimeras. All of the $sLe^x$-expression cells ($67.3 \pm 2.2\%$ of positive cells) showed maximal binding to the P- and E-selectin chimera ($61.7 \pm 2.2\%$ and $68.3 \pm 4.4\%$ of input, respectively). In addition, the epitope expression was found to be at a lower level ($5.4 \pm 0.1\%$ of positive cells) in the absence of the MFP, where the background binding of cells to selectins could have been approximately 14–15% of the input cell. These results demonstrated that $sLe^x$-expression on the cell was correlated with the adhesion strength of the cell.

Panosialin K is a useful tool to study the consequences of the FucT-VII inhibitor in the cell-based system. Panosialin K is a preferential FucT-VII inhibitor displaying only very low activity towards the related α1-3 galactosyltransferase enzymes. In the GeneSwitch-based cell assay, we showed that panosialin K had a negligible effect at the mRNA level of FucT-VII, whereas the expression of $sLe^x$-epitopes and selectin ligand was dramatically decreased on MFP-induced Molt-4 8H12G in a dose-dependent manner. These data suggest that the effect of panosialin K was specifically mediated via FucT-VII inhibition. Although panosialin K is a strong FucT-VII inhibitor, panosialin was also shown to be an inhibitor

of viral sialidase, acid phosphatase, polygalacturonase, and glycosidase [36–38]. Recently, a potent and highly selective inhibitor of human α1-3 FucT was identified via click chemistry [39], and the optimization of these non-selective inhibitors will be of great importance to the discovery of selective FucT-VII inhibitors.

In summary, we have established a rapid and sensitive cell-based assay of FucT-VII using the GeneSwitch system for the first time. These data support the idea that the human FucT-VII activity is critical for the rapid expression of $sLe^x$-epitopes and E-/P-selectin ligands. The pivotal role of FucT-VII in the synthesis of P- and E-selectin ligands suggests that blockage of its activity and/or transcription provides a novel approach to treat selectin-mediated immune disorders. This method will accelerate the finding of the selective inhibitors of FucT-VII as anti-inflammatory agents.

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