Supporting Information

Sakuma et al. 10.1073/pnas.1111135109

SI Materials and Methods

Antibodies and Reagents. Monoclonal antibodies against sLe^x (clone SNH-3, murine IgM), sLe^a (clone 2D3, murine IgM), and Le^y (clone AH-6, murine IgM) were prepared as described previously (1–3). Anti-Le^b antibody (clone 2-25LE, murine IgG1) was obtained from Seikagaku Biobusiness. Recombinant human E/P-selectins Fc chimera and FITC-conjugated antihuman IgG Fc antibody were obtained from R&D Systems and Sigma, respectively. Antibodies against c-Myc (clone 9E11) and SNAIL1, those against CDX2 and ZEB1, and those against phospho–c-$Myc^{Ser62/Thr58}$ and phospho-$GSK3\beta^{Ser9}$ (clone D85E12) were obtained from Abcam, Santa Cruz, and Cell Signaling Technology, respectively.

Flow Cytometry. For flow cytometric analyses, cells were harvested and stained with respective primary antibodies using culture supernatants at a dilution of 1:5 or purified antibodies at 1 μg/mL. The cells were then stained with a 1:300 dilution of FITC-conjugated secondary antibody. For selectin-binding assays, cells were incubated with recombinant selectins at 20 μg/mL followed by incubation with FITC-conjugated secondary antibody at 2 μg/mL, with all of the reactions performed in calcium containing Dulbecco's PBS except experiments in the presence of EDTA at a final concentration of 1 mM. For blocking experiments, cells were pretreated with SNH-3 and/or 2D3 antibodies at a final concentration of 100 μg/mL before incubation with recombinant selectins. Propidium iodide was used at 1 μg/mL to identify living cells. Cells were analyzed with a FACSCalibur (BD Biosciences).

Conventional and Quantitative RT-PCR Analysis. Total cellular RNA was isolated with ISOGEN reagent (Nippon Gene). First strand cDNA was prepared from the total RNA (5 μg) with SuperScript II Reverse Transcriptase (Invitrogen). Conventional RT-PCR analysis was performed with KOD-Plus- (Toyobo) and primers listed in Table S1 using a GeneAmp PCR system 9700 (Applied Biosystems). For quantitative RT-PCR, cDNA samples were mixed with FAM-labeled TaqMan gene expression assays (Table S2), VIC-labeled TaqMan MGB probe for β-actin, and TaqMan gene expression master mix (Applied Biosystems), followed by amplification using 7500 Fast Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. Results were calculated by the comparative CT method, with relative transcript levels determined as $2^{-\Delta\Delta CT}$.

ChIP Assay. Chromatin samples were isolated from cells with a ChIP-IT Express kit (Active Motif) according to the manufacturer's protocol. Samples (60 μL) were incubated with antibodies (1 μg) for 8 h at 4 °C. The primers for detection of the promoter sequences were designed in the 5′-regulatory regions of *ST3GAL1/3/4*, *FUT3*, and *FUT2*, all of which were previously reported (4–9). The primers used are listed in Table S3.

RNA Knockdown and Forced Expression. The pLKO.1-based lentiviral shRNA, the nontarget shRNA, packaging and envelope plasmids were obtained from Sigma. The plasmids were co-transfected into HEK293T cells to produce viral particles using FuGENE 6 (Promega). HT29 and DLD-1 cells were infected with the viral supernatants in the presence of 8 μg/mL of polybrene (Sigma). After selection with 5 μg/mL of puromycin (Sigma), total RNA was extracted and analyzed by quantitative RT-PCR. For c-Myc knockdown, TRCN0000010389 having the sequence CCGGAACTATGACCTCGACTACGACTCGAGTCGTAGTCGAGGTCATAGTTCTTTTTG gave the best results among the five tested shRNA vectors. For CDX2 knockdown, TRCN0000013687 having the sequence CCGGAGCCCTTGAGTCCGGTGTCTTCTCGAGAAGACACCGGACTCAAGGGCTTTTTT gave the best results among the five tested shRNA vectors. For CDX2 forced expression, a cDNA expression vector was purchased from OriGene and introduced into HT29 and DLD-1 cells using FuGENE 6 reagent. After a 3-d culture, total RNA was extracted and analyzed by quantitative RT-PCR.

Cell Migration Assay. Cell migration activity was determined using Biocoat Matrigel invasion chambers (BD Biosciences) according to the manufacturer's instructions. Briefly, cells (2×10^5) were seeded in serum-free culture medium onto the Matrigel-coated filters. Culture medium supplemented with 10% (vol/vol) FBS was added to the lower part of the chambers. After a 24-h incubation period at 37 °C, the filters were stained with Diff-Quik dye (Sysmex International Reagents) and migrated cells were counted in five randomly chosen fields under a light microscope.

ELISA. Culture supernatant VEGF levels were measured by ELISA using a Milliplex MAP Human Cytokine/Chemokine kit (Millipore). Samples were collected on the third day from the medium replacement and subjected to ELISA at a dilution of 1:4. All of the assays were performed in triplicate.

Western Blotting. Cell lysates were prepared with RIPA buffer (Thermo Scientific) containing blends of protease and phosphatase inhibitors (Roche Applied Science). Samples were subjected to SDS/PAGE, followed by transfer onto PVDF membrane (Bio-Rad). After blocking, the blots were incubated with primary antibodies and HRP-conjugated secondary antibody (SouthernBiotech) at appropriate dilutions. The signal was detected with an ECL kit (GE Healthcare).

Immunohistochemistry. Colon cancer tissues were obtained from patients in Kyoto University Hospital under informed consent, and investigations were conducted according to the Declaration of Helsinki principles. Frozen sections of 10-μm thickness were fixed with ice-cold acetone. After blocking with PBS(−) including 5% (vol/vol) normal goat serum and 0.3% Triton X-100 for 60 min, the sections were incubated with primary and secondary antibodies, and Hoechst 33342 (Dojindo) according to the manufacturer's instructions. Stained sections were observed with a LSM510 confocal microscope (Carl Zeiss).

1. Abe K, McKibbin JM, Hakomori S (1983) The monoclonal antibody directed to difucosylated type 2 chain (Fuc alpha 1 leads to 2Gal beta 1 leads to 4[Fuc alpha 1 leads to 3]GlcNAc; Y Determinant). *J Biol Chem* 258:11793–11797.
2. Phillips ML, et al. (1990) ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Lex. *Science* 250:1130–1132.
3. Takada A, et al. (1991) Adhesion of human cancer cells to vascular endothelium mediated by a carbohydrate antigen, sialyl Lewis A. *Biochem Biophys Res Commun* 179:713–719.
4. Taniguchi A, Yoshikawa I, Matsumoto K (2001) Genomic structure and transcriptional regulation of human Galbeta1,3GalNAc alpha2,3-sialyltransferase (hST3Gal I) gene. *Glycobiology* 11:241–247.

5. Taniguchi A, Saito K, Kubota T, Matsumoto K (2003) Characterization of the promoter region of the human Galbeta1,3(4)GlcNAc alpha2,3-sialyltransferase III (hST3Gal III) gene. *Biochim Biophys Acta* 1626:92–96.
6. Taniguchi A, Matsumoto K (1999) Epithelial-cell-specific transcriptional regulation of human Galbeta1,3GalNAc/Galbeta1,4GlcNAc alpha2,3-sialyltransferase (hST3Gal IV) gene. *Biochem Biophys Res Commun* 257:516–522.
7. Cameron HS, Szczepaniak D, Weston BW (1995) Expression of human chromosome 19p alpha(1,3)-fucosyltransferase genes in normal tissues. Alternative splicing, polyadenylation, and isoforms. *J Biol Chem* 270:20112–20122.
8. Dabrowska A, Baczyńska D, Widerak K, Laskowska A, Ugorski M (2005) Promoter analysis of the human alpha1,3/4-fucosyltransferase gene (FUT III). *Biochim Biophys Acta* 1731:66–73.
9. Koda Y, Soejima M, Wang B, Kimura H (1997) Structure and expression of the gene encoding secretor-type galactoside 2-alpha-L-fucosyltransferase (FUT2). *Eur J Biochem* 246:750–755.

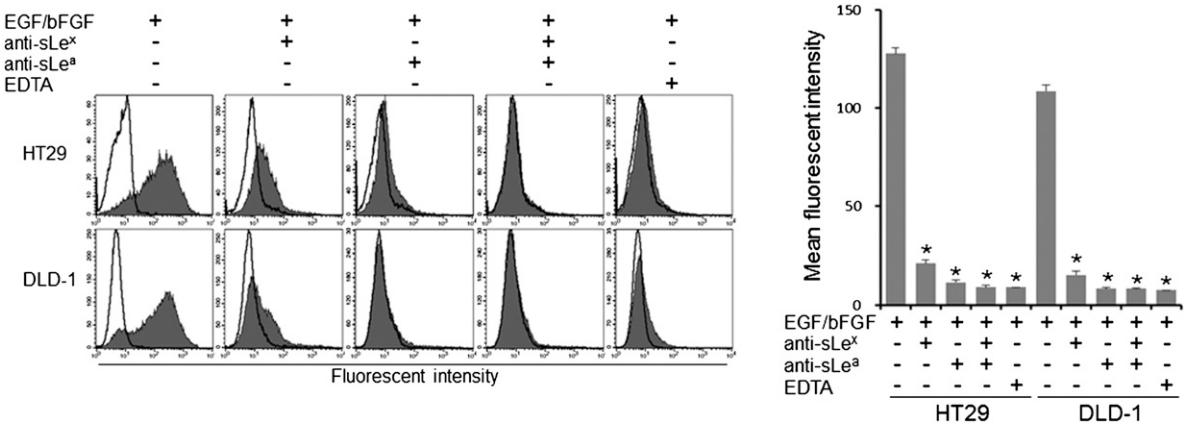

Fig. S1. Contribution of sLe$^{x/a}$ to E-selectin binding activity. E-selectin binding activity was examined by flow cytometry using recombinant E-selectin. The EGF/bFGF-treated cells were pretreated with or without anti-sLe^x and/or anti-sLe^a antibodies at a final concentration of 100 μg/mL or pretreated with or without 1 mM EDTA before incubation with recombinant E-selectin. Recombinant P-selectin was used as a negative control. Statistic analysis was performed in three independent experiments by *t* test. Bold lines, staining control; error bars, SD; asterisks, $P < 0.000005$ compared with the cells without pretreatment.

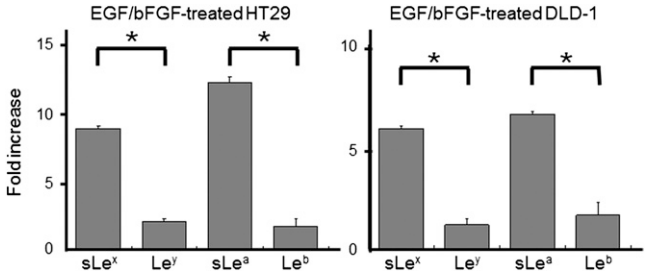

Fig. S2. Contribution of *FUT2* down-regulation to the preferential increase of sLe$^{x/a}$ compared with Le$^{y/b}$. Expression levels of sLe^x, Le^y, sLe^a, and Le^b were determined by flow cytometry. Results from three independent experiments are shown by fold increases of the mean fluorescent intensity in the EGF/bFGF-treated cells compared with that in the untreated cells. Statistic analysis was performed in three independent experiments by *t* test. Error bars, SD; asterisks, $P < 0.01$.

A ST3GAL1

TTCCTTAGCCCCGCCAGCTTGAGGGCCG
CGTCCAGAGAGCGGGGAGCTCCTCTCGG
GGCGCCGATCAGGTCCCCGCGCCGCAGC
CGCCGCCTCCGGCGTCTCCCAGGCTCGG
CCTCTGCCGAGCCCGCTGCGGTCCACGT
GGCTTGGCAGAGCTAAATTCGGCTTGCAG
GAAGCCCGGAGCCAAGGG**AAAGCCGAG**
CCAAGTTGGGGTG

B ST3GAL3

AGTGGCCGTGACTACCCTAGCACTTTGC
GTTCCCCGCGCGGGCGACGAAACACGCT
CTCGAGGGGAGAGAGCCGTGCGTCCTCT
GGGCCGTGAAGCCAGGGGAAGAGGGTTC
CTCTAGACAGCTCGATGTGCCCGGGAGA
GACACCCACGCTGAACCTGCAAGCTGGG
GTGCTCCACCAGCGCGAACCTCTCGCCC
CGCACTCGCCGCAGCACTGCTCTCGTGG
TAGGCGGGGTGGGCGGGACGGCA**AATCC**
GCGGCTCCTGCGTCTA

C ST3GAL4

GGCTCACCTGGATCCTTAATGCCGCCCCT
TGGAGGAGTTAGGAGGATCCTGGATGAG
AAAACTCACCCTCAGGATGATTGCCCCCA
GGGAGCAGCTTCCTGCTTTCTGGTGGAAG
GGAGGGGCAGACAGTGGGTGTGTCCTGC
TCCAGTGTCTAGGCAGGAGAGTTTGT**GAA**
GCTGACCGGACACCTGTG

D FUT3

GGTCTCACAGGCGAGATTAGGACACCCC
GGAAACTGGCTTCCAGACAATATCCCTGC
TGCAGGGGAGAACACCCTAGGTCACCTG
GTGACAGGTG**TGTGCTGCAATGTACACGT**
ACTTGTTCC

E FUT2

CTTTCTGTTGGGGCATCACAACAGTTCCC
CAAGGAAGACCCTCGGGGACCCGGATGG
GGGATGCGACCTTGTCCTGCCTCTCTCCC
CCACCCTTATGGCCAGGCTTGGGGTGCCT
GGTGCAGGTGGAGGAGCTAAGG**GTAGAT**
AACAAGATGGACTTTGTGGCCG

Fig. S3. 5′-regulatory regions of *ST3GAL1/3/4*, *FUT3*, and *FUT2*. (*A–E*) DNA sequences of the 5′-regulatory regions of *ST3GAL1/3/4*, *FUT3*, and *FUT2* are shown. Bold letters, primer-targeted sequences for ChIP assay; single underlines, potential c-Myc binding sites; double underlines, potential binding sites for CDX1 and CDX2.

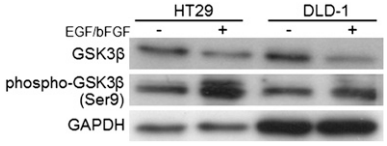

Fig. S4. EGF/bFGF-induced alteration in the levels of GSK3β and phospho-GSK3β^{Ser9}. The levels of GSK3β and phospho-GSK3β^{Ser9} in the untreated and EGF/bFGF-treated cells were determined by Western blotting.

Table S1. Primers used for conventional RT-PCR

Gene	Primer sequence	Length	Annealing, °C	Cycles
β-actin	F: CGTGCGTGACATTAAGGAGAAGC	305	57	22
	R: CAATGCCAGGGTACATGGTGGT			
ST3GAL1	F: TGGTCCTGGAGCTCTCCGAGAA	363	58	30
	R: GACTGTCTATCTCAGGCCCATAAGAAGA			
ST3GAL2	F: GATGATGCTGCAGCCCCAGTTC	237	58	30
	R: ACATCCTGCTCAAAGCCCACGGTT			
ST3GAL3	F: CGGATGGCTTCTGGAAATCTGT	300	55	30
	R: TTGTGCGTCCAGGACTCTTTGA			
ST3GAL4	F: TCCAGGGTGAGGCAGAGAGCAA	190	58	27
	R: TTGGGGATGGAGGAGCTGGTGA			
FUT1	F: CACGAAAAGCGGACTGTGGATCTG	172	58	31
	R: GACACAGGATCGACAGGCCTAG			
FUT2	F: CCTTCAGCAGGACCAGGTGAGA	198	58	31
	R: GGTCCCAGTGCCTTTGATGTTGAG			
FUT3	F: TGTTTCTTCTCCTACCTGCGTGTGTC	230	58	30
	R: GTGTCTGCCTGTGGGTACACCT			
FUT4	F: CAACATGTGACCGTGGACGTGTTC	135	58	27
	R: GGTGATATAATCCAGGTGCTGCGAGTT			
FUT6	F: CATCTCAAGGTGGACGTGTACGGA	215	58	30
	R: GGTGGCAGGAACCTCTCGTAGT			
FUT7	F: CCTGGGAGACTGTGGATGAATAATGCT	174	58	32
	R: GTGCCAGACAAGGATGGTGATCGT			
E-cadherin	F: CAGAGCCTCTGGATAGAGAACGCA	245	58	30
	R: GGCATTGTAGGTGTTCACATCATCGTC			
SNAIL1	F: TATGCTGCCTTCCCAGGCTTG	143	57	30
	R: ATGTGCATCTTGAGGGCACCC			
ZEB1	F: CCAGTGGTCATGATGAAAATGGAACACC	243	58	33
	R: CAGACTGCGTCACATGTCTTTGATCTC			
Vimentin	F: GGCTCAGATTCAGGAACAGC	373	55	30
	R: CTGAATCTCATCCTGCAGGC			
MUC2	F: CCGTCCTCCTACCACATCAT	149	55	30
	R: CTCTCCAGGCCGTTGAAGT			
ALPI	F: GCAACCCTGCAACCCACCCAAGGA	278	62	30
	R: CCAGCATCCAGATGTCCCGGGAG			
c-Myc	F: TCCGTCCTCGGATTCTCTGCTCT	208	58	30
	R: GCCTCCAGCAGAAGGTGATCCA			
CDX1	F: AGGACAAGTACCGCGTGGTCTA	670	57	35
	R: CCTCTGAACGTATGGAGGAGGA			
CDX2	F: CAGTCGCTACATCACCATCCG	384	57	28
	R: GCAGAGTCCACGCTCCTCAT			

F, forward primer; R, reverse primer.

Table S2. TaqMan gene expression assays used for quantitative RT-PCR

Gene	Assay ID
ST3GAL1	Hs00161688_m1
ST3GAL3	Hs00544033_m1
ST3GAL4	Hs00920871_m1
FUT2	Hs00704693_s1
FUT3	Hs01868572_s1
FUT6	Hs03026676_s1
c-Myc	Hs00905030_m1
CDX2	Hs01078080_m1

Table S3. Primers used for ChIP assay

Gene	Primer sequence	Product length	Annealing, °C
ST3GAL1	F: TTCCTTAGCCCCGCCAGCTTGA	209	58
	R: CACCCCAACTTGGCTCGGCTTT		
ST3GAL3	F: AGTGGCCGTGACTACCCTAGCAC	268	62
	R: TAGACGCAGGAGCCGCGGATT		
ST3GAL4	F: GGCTCACCTGGATCCTTAATGCC	189	58
	R: CACAGGTGTCCGGTCAGCTTCA		
FUT2	F: CTTTCTGTTGGGGCATCACAACAGTTC	165	58
	R: CGGCCACAAAGTCCATCTTGTTATCTAC		
FUT3	F: GGTCTCACAGGCGAGATTAGGACA	123	58
	R: GGAACAAGTACGTGTACATTGCAGCACA		

F, forward primer; R, reverse primer.