

Figure 7 Localization of EB1 and DLG5 in the vascular endothelial cells of inflamed colon of F344 rats. Representative images of CD31 and EB1 (A), DLG1 (B) or DLG5 (C) co-fluorescent staining of distal colonic lesions from F344 rats immediately after DSS treatment (week 1). EB1 and DLG5, but not DLG1, were highly expressed in VECs at the submucosa. Scale bar = 50 μ m. Images in the **right column** are magnified from the **boxed areas** of the merged images. Scale bar = 20 μ m.

Fluorescent IHC analysis of inflamed colonic tissue at week 1, immediately after DSS treatment, revealed that EB1 and DLG5, but not DLG1, were expressed in the VECs (Figure 7). EB1 was also expressed in the severely ulcerated mucosal region (Figure 7A). DLG1, DLG5, and EB1 were not expressed in the VECs of the colon before the DSS treatment (data not shown). Thus, these findings indicate that the expression of EB1 and DLG5 was induced in VECs when colon inflammation occurred and that DLG5, rather than EB1, showed a pattern of expression similar to that of the APC protein (Figure 3).

Discussion

APC has been identified as the causative gene of the familial adenomatous polyposis of the colon. APC negatively regulates Wnt signaling by interacting with β -catenin via its

β -catenin binding sites. Truncation of the APC protein at β -catenin binding sites result in the activation of Wnt signaling, which is a hallmark of the initiation of carcinogenesis in colonic epithelial cells.^{12,28,29} In KAD rats (homozygous for the *Apc* ^{Δ 2523} mutation), Wnt signaling is normal because the β -catenin binding sites are intact. Thus, we could exclude the effects of Wnt signaling when we compared physiologic characteristics found in KAD and control F344 rats. Alternatively, we could ascribe characteristic differences to the physiologic function of the C terminus of APC.

Even 3 weeks after terminating DSS treatment, KAD rats had continuous elevation of the expression of inflammatory-related genes, such as *Cox2* and *Ptges*, and enhanced cell proliferation, which were coincident with persistent inflammation. COX-2 and PTGES are functionally coupled for the biosynthesis of prostaglandin E₂,³⁰ which plays a critical role during tumorigenesis in the gastrointestinal tract.^{31,32} Thus, these findings can explain the significantly

higher incidences and multiplicities of colon tumors found in KAD rats compared with the control F344 rats.¹⁶

Immediately after DSS treatment, KAD rats had an abnormal deposition of fibrin in microvessels, instead of a normal formation of fibrin layers to cover the damaged mucosa. KAD rats also had decreased microvessel angiogenesis in the damaged colonic mucosa immediately after DSS treatment. Given the evident increase in expression of APC in VECs of the edematous submucosa, it is likely that APC has a critical role in angiogenesis that is driven in response to acute inflammation of the colon and that KAD rats have defects in such angiogenesis. Such defects may result in a delay in the repair process of the damaged mucosa possibly because of the shortage of oxygen and nutrition supply and therefore a persistence of colitis in KAD rats.

To determine how APC is involved in angiogenesis, we characterized the physiologic function of VECs of KAD rats. KAD rat VECs had stronger adhesion and formed abnormal tube morphology, which was characterized by a smaller number of branching points and a shorter length when VECs were stimulated to form tubes on Matrigel. The adhesion activity of VECs plays an important role in tube formation.^{33,34} Therefore, it is likely that the enhanced adhesion activity may result in the low branched and short tubes found in KAD rat VECs. Although VECs were prepared from the thoracic aorta in this study, defects in tube formation may explain the decreased number of microvessels found in the damaged colonic mucosa of KAD rats.

Expression of both APC and DLG5 was specifically induced in the VEC of the inflamed colon at week 1, immediately after DSS treatment. DLG5 interacted with the C terminus of APC, probably via its PDZ domains. The DLG protein family interacts with transmembrane proteins in endothelial cells and is associated with angiogenesis.³⁵ Thus, we consider that the APC-DLG5 molecular pathway may function in the regulation of angiogenesis and cell adhesion of VECs.

It is known that abnormal angiogenesis contributes to the initiation and perpetuation of IBD and that microvessel dysfunction causes poor mucosal healing in IBD.^{36,37} Similar to IBD patients, KAD rats had reduced angiogenesis in the inflamed mucosa and the KAD VECs had reduced adhesion activity. The human *DLG5* locus is associated with the pathogenesis of IBD.²⁷ In rats, expression of *DLG5* can be induced by DSS treatment, and *DLG5* binds to the C terminus of APC, which is absent in KAD rats. These results suggest that both APC and *DLG5* may be involved in the pathogenesis of IBD. Further genetic analysis of relevant human populations may clarify the involvement of APC in the development of colitis.

It is expected that mutant APC derived from the *Apc*^{A2523} allele may coexist with wild-type APC in the heterozygous KAD (*Apc*^{A2523/+}) rat because transcripts from the mutant allele can escape nonsense-mediated decay. Thus, further study using heterozygous rats may reveal dominant negative functions of the mutant APC.

In summary, we found a new function of APC in the pathogenesis of colonic inflammation, which appears to be mediated by the regulation of cellular adhesion activity of VECs. Molecular interaction of APC with *DLG5* may function in the regulation of angiogenesis and cell adhesion of VECs. Our findings suggest that APC may contribute to the pathogenesis of IBD, in which patients are more susceptible to colorectal cancers.

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Supplemental Data

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References

- Wallace JL, Vong L, Dharmani P, Srivastava V, Chadee K: Muc-2-deficient mice display a sex-specific: COX-2-related impairment of gastric mucosal repair. *Am J Pathol* 2011, 178:1126–1133
- Tonnesen MG, Feng X, Clark RA: Angiogenesis in wound healing. *J Investig Dermatol Symp Proc* 2000, 5:40–46
- Tarnawski AS: Cellular and molecular mechanisms of gastrointestinal ulcer healing. *Dig Dis Sci* 2005, 50(Suppl 1):S24–S33
- Podolsky DK: Inflammatory bowel disease. *N Engl J Med* 2002, 347:417–429
- Bernstein CN, Blanchard JF, Kliever E, Wajda A: Cancer risk in patients with inflammatory bowel disease: a population-based study. *Cancer* 2001, 91:854–862
- Danese S, Sans M, Fiocchi C: Inflammatory bowel disease: the role of environmental factors. *Autoimmun Rev* 2004, 3:394–400
- Cooney R, Jewell D: The genetic basis of inflammatory bowel disease. *Dig Dis* 2009, 27:428–442
- Kawada M, Arihiro A, Mizoguchi E: Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease. *World J Gastroenterol* 2007, 13:5581–5593
- Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R: A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 1990, 98:694–702
- Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, Joslyn G, Stevens J, Spirio L, Robertson M, Sargeant L, Krapcho K, Wolff E, Burt R, Hughes JP, Warrington J, McPherson J, Wasmuth J, Le Paslier D, Abderrahim H, Cohen D, Leppert M, White R: Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 1991, 66:589–600
- Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C, Taketo M: Loss of *Apc* heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated *Apc* gene. *Proc Natl Acad Sci U S A* 1995, 92:4482–4486
- Aoki K, Taketo MM: Adenomatous polyposis coli (APC): a multifunctional tumor suppressor gene. *J Cell Sci* 2007, 120:3327–3335
- Beroud C, Soussi T: APC gene: database of germline and somatic mutations in human tumors and cell lines. *Nucleic Acids Res* 1996, 24:121–124
- Smits R, Kielman MF, Breukel C, Zurcher C, Neufeld K, Jagmohan-Changur S, Hofland N, van Dijk J, White R, Edelmann W,

- Kucherlapati R, Khan PM, Fodde R: Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes Dev* 1999, 13: 1309–1321
15. Yokoyama A, Nomura R, Kurosumi M, Shimomura A, Onouchi T, Iizuka-Kogo A, Smits R, Oda N, Fodde R, Itoh M, Senda T: The C-terminal domain of the adenomatous polyposis coli (Apc) protein is involved in thyroid morphogenesis and function. *Med Mol Morphol* 2011, 44:207–212
 16. Yoshimi K, Tanaka T, Takizawa A, Kato M, Hirabayashi M, Mashimo T, Serikawa T, Kuramoto T: Enhanced colitis-associated colon carcinogenesis in a novel Apc mutant rat. *Cancer Sci* 2009, 100:2022–2027
 17. Yoshimi K, Hashimoto T, Niwa Y, Hata K, Serikawa T, Tanaka T, Kuramoto T: Use of a chemically induced-colon carcinogenesis-prone Apc-mutant rat in a chemotherapeutic bioassay. *BMC Cancer* 2012, 12:448
 18. Melgar S, Karlsson A, Michaelsson E: Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2005, 288:G1328–G1338
 19. Vermeulen PB, Gasparini G, Fox SB, Toi M, Martin L, McCulloch P, Pezzella F, Viale G, Weidner N, Harris AL, Dirix LY: Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. *Eur J Cancer* 1996, 32A: 2474–2484
 20. Feroze-Merzoug F, Berquin IM, Dey J, Chen YQ: Peptidylprolyl isomerase A (PPIA) as a preferred internal control over GAPDH and beta-actin in quantitative RNA analyses. *Biotechniques* 2002, 32: 776–778, 780, 782
 21. Shi W, Wang NJ, Shih DM, Sun VZ, Wang X, Lusis AJ: Determinants of atherosclerosis susceptibility in the C3H and C57BL/6 mouse model: evidence for involvement of endothelial cells but not blood cells or cholesterol metabolism. *Circ Res* 2000, 86:1078–1084
 22. Kroboth K, Newton IP, Kita K, Dikovskaya D, Zumburn J, Waterman-Storer CM, Nathke IS: Lack of adenomatous polyposis coli protein correlates with a decrease in cell migration and overall changes in microtubule stability. *Mol Biol Cell* 2007, 18:910–918
 23. Matsumoto S, Fumoto K, Okamoto T, Kaibuchi K, Kikuchi A: Binding of APC and dishevelled mediates Wnt5a-regulated focal adhesion dynamics in migrating cells. *EMBO J* 2010, 29:1192–1204
 24. Shimizu T, Suzuki M, Fujimura J, Hisada K, Yoshikazu O, Obinata K, Yamashiro Y: The relationship between the concentration of dextran sodium sulfate and the degree of induced experimental colitis in weanling rats. *J Pediatr Gastroenterol Nutr* 2003, 37:481–486
 25. Ingber DE: Mechanical signaling and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology. *Circ Res* 2002, 91:877–887
 26. Li D, Xie S, Ren Y, Huo L, Gao J, Cui D, Liu M, Zhou J: Microtubule-associated deacetylase HDAC6 promotes angiogenesis by regulating cell migration in an EB1-dependent manner. *Protein Cell* 2011, 2: 150–160
 27. McGovern DP, Gardet A, Torkvist L, Goyette P, Essers J, Taylor KD, Neale BM, Ong RT, Lagace C, Li C, Green T, Stevens CR, Beauchamp C, Fleshner PR, Carlson M, D'Amato M, Halfvarson J, Hibberd ML, Lordal M, Padyukov L, Andriulli A, Colombo E, Latiano A, Palmieri O, Bernard EJ, Deslandres C, Hommes DW, de Jong DJ, Stokkers PC, Weersma RK, Sharma Y, Silverberg MS, Cho JH, Wu J, Roeder K, Brant SR, Schumm LP, Duerr RH, Dubinsky MC, Glazer NL, Haritunians T, Ippoliti A, Melmed GY, Siscovick DS, Vasiliauskas EA, Targan SR, Annesse V, Wijmenga C, Pettersson S, Rotter JJ, Xavier RJ, Daly MJ, Rioux JD, Seielstad M: Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet* 2010, 42:332–337
 28. Akiyama T, Kawasaki Y: Wnt signalling and the actin cytoskeleton. *Oncogene* 2006, 25:7538–7544
 29. Senda T, Iizuka-Kogo A, Onouchi T, Shimomura A: Adenomatous polyposis coli (APC) plays multiple roles in the intestinal and colorectal epithelia. *Med Mol Morphol* 2007, 40:68–81
 30. Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh S, Kudo I: Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem* 2000, 275:32783–32792
 31. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM: Suppression of intestinal polyposis in Apc^{Δ716} knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996, 87:803–809
 32. Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, Taketo MM: Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc^{Δ716} knockout mice. *Nat Med* 2001, 7:1048–1051
 33. Drake CJ, Hungerford JE, Little CD: Morphogenesis of the first blood vessels. *Ann N Y Acad Sci* 1998, 857:155–179
 34. Davis GE, Stratman AN, Sacharidou A, Koh W: Molecular basis for endothelial lumen formation and tubulogenesis during vasculogenesis and angiogenic sprouting. *Int Rev Cell Mol Biol* 2011, 288: 101–165
 35. Yamamoto Y, Irie K, Asada M, Mino A, Mandai K, Takai Y: Direct binding of the human homologue of the Drosophila disc large tumor suppressor gene to seven-pass transmembrane proteins, tumor endothelial marker 5 (TEM5), and a novel TEM5-like protein. *Oncogene* 2004, 23:3889–3897
 36. Koutroubakis IE, Tsiolakidou G, Karmiris K, Kouroumalis EA: Role of angiogenesis in inflammatory bowel disease. *Inflamm Bowel Dis* 2006, 12:515–523
 37. Papa A, Scaldaferrri F, Danese S, Guglielmo S, Roberto I, Bonizzi M, Mocchi G, Felice C, Ricci C, Andrisani G, Fedeli G, Gasbarrini G, Gasbarrini A: Vascular involvement in inflammatory bowel disease: pathogenesis and clinical aspects. *Dig Dis* 2008, 26:149–155

Transcription factors c-Myc and CDX2 mediate E-selectin ligand expression in colon cancer cells undergoing EGF/bFGF-induced epithelial–mesenchymal transition

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Sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a) glycans are expressed on highly metastatic colon cancer cells. They promote extravasation of cancer cells and tumor angiogenesis via interacting with E-selectin on endothelial cells. Recently, epithelial–mesenchymal transition (EMT) has been noted as a critical phenotypic alteration in metastatic cancer cells. To address the association between sLe^{x/a} expression and EMT, we assessed whether sLe^{x/a} are highly expressed on colon cancer cells undergoing EMT. Treatment of HT29 and DLD-1 cells with EGF and/or basic FGF (bFGF) induced EMT and significantly increased sLe^{x/a} expression resulting in enhanced E-selectin binding activity. The transcript levels of the glycosyltransferase genes *ST3GAL1/3/4* and *FUT3* were significantly elevated and that of *FUT2* was significantly suppressed by the treatment. We provide evidence that *ST3GAL1/3/4* and *FUT3* are transcriptionally up-regulated by c-Myc with probable involvement of Ser62 phosphorylation, and that *FUT2* is transcriptionally down-regulated through the attenuation of CDX2. The contribution of c-Myc and CDX2 to the sLe^{x/a} induction was proved to be significant by knockdown or forced expression experiments. Interestingly, the cells undergoing EMT exhibited significantly increased VEGF secretion, which can promote tumor angiogenesis in cooperation with sLe^{x/a}. Finally, immunohistological study indicated high E-selectin ligand expression on cancer cells undergoing EMT in vivo, supporting their coexistence observed in vitro. These results suggest a significant link between sLe^{x/a} expression and EMT in colon cancer cells and a pivotal role of c-Myc and CDX2 in regulating sLe^{x/a} expression during EMT.

Colon cancer is one of the most prevalent cancers worldwide, with more than 1,200,000 new cases and over 600,000 deaths estimated to have occurred in 2008 (1). Although early detection, increased awareness, and developments in treatment have increased complete cure rates especially in some advanced countries, distant metastasis is still a critical event that makes colon cancer a lethal disease. Therefore, novel therapeutic approaches to inhibit metastasis are required.

Sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a) are E-selectin ligand glycans expressed on the surface of many types of cancer cells, including colorectal, pancreatic, gastric, breast, prostate, and lung cancer (2, 3). These glycans play crucial roles in hematogenous metastasis through interaction with endothelial cells. The most established role is promoting extravasation of cancer cells: circulating cancer cells in blood flow arrest at distant sites by adhering to endothelial cells, which enables their movement out of the vasculature (2, 3). Importantly, the interaction between sLe^{x/a} and E-selectin exclusively mediates the adhesion of most epithelial cancer cells to endothelial cells, whereas sLe^{x/a}-independent interaction with endothelial ICAM-1 and VCAM-1 mediates the adhesion of nonepithelial malignant cells, such as leukemia and some sarcoma cells, to endothelial cells (4). Another important role of sLe^{x/a} in hematogenous metastasis is tumor angiogenesis (3, 5), which can facilitate intravasation and postextravasational proliferation of cancer cells (6–8). In line with these observations, high sLe^{x/a} expression levels in colon cancer patients are correlated with poor prognosis (2). Therefore, these

glycans are frequently evaluated as tumor markers. Whereas the diagnostic utility of sLe^{x/a} has been well established, therapeutic approaches targeting these glycans are not well developed, partly because molecular mechanisms of their expression have been only partially elucidated (9–11).

Recently, epithelial–mesenchymal transition (EMT) has been noted as a critical event in the early step of cancer metastasis (12, 13). It is also notable that EMT is known to be associated with cancer stem cells (14, 15). EMT is defined as a transitional process from epithelial to mesenchymal phenotype, including fibroblast-like morphology, down-regulation of *E-cadherin* by transcriptional repressors such as *SNAIL1*, *ZEB1*, and *TWIST*, mesenchymal marker expression such as Vimentin, Fibronectin, and N-cadherin, and enhanced cell motility. A variety of EMT inducers have been reported, including TGF- β and receptor tyrosine kinase (RTK) growth factors such as hepatocyte growth factor (HGF), EGF, and basic FGF (bFGF). Although many studies have focused on TGF- β (16), the TGF- β signaling pathway is frequently inactivated in colon cancer due to loss-of-function mutations in *TGFBR2* and *SMAD* genes (17). Therefore, RTK growth factors are likely to figure more heavily than TGF- β in EMT of colon cancer cells. Several clinical studies have suggested the correlation between RTK signaling and metastasis. EGFR was expressed in ~85% of patients with metastatic colon cancer (18) and its expression level and function in colon cancer cells were correlated with metastatic potential (19, 20). Plasma bFGF levels were significantly higher in patients with metastatic colon cancer than in normal controls, whereas those levels were comparable between patients with nonmetastatic colon cancer and normal controls (21). Sato et al. demonstrated by quantitative RT-PCR that the transcript levels of *FGFR1* in colon cancer tissues were significantly higher in patients with liver metastasis than in those without liver metastasis (22).

Despite the significant roles of sLe^{x/a} and EMT in cancer metastasis, their association remains unknown. To address this issue, we assessed whether sLe^{x/a} is highly expressed on cancer cells undergoing EMT.

Results

Induction of EMT in Colon Cancer Cells by EGF or bFGF. To prepare colon cancer cells undergoing EMT, we treated HT29 and DLD-1 cells with EGF (20 ng/mL) and/or bFGF (10 ng/mL) in serum-deprived medium. Treatment with either EGF or bFGF alone transiently induced a fibroblast-like appearance (Fig. 1A); however, it was very difficult to maintain the cells for further experiments. Treatment with both EGF and bFGF (hereafter EGF/bFGF treatment) permitted better cell survival and induced a fibroblast-

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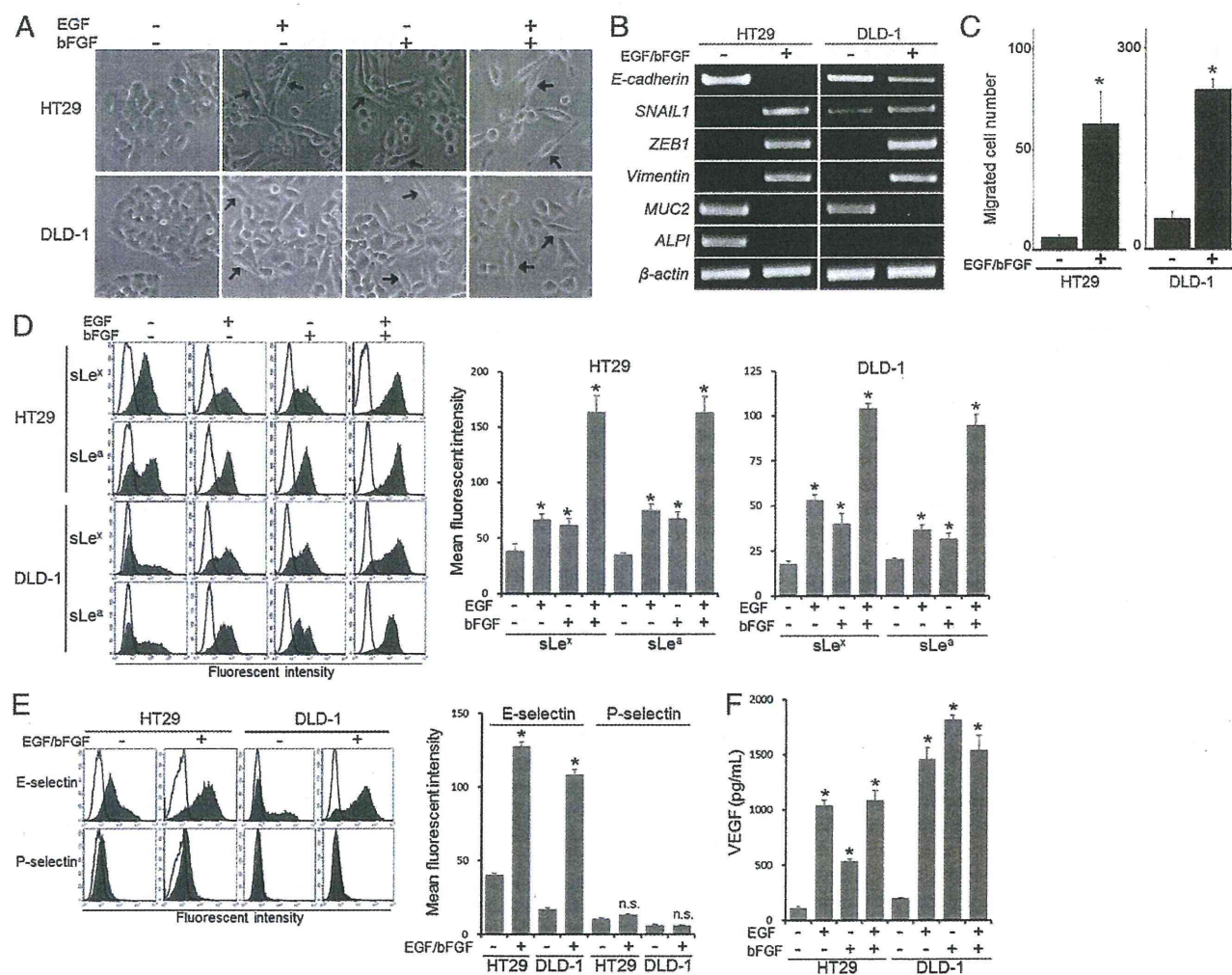


Fig. 1. Induction of EMT and $sLe^{x/a}$ expression in colon cancer cells by EGF or bFGF. (A) HT29 and DLD-1 cells were maintained in culture medium with 10% FBS or in serum-free medium supplemented with EGF (20 ng/mL) and/or bFGF (10 ng/mL). After culture for 7 d, cells were observed under a phase-contrast microscope. (Arrows) Cells exhibiting fibroblastic morphology. (B) Expression of marker genes for EMT and colon-epithelial differentiation was examined by conventional RT-PCR. (C) Cell migration activity was determined with Biocoat Matrigel invasion chambers. (D) Expression levels of $sLe^{x/a}$ were determined by flow cytometry. Bold lines, staining control. (E) Selectin-binding activity was determined by flow cytometry. Bold lines, staining control. (F) Culture supernatant VEGF levels were measured by ELISA. (C–F) Statistic analysis was performed in three independent experiments by *t* test. Error bars, SD; asterisks, $P < 0.05$ (C), $P < 0.01$ (D), $P < 0.000005$ (E), and $P < 0.0001$ (F) compared with the untreated cells; NS, not significant ($P > 0.05$).

like appearance (Fig. 1A). The EGF/bFGF treatment increased the levels of the mesenchymal marker genes *SNAIL1*, *ZEB1*, and *Vimentin*, whereas it reduced the level of *E-cadherin* and the colon-epithelial differentiation marker genes *MUC2* (mucin 2) and *ALPI* (intestinal alkaline phosphatase) (Fig. 1B). Functionally, the treated cells showed significantly enhanced migration activity ($P < 0.05$; Fig. 1C). These results indicated that the EGF/bFGF treatment induced EMT in HT29 and DLD-1 cells.

Induction of $sLe^{x/a}$ Expression and E-Selectin Binding Activity in Colon Cancer Cells by EGF or bFGF. We then evaluated $sLe^{x/a}$ expression levels on the cells undergoing EMT by flow cytometry. The results indicated significantly increased $sLe^{x/a}$ expression on the EGF/bFGF-treated cells as well as on the cells treated with either factor alone compared with the untreated cells ($P < 0.01$; Fig. 1D). To test whether the increased $sLe^{x/a}$ expression could contribute to the interaction with E-selectin, we examined binding activity of the treated cells to recombinant E-selectin. The cells exhibited significantly enhanced E-selectin binding activity ($P < 0.000005$), whereas no significant binding activity was detected for P-selectin ($P > 0.05$;

Fig. 1E), which selectively binds to sLe^x determinants carried on P-selectin glycoprotein ligand 1 (PSGL-1), generally expressed on leukocytes (23). Furthermore, the E-selectin binding activity was significantly inhibited by anti- sLe^x antibody, anti- sLe^a antibody, or EDTA ($P < 0.000005$; Fig. S1), indicating that recombinant E-selectin did bind to the cells through the interaction with $sLe^{x/a}$.

We previously demonstrated that $sLe^{x/a}$ expressed on cancer cells promote tumor angiogenesis through interacting with E-selectin on endothelial cells (5). Because E-selectin is known to be induced by VEGF (24), we examined whether VEGF was secreted into the supernatant of the cells treated with EGF and/or bFGF. Results of ELISA indicated that the VEGF level was significantly increased by the treatment ($P < 0.0001$; Fig. 1F). These results suggest that EGF and/or bFGF can strongly promote angiogenesis synergistically by inducing $sLe^{x/a}$ and E-selectin expression on colon cancer cells and endothelial cells, respectively.

Altered Expression of *ST3GAL1/3/4*, *FUT3*, and *FUT2* Induced by EGF or bFGF. To address the molecular mechanism underlying the EMT-associated $sLe^{x/a}$ expression, we focused on the glycosyltrans-

ferase genes. Sialyltransferases and fucosyltransferases are essential enzymes for the synthesis of sialic acid and fucose residues of sLe^{x/a}, respectively. Screening of the genes involved in the sLe^{x/a} synthesis by conventional RT-PCR using primers listed in Table S1 revealed that the levels of *ST3GAL1/3/4* and *FUT3/6* were increased, whereas that of *FUT2* was decreased by the EGF/bFGF treatment of HT29 and DLD-1 cells (Fig. 2A). Quantitation of the expression levels of these genes by real-time RT-PCR using the assays listed in Table S2 indicated that the EGF/bFGF treatment induced significant increases in the *ST3GAL1/3/4* and *FUT3* levels ($P < 0.005$; Fig. 2B) and a significant decrease in the *FUT2* level ($P < 0.00005$; Fig. 2C). In addition, these alterations were also induced by treatment with EGF or bFGF alone (Fig. 2B and C). However, the *FUT6* level was not significantly changed by any of the treatment ($P > 0.05$; Fig. 2B). Therefore, we focused on *ST3GAL1/3/4*, *FUT3*, and *FUT2* for further experiments. *ST3GAL1/3/4* catalyze the addition of *N*-acetylneuraminic acid (NeuAc) to the nonreducing terminal galactose (Gal) residue of glycans, and *FUT3* catalyzes addition of fucose (Fuc) to the *N*-acetylglucosamine (GlcNAc) residue (Fig. 2D). Therefore, up-regulation of *ST3GAL1/3/4* and *FUT3* results in increased sLe^{x/a} expression. In contrast, *FUT2* catalyzes addition of Fuc to the nonreducing terminal Gal, competing with NeuAc addition by sialyltransferases (Fig. 2D). Down-regulation of *FUT2* thus contributes to increased sLe^{x/a} expression. As expected, quantitative analysis by flow cytometry indicated that EGF/bFGF treatment induced significantly higher increase in the levels of sLe^x and sLe^a compared with those of Le^y and Le^b, respectively ($P < 0.01$; Fig. S2).

Involvement of c-Myc in the Induction of *ST3GAL1/3/4* and *FUT3* Expression by EGF/bFGF. To explore the mechanism of the EGF/

bFGF-induced alteration in the glycogene transcription, we next searched for potential transcription factor binding sites in the 5'-regulatory regions of the glycogenes identified above and noticed potential c-Myc binding sites in the promoters of *ST3GAL1/3/4* and *FUT3* (Fig. S3 A–D). ChIP assays using primers listed in Table S3 indicated increased recruitment of c-Myc to their promoters in the EGF/bFGF-treated cells (Fig. 3A). To determine the role of c-Myc in the sLe^{x/a} induction, we performed c-Myc knockdown experiments. Namely, we introduced a c-Myc shRNA-expressing vector into HT29 and DLD-1 cells (Fig. 3B) and treated the cells with EGF/bFGF. Knockdown of c-Myc significantly inhibited the maximal induction of *ST3GAL1/3/4* and *FUT3* expression ($P < 0.05$; Fig. 3C). Consequently, the maximal sLe^{x/a} induction was also inhibited ($P < 0.0005$; Fig. 3D). These results suggested a pivotal role of c-Myc in the sLe^{x/a} induction through the transcriptional regulation of *ST3GAL1/3/4* and *FUT3* under the EGF/bFGF treatment. To gain insights into the molecular mechanism by which the EGF/bFGF treatment induced the glycogenes through c-Myc, we performed Western blot analysis. Unexpectedly, the level of total c-Myc was reduced by the treatment in HT29 cells (Fig. 3E) likely caused by a decrease in the transcript level (Fig. 3F). However, the level of phospho-c-Myc^{Ser62/Thr58} was strongly enhanced by the treatment both in HT29 and DLD-1 cells (Fig. 3E). It is known that a priming phosphorylation of c-Myc at Ser62 can be followed by phosphorylation at Thr58 by GSK3 β (25). Because Western blotting revealed a decrease in the level of total GSK3 β and an increase in that of phospho-GSK3 β ^{Ser9}, the inactivated form of GSK3 β (Fig. S4), the increase in the phospho-c-Myc^{Ser62/Thr58} level most likely reflects the hyperphosphorylation of the Ser62 site, which has been implicated in the enhanced recruitment of c-Myc to the promoter of its target

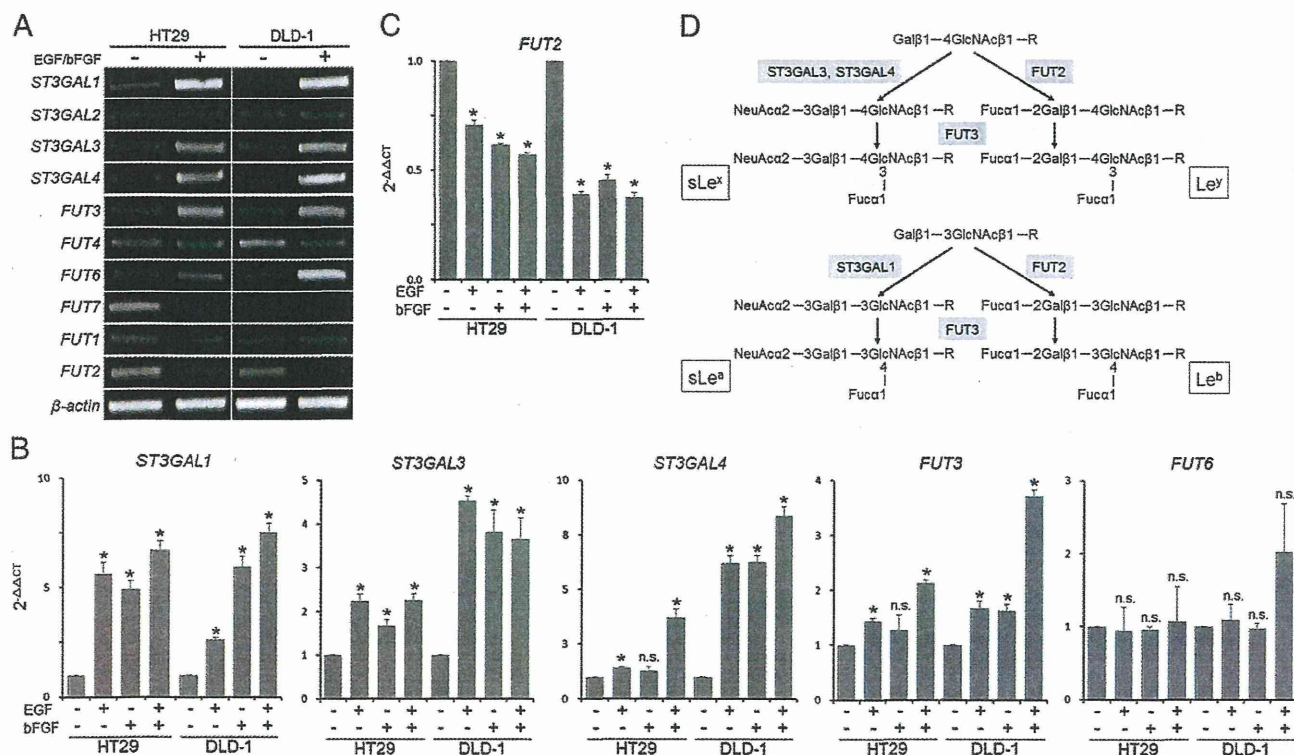


Fig. 2. Altered glycogene expression induced by EGF or bFGF. (A) Expression of the sialyltransferase and fucosyltransferase genes involved in the sLe^{x/a} synthesis was screened by conventional RT-PCR. (B and C) Expression levels of *ST3GAL1/3/4*, *FUT3/6* (B), and *FUT2* (C) were determined by quantitative RT-PCR. The mean $2^{-\Delta\Delta CT}$ values \pm SD from three independent experiments are shown. Statistical analysis was performed by *t* test. Asterisks, $P < 0.005$ (B) and $P < 0.00005$ (C) compared with the untreated cells; NS, not significant ($P > 0.05$). (D) Scheme of the sLe^x, Le^y, sLe^a, and Le^b synthetic pathways showing the roles of *ST3GAL1/3/4*, *FUT3*, and *FUT2*. Note that *FUT2* competes with *ST3GAL3/4* for sLe^x synthesis and with *ST3GAL1* for sLe^a synthesis, respectively.

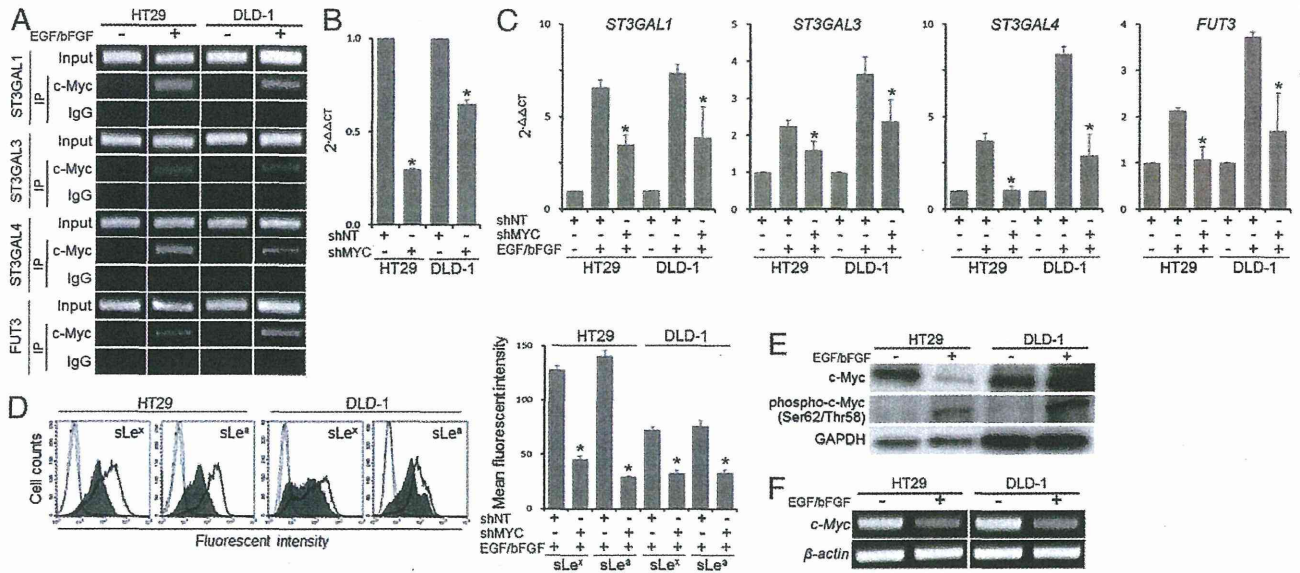


Fig. 3. Involvement of c-Myc in the transcriptional regulation of *ST3Gal1/3/4* and *FUT3*. (A) ChIP assays were performed to examine the binding of c-Myc to the 5'-regulatory regions of *ST3Gal1/3/4* and *FUT3*. (B) Effect of c-Myc shRNA (shMYC) on the transcript level of c-Myc was evaluated by quantitative RT-PCR. (C) Effects of shMYC on the expression levels of *ST3Gal1/3/4* and *FUT3* were examined by quantitative RT-PCR. (D) Expression levels of sLe^{x/a} were examined by flow cytometry. Dotted lines, staining control; bold lines, nontarget shRNA (shNT)-introduced cells; filled histogram, shMYC-introduced cells. (E) Levels of c-Myc and phospho-c-Myc^{Ser62/Thr58} were determined by Western blotting. (F) Expression of c-Myc was examined by conventional RT-PCR. (B–D) Statistic analysis was performed in three independent experiments by *t* test. Error bars, SD; asterisks, $P < 0.00001$ (B), $P < 0.05$ (C), and $P < 0.00005$ (D) compared with the shNT-transfected cells (B) or to the shNT-introduced cells treated with EGF/bFGF (C and D).

genes as well as in the enhanced transcriptional activity of c-Myc (26, 27).

Involvement of CDX2 in the EGF/bFGF-Induced Transcriptional Suppression of *FUT2*. We next examined the mechanism of the

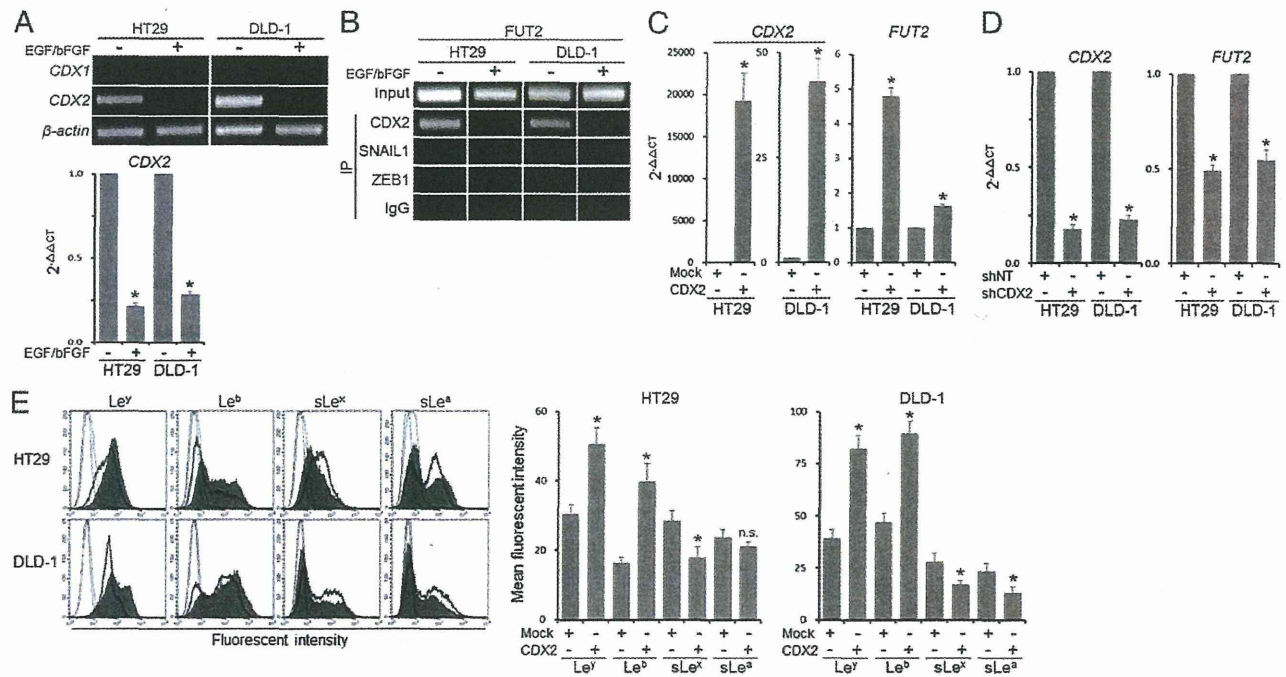


Fig. 4. Involvement of CDX2 in the transcriptional regulation of *FUT2*. (A) Expression of *CDX1* and *CDX2* was examined by conventional RT-PCR. The transcript level of *CDX2* was determined by quantitative RT-PCR. (B) ChIP assays were performed to examine the binding of CDX2, SNAIL1, and ZEB1 to the 5'-regulatory region of *FUT2*. (C and D) Effect of CDX2 forced expression (C) or CDX2 shRNA (shCDX2; D) on the expression levels of *CDX2* and *FUT2* were determined by quantitative RT-PCR. (E) Expression levels of Le^{y/b} and sLe^{x/a} were examined by flow cytometry. Dotted lines, staining control; bold lines, mock vector-introduced cells; filled histogram, CDX2 expression vector-introduced cells. (A and C–E) Statistic analysis was performed in three independent experiments by *t* test. Error bars, SD; asterisks, $P < 0.000001$ (A), $P < 0.00005$ (C and D), and $P < 0.05$ (E) compared with the untreated cells (A), to the mock vector-transfected cells (C and E) or to the nontarget shRNA (shNT)-transfected cells (D); NS, not significant ($P > 0.05$).

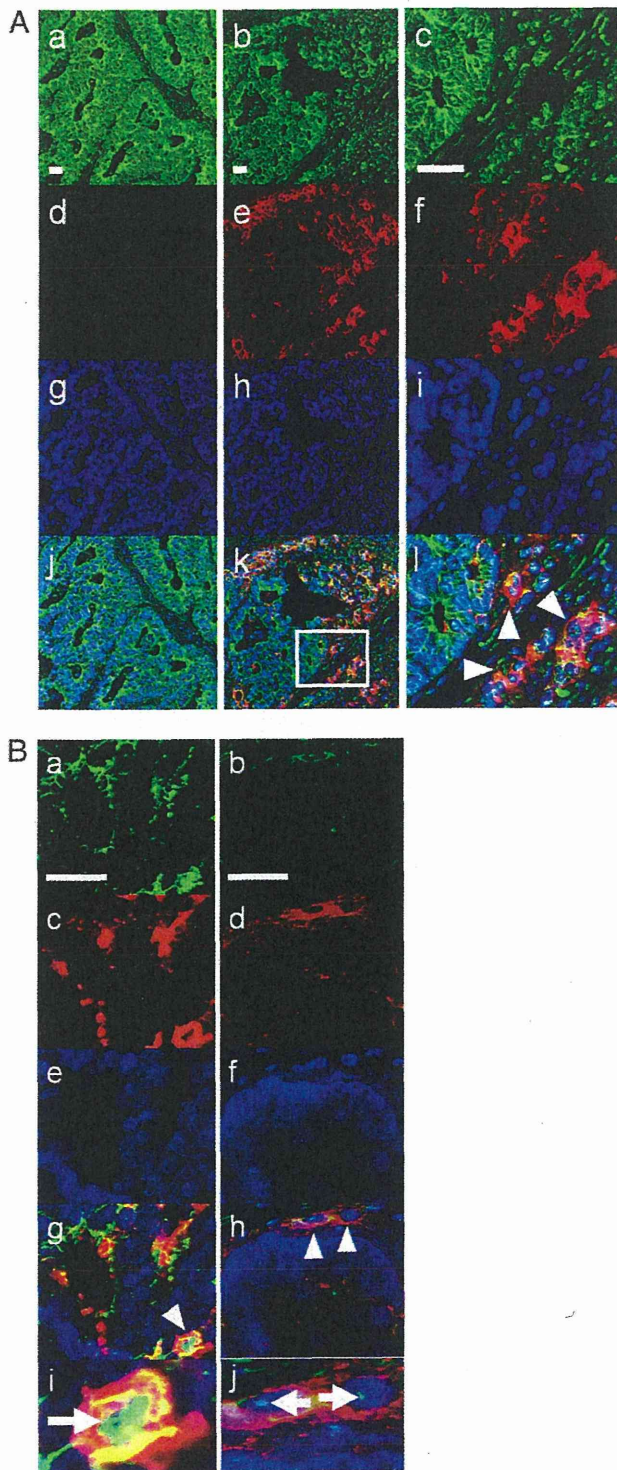


Fig. 5. E-selectin ligand glycan expression on colon cancer cells undergoing EMT in vivo. (A) Expression of E-cadherin (green) and sLe^a (red) was examined on human colon cancer sections by immunohistochemistry. (Left) Region with cancer cells exhibiting high cell-surface E-cadherin expression. (Center) Another region in the same section containing cancer cells without cell-surface E-cadherin expression, a part of which are surrounded by a square (k) and magnified in the Right column. Arrowheads indicate cells with decreased E-cadherin and increased sLe^a expression on the cell surface. (Blue) Hoechst 33342. (B) Expression of SNAIL1 (a, green), ZEB1 (b, green), and sLe^a (c and d, red) was examined on sections from the same patient. (e and f)

EGF/bFGF-induced transcriptional suppression of *FUT2*. We noticed potential binding sites for CDX1 and CDX2, transcription factors known to regulate several colon-specific genes (28–31), in the 5'-regulatory region of *FUT2* (Fig. S3E). Both HT29 and DLD-1 cells showed good levels of *CDX2* expression, which were significantly reduced by EGF/bFGF treatment ($P < 0.000001$; Fig. 4A). ChIP assays revealed that binding of CDX2 to the *FUT2* promoter was abolished by the treatment (Fig. 4B). On the other hand, SNAIL1 and ZEB1, EMT-related transcriptional repressors, were not recruited to the promoter (Fig. 4B). To determine the role of CDX2 in the transcriptional regulation of *FUT2*, we introduced a CDX2 expression vector into HT29 and DLD-1 cells. The cells exhibited significantly elevated *FUT2* expression compared with the mock vector-transfected cells ($P < 0.0005$; Fig. 4C). In contrast, HT29 and DLD-1 cells introduced with CDX2 shRNA showed significantly reduced level of *FUT2* ($P < 0.0005$; Fig. 4D). Furthermore, forced expression of *CDX2* elevated Le^{y/b} expression and suppressed sLe^{x/a} expression in HT29 and DLD-1 cells (Fig. 4E). These results suggest that the EGF/bFGF-induced down-regulation of *CDX2* contributes to the sLe^{x/a} induction via suppression of *FUT2* transcription.

E-Selectin Ligand Glycan Expression on Colon Cancer Cells Undergoing EMT in Vivo. Finally, we examined association between E-selectin ligand glycan expression and EMT in clinical samples by immunohistochemical analysis. We focused on sLe^a in this experiment, because this glycan is preferentially expressed on cancer cells, whereas sLe^x is broadly expressed on various normal cells including leukocytes and might complicate the results. We performed double staining with antibodies against sLe^a and E-cadherin on sections from five colorectal cancer patients. In one section from a 70-y-old male patient with colon cancer, we identified a small area of cancer cells that lacked cell-surface E-cadherin expression at the invasion front (Fig. 5A). Most interestingly, these cancer cells exhibited high sLe^a expression, whereas cancer cells with cell-surface E-cadherin exhibited no sLe^a expression (Fig. 5A). Furthermore, double staining with antibodies against SNAIL1 and sLe^a identified a subset of cancer cells that coexpressed nuclear SNAIL1 and a high level of sLe^a (Fig. 5B). Similar results were obtained by double staining with antibodies against ZEB1 and sLe^a (Fig. 5B). These results supported the coincidence of E-selectin ligand expression and EMT observed in vitro.

Discussion

The major findings of this study are as follows: (i) sLe^{x/a} expression is strongly induced during EMT of colon cancer cells triggered by EGF or bFGF, and (ii) c-Myc and CDX2 play key roles in the sLe^{x/a} induction by EGF or bFGF.

Our present results demonstrate that c-Myc contributes to sLe^{x/a} expression by transcriptional induction of *ST3GAL1/3/4* and *FUT3* in the EGF/bFGF-treated cells. Although the detailed mechanism of this glycoenzyme induction by c-Myc remains unclear, we speculate the possible involvement of Ser62 phosphorylation of c-Myc as described above (Fig. 3E). Ser62 of c-Myc is known to be phosphorylated by ERK or cyclin-dependent kinase (CDK) 2 (25–27). The kinase that contributed to this phosphorylation in the EGF/bFGF-treated cells remains to be identified.

In this study, we demonstrated that the transcription of *CDX2* was down-regulated by the EGF/bFGF treatment, which resulted in a decrease in the transcript level of *FUT2*. Although the mechanism underlying the down-regulation of *CDX2* by the treatment remains unknown, SNAIL1 may be involved because transcription of *CDX2* is known to be repressed by SNAIL1 (32), and *SNAIL1* expression was increased by EGF/bFGF (Fig. 1B). Although several lines of evidence indicate that *CDX2* is a tumor suppressor (33), the association between *CDX2* and metastasis

Hoechst 33342. Cells with arrowheads (g and h) are magnified (i and j), with arrows showing nuclear SNAIL1 and ZEB1, respectively. (Scale bars, 50 μm.)

has been unclear. Clinically, Baba et al. reported that the loss of CDX2 expression in colon cancer tissues was significantly correlated with stage IV disease (34). Our present findings may explain at least a part of the mechanisms by which the loss of CDX2 contributes to metastasis.

We previously reported that hypoxia induced sLe^{x/a} expression in colon cancer cells (9). In that report, we documented that the transcription of *ST3GALI*, *FUT7*, and *UGT1* (UDP-galactose transporter 1), which are all involved in the E-selectin ligand glycan synthesis, was elevated under a hypoxic condition. Hypoxia-inducible factor-1 α (HIF-1 α) was involved in the induction of these glycozymes. The present study provides additional information on the transcriptional regulation of the sLe^{x/a} synthesis-related glycozymes.

Recently, Guan et al. reported a significant association between glycans and EMT, demonstrating that the expression levels of GM2 and Gg4 glycosphingolipids were significantly decreased during TGF- β -induced EMT and that the glucosylceramide synthase inhibitor EtDO-P4 induced EMT (35). From their subsequent observations demonstrating that exogenous addition of Gg4 abrogated the EMT process and that Gg4 was closely associated with E-cadherin and β -catenin, they proposed that Gg4 may be important in maintaining epithelial cell membrane organization (36). Together with these reports, our present study demonstrates a drastic alteration in the glycan expression during the EMT process. It remains an interesting issue whether the alteration in sLe^{x/a} expression further promotes the EMT process as the alteration in the Gg4 expression did.

We demonstrated that sLe^a was preferentially expressed on the cancer cells with low expression of membranous E-cadherin, nuclear SNAIL1, and nuclear ZEB1 in a clinical sample of colon cancer. These results are consistent with the coincidence of sLe^{x/a} expression and EMT observed in vitro and suggest that these glycans may serve as a good marker of EMT in cancer patients. Our results indicate that RTK signaling activation confers both EMT and sLe^{x/a} expression on cancer cells. As RTK signaling pathways provide effective therapeutic targets, these glycans may serve as surrogate markers for evaluating therapeutic effects of such modalities.

Materials and Methods

Additional information can be found in *SI Materials and Methods*.

Human colon cancer cell lines, HT29 and DLD-1, were maintained in DMEM and RPMI1640 medium (Invitrogen), respectively, supplemented with 10% (vol/vol) FBS. For treatment with EGF and/or bFGF, recombinant human EGF (Sigma; 20 ng/mL) and/or recombinant human bFGF (Sigma; 10 ng/mL) were added to the serum-free medium with recombinant human insulin (Sigma; 25 μ g/mL), human holo-transferrin (Sigma; 100 μ g/mL), putrescine dihydrochloride (Sigma; 10 μ g/mL), and sodium selenite (Sigma; 5 ng/mL).

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- Jemal A, et al. (2011) Global cancer statistics. *CA Cancer J Clin* 61:69–90.
- Kannagi R (1997) Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. *Glycoconj J* 14:577–584.
- Kannagi R, Izawa M, Koike T, Miyazaki K, Kimura N (2004) Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. *Cancer Sci* 95:377–384.
- Takada A, et al. (1993) Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. *Cancer Res* 53:354–361.
- Tei K, et al. (2002) Roles of cell adhesion molecules in tumor angiogenesis induced by cotransplantation of cancer and endothelial cells to nude rats. *Cancer Res* 62:6289–6296.
- Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature* 407:249–257.
- Tien YW, et al. (2001) Tumor angiogenesis and its possible role in intravasation of colorectal epithelial cells. *Clin Cancer Res* 7:1627–1632.
- Zetter BR (1998) Angiogenesis and tumor metastasis. *Annu Rev Med* 49:407–424.
- Koike T, et al. (2004) Hypoxia induces adhesion molecules on cancer cells: A missing link between Warburg effect and induction of selectin-ligand carbohydrates. *Proc Natl Acad Sci USA* 101:8132–8137.
- Miyazaki K, et al. (2004) Loss of disialyl Lewis(a), the ligand for lymphocyte inhibitory receptor sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7) associated with increased sialyl Lewis(a) expression on human colon cancers. *Cancer Res* 64:4498–4505.
- Yusa A, Miyazaki K, Kimura N, Izawa M, Kannagi R (2010) Epigenetic silencing of the sulfate transporter gene *DTDST* induces sialyl Lewisx expression and accelerates proliferation of colon cancer cells. *Cancer Res* 70:4064–4073.
- Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. *J Clin Invest* 119:1420–1428.
- Thiery JP, Acloque H, Huang RY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. *Cell* 139:871–890.
- Mani SA, et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133:704–715.
- Radisky DC, LaBarge MA (2008) Epithelial-mesenchymal transition and the stem cell phenotype. *Cell Stem Cell* 2:511–512.
- Ikushima H, Miyazono K (2010) TGF β signaling: A complex web in cancer progression. *Nat Rev Cancer* 10:415–424.
- Xu Y, Pasche B (2007) TGF- β signaling alterations and susceptibility to colorectal cancer. *Hum Mol Genet* 16(Spec No 1):R14–R20.
- Normanno N, et al. (2009) Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat Rev Clin Oncol* 6:519–527.
- Radinsky R, et al. (1995) Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin Cancer Res* 1:19–31.
- Goldstein NS, Armin M (2001) Epidermal growth factor receptor immunohistochemical reactivity in patients with American Joint Committee on Cancer Stage IV colon adenocarcinoma: Implications for a standardized scoring system. *Cancer* 92:1331–1334.
- George ML, Tutton MG, Abulafi AM, Eccles SA, Swift RI (2002) Plasma basic fibroblast growth factor levels in colorectal cancer: A clinically useful assay? *Clin Exp Metastasis* 19:735–738.
- Sato T, et al. (2009) Overexpression of the fibroblast growth factor receptor-1 gene correlates with liver metastasis in colorectal cancer. *Oncol Rep* 21:211–216.
- McEver RP, Cummings RD (1997) Perspectives series: Cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest* 100:485–491.
- Kim I, et al. (2001) Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem* 276:7614–7620.
- Sears R, et al. (2000) Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev* 14:2501–2514.
- Benassi B, et al. (2006) c-Myc phosphorylation is required for cellular response to oxidative stress. *Mol Cell* 21:509–519.
- Hydbring P, et al. (2010) Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation. *Proc Natl Acad Sci USA* 107:58–63.
- Dang DT, Mahatan CS, Dang LH, Agboola IA, Yang VW (2001) Expression of the gut-enriched Krüppel-like factor (Krüppel-like factor 4) gene in the human colon cancer cell line RKO is dependent on CDX2. *Oncogene* 20:4884–4890.
- Yamamoto H, Bai YQ, Yuasa Y (2003) Homeodomain protein CDX2 regulates goblet-specific MUC2 gene expression. *Biochem Biophys Res Commun* 300:813–818.
- Chan CW, et al. (2009) Gastrointestinal differentiation marker Cytokeratin 20 is regulated by homeobox gene CDX1. *Proc Natl Acad Sci USA* 106:1936–1941.
- Kakizaki F, et al. (2010) CDX transcription factors positively regulate expression of solute carrier family 5, member 8 in the colonic epithelium. *Gastroenterology* 138:627–635.
- Gross I, et al. (2008) The intestine-specific homeobox gene *Cdx2* decreases mobility and antagonizes dissemination of colon cancer cells. *Oncogene* 27:107–115.
- Guo RJ, Suh ER, Lynch JP (2004) The role of Cdx proteins in intestinal development and cancer. *Cancer Biol Ther* 3:593–601.
- Baba Y, et al. (2009) Relationship of CDX2 loss with molecular features and prognosis in colorectal cancer. *Clin Cancer Res* 15:4665–4673.
- Guan F, Handa K, Hakomori SI (2009) Specific glycosphingolipids mediate epithelial-to-mesenchymal transition of human and mouse epithelial cell lines. *Proc Natl Acad Sci USA* 106:7461–7466.
- Guan F, Schaffer L, Handa K, Hakomori SI (2010) Functional role of gangliosylceramide in epithelial-to-mesenchymal transition process induced by hypoxia and by TGF- β . *FASEB J* 24(12):4889–4903.

Supporting Information

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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 β ^{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 μ g/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 μ g/mL followed by incubation with FITC-conjugated secondary antibody at 2 μ g/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 μ g/mL before incubation with recombinant selectins. Propidium iodide was used at 1 μ g/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 μ g) 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 μ L) were incubated with antibodies (1 μ g) for 8 h at 4 $^{\circ}$ 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 μ g/mL of polybrene (Sigma). After selection with 5 μ g/mL of puromycin (Sigma), total RNA was extracted and analyzed by quantitative RT-PCR. For c-Myc knockdown, TRCN0000010389 having the sequence CCGGAACATATGACCTCGACTACGACTCGAGTCGTAGT-CGAGGTCATAGTTCTTTTTG gave the best results among the five tested shRNA vectors. For CDX2 knockdown, TRCN0000013687 having the sequence CCGGAGCCCTTGA-GTCCGGTGTCTTCTCGAGAAGACACCGGACTCAAGG-GCTTTTTT 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 $^{\circ}$ 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- μ m 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 X. *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.