

Expression of Human $\alpha(1,3)$ Fucosyltransferase Antisense Sequences Inhibits Selectin-mediated Adhesion and Liver Metastasis of Colon Carcinoma Cells¹

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ABSTRACT

The initial steps of leukocyte and tumor cell adhesion involve selectin receptor/ligand interactions. The selectin ligand components sialyl Lewis x and sialyl Lewis a are oncodevelopmental antigens involved in progression of adenocarcinoma. Interrupting biosynthesis of these surface glycans by inhibition of $\alpha(1,3)$ fucosyltransferase (FUT) gene expression is an attractive goal for functional and therapeutic studies. We report here the inhibition of E-selectin-mediated adenocarcinoma cell adhesion by stable transfection of antisense sequences directed at the human Lewis $\alpha(1,3/1,4)$ fucosyltransferase gene, *FUT3*. The metastatic parental cell line, HT-29LMM, expressed high levels of sialyl Lewis x, sialyl Lewis a, $\alpha(1,3/1,4)$ fucosyltransferase activity, and *FUT3* transcript, but antisense transfectant cell lines did not. When injected into the spleens of nude mice, the stable antisense clones were unable to colonize the liver. These results provide target validation for inhibition of carcinoma metastasis with antisense FUT sequences and confirm the primacy of $\alpha(1,3)$ fucosyltransferases in the synthesis of selectin ligands.

INTRODUCTION

Hematogenous metastases adversely affect the prognosis of colon carcinoma, and liver is the primary site of metastasis. During metastasis, tumor cells separate from the primary site, travel through the bloodstream, marginate and adhere to vascular endothelia, and transmigrate into extravascular site(s), where colonization occurs. Diapedesis of tumor cells from the circulation into secondary sites is believed to occur by a mechanism similar to leukocyte extravasation, in which cells must first contact and “roll” along the endothelial cell layer. Rolling requires interactions between selectin cell adhesion molecules and their ligands (reviewed in Refs. 1 and 2).

Selectin-mediated tumor cell adhesion has been modeled *in vitro*, with colorectal adenocarcinoma cells binding to cytokine-activated vascular endothelium (3, 4). The fucosylated ligand components sLex³ and sLea on the surface of circulating adenocarcinoma cells have been shown to bind endothelial selectins (3–8). The specificity of this interaction is supported by studies which show that anti-sLex (7) and anti-sLea (3) antibodies block adhesion of colon carcinoma

cell lines to HUVEC, with sLea possibly being the more predominant ligand in this setting (8).

Selectin-mediated interactions may also be important in colonization of tumor rests at distant site(s), particularly the liver. The “seed and soil” hypothesis, stating that tumor cells will thrive only in conditions favorable to their growth, has been shown to apply to hepatic metastasis of colon carcinoma (9, 10). Several recent experimental models support this hypothesis in the context of selectins and their ligands. Tail vein injections into transgenic mice constitutively expressing E-selectin demonstrate the primacy of sLea interactions to hepatic localization. In this model, B16F10 melanoma cells, which normally migrate to the lungs, were redirected to the liver when cell surface sLea expression was introduced (11). Although constitutive hepatic expression of E-selectin is not physiological, liver inflammation is a frequent occurrence in clinical oncology and forms a suitable microenvironment for carcinoma adherence (12–16).

In another model, KM12-HX colon carcinoma cells, selected for high sLex expression, bound avidly to endothelial cells and colonized mouse liver more efficiently than KM12-LX cells, their unselected counterparts (17). Using the opposite approach, another colon cancer cell line was selected for high liver metastatic capacity (OCUC-LM1) and then found to express more surface sLea than its parental cell line (18). Furthermore, *i.p.* injection of monoclonal anti-sLea blocks carcinoma metastasis to the liver in nude mice (19). After examining several adhesion markers, expression of sLea was found to be the most significant predictor of hepatic metastasis in this model. Thus, in addition to facilitating rolling and subsequent extravasation of carcinoma cells, sLea and sLex may augment metastasis by conferring the ability to flourish in the hepatic microenvironment.

Clinical correlates demonstrate that sLea and sLex expression on adenocarcinoma cells is associated with advanced stage disease and poor patient prognosis (20–24). Greater surface expression of dimeric sLex, an aberrant difucosylated antigen (23), correlates with shorter survival time, presumably related to venous invasiveness and/or metastatic potential (23–25). In another study, metastatic liver specimens were shown to express higher levels of sLex and sLea when compared with their matched primary tumors, with highest expression on the advancing edges of the tumor (26). Although these correlative studies establish a pathophysiological role for fucosylated antigens, regulatory mechanisms that determine glycan expression are not known.

Biosynthesis of sLex and sLea is dependent upon the addition of fucose in $\alpha(1,3)$ and $\alpha(1,4)$ linkage to sialylated precursors by cell type-specific and developmental stage-specific fucosyltransferases (27–32). Enzymatic studies using synthetic carbohydrate acceptors characterized fucosyltransferases as $\alpha(1,3)$ and/or $\alpha(1,4)$ activities present in different tissues (27–32) by assessing their ability to modify type I and/or type II precursors. Several human $\alpha(1,3)$ fucosyltransferase genes have been cloned and characterized to date (33–41). Expression of these genes (*FUT 3–7*) and their enzyme products (Fuc-TIII-VII) is tissue specific (40, 42).

The human fucosyltransferases responsible for $\alpha(1,3)$ activity in

Received 12/8/98; accepted 3/3/99.

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¹ Supported by NIH-National Cancer Institute Grant 5K08-CA01758-05, the Council for Tobacco Research Scholar Award SA059R1 (to B. W. W.), and NIH-National Cancer Institute Grant 1R55-CA755528-01 (to J. C. C.).

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³ The abbreviations used are: sLex, sialyl Lewis x, NeuNAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ [Fuc $\alpha 1 \rightarrow 3$]GlcNAc; sLea, sialyl Lewis a, NeuNAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 3$ [Fuc $\alpha 1 \rightarrow 4$]GlcNAc; Lewis x, Gal $\beta 1 \rightarrow 4$ [Fuc $\alpha 1 \rightarrow 3$]GlcNAc; LacNAc, *N*-acetylactosamine, Gal $\beta 1 \rightarrow 4$ GlcNAc; Lacto-N-biose I, Gal $\beta 1 \rightarrow 3$ GlcNAc; sialyl LacNAc, $\alpha(2,3)$ sialyl-*N*-acetylactosamine, NeuNAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ GlcNAc; $\alpha(1,3)$ fucosyltransferase, GDP-fucose: β -D-*N*-acetylglucosaminide 3- α -L-fucosyltransferase; RT-PCR, reverse transcription-mediated PCR; HUVEC, human umbilical vein endothelial cells; TNF, tumor necrosis factor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; CEA, carcinoembryonic antigen; ODN, oligodeoxynucleotide.

epithelial tissues are encoded by the chromosome 19p genes *FUT3* and *FUT6* (42). *FUT3* [also called the Lewis $\alpha(1,3/1,4)$ fucosyltransferase gene] and *FUT6* [the plasma $\alpha(1,3)$ fucosyltransferase gene] transcripts are present in both normal and transformed tissues. Differentially spliced species of both of these enzymes are expressed in specific patterns in kidney, liver, and colon tissues (42). In addition, Lewis and plasma fucosyltransferase transcripts are prevalent in numerous adenocarcinoma cell lines, with notably high expression of *FUT3* in colon carcinoma (40, 43).

To develop a model to test the function of $\alpha(1,3)$ fucosyltransferases *in vivo*, we sought a metastatic human colon carcinoma cell line that expressed high levels of *FUT3* transcript but lacked significant expression of *FUT4-7*. Mannori *et al.* (44) examined selectin-mediated adhesion of several mucinous and nonmucinous cell lines that express sLex and/or sLea. HT-29, a particularly well-studied line, binds E-selectin but not P- or L-selectin, through nonmucinous ligands carrying these epitopes (44). HT-29 cells express moderately high levels of Fuc-TIII enzyme activity and *FUT3* transcript; there is much less expression of Fuc-TIV-VII (45). A variant cell line, HT-29LMM (23, 46, 47), expresses high surface levels of fucosylated ligands. This cell line was derived from metastatic lesions resulting from intrasplenic injections of HT-29 cells in nude mice (46). In this report, we will show that this variant cell line expresses increased levels of surface sLex and sLea, Fuc-TIII enzyme activity, and *FUT3* transcript.

HT-29LMM provide an attractive target for antisense studies to examine the putative role of *FUT* genes in the metastasis of colon carcinoma cells. Antisense technology has been effective both *in vitro* and *in vivo* for determining the functions of a wide array of genes (48). More recently, antisense methods have been explored as therapeutic strategies, both to down-regulate specific genes (49) and to restore gene function (50). For our investigations, plasmid-based *FUT3* antisense experiments are reported to examine the function of human fucosyltransferases and to provide target validation for potential molecular therapy.

MATERIALS AND METHODS

Cell Culture. HT-29, HT-29LMM, and HUVEC were isolated and propagated as described previously (46, 47, 51).

Antibodies. Mouse IgG1/IgG2 control and anti-CD18 antibodies were obtained through Becton Dickinson (San Jose, CA). Anti-Lewis x antibody (anti-SSEA-1, mouse IgM) was obtained through the Developmental Studies Hybridoma Bank (Iowa City, IA). A monoclonal anti-VIM-2 antibody (IgM) was purchased from Immunotech (Westbrook, ME). Monoclonal anti-sLea antibody (CSLEA-1, mouse IgG3) was a gift from Dr. P. Terasaki (University of California at Los Angeles, Los Angeles, CA). Sources for the anti-sLex IgM monoclonal antibodies CSLEX1 and KM93 were Becton Dickinson and Kamiya Biomedical (Seattle, WA), respectively. For blocking studies, anti-E-selectin antibody (CD62E, 1.2B, mouse IgG1) was obtained from Serotec (Raleigh, NC). Fluorescein-conjugated goat anti-mouse IgM and IgG antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

Flow Cytometry Analysis. HT-29, HT-29LMM, and cloned cell lines were prepared and stained with monoclonal anti-carbohydrate antibodies at saturating concentrations as described (37, 38). Anti-SSEA-1 was used at a dilution of 1:1000 and anti-VIM-2 was used at 1:50. Anti-sLex (CSLEX-1) was used at 10 μ g/ml. Anti-sLea (CSLEA-1) was used at 1:500. Cells were then stained with FITC-conjugated goat anti-mouse IgM or anti-mouse IgG. Mouse IgG1/IgG2 and anti-CD18 antibodies (negative controls throughout) were used according to the manufacturer's instructions. Cells were analyzed on a Becton Dickinson FACScan as described previously (37, 38).

Fucosyltransferase Assays. Triton X-100 extracts were prepared from HT-29, HT-29LMM, and transfected cells as described (37, 38). Fucosyltransferase assays were performed in a volume of 20 μ l and contained 25 mM sodium cacodylate (pH 6.2), 5 mM ATP, 10 mM L-fucose, 20 mM MnCl₂, 3 μ M GDP-[¹⁴C]fucose (Amersham), and 10 μ g of cell extract protein. Acceptor

substrates (LacNAc, Lacto-N-biose I, or sialyl LacNAc) were added to a final concentration of 20 mM. Control assays with no added acceptor were performed using the same conditions. Reactions were incubated at 37°C for 1 h. Assays were terminated with 580 μ l of 5 mM sodium phosphate, pH 6.8. The terminated assays were centrifuged, and the supernatants were collected. An aliquot of each terminated reaction supernatant was subjected to scintillation counting. Another aliquot was applied to a column containing either Dowex 1X2-400, formate form or a Dowex 1-X8 (PO₄⁻²) column equilibrated as described previously (37, 38). To quantitate incorporation of radioactive fucose into product, the flow-through fraction and 2 ml of a subsequent water elution were collected, pooled, and counted. A portion of this material was assayed by high-performance liquid chromatography as described (37, 38).

Sialyltransferase Assays. Sialyltransferase assays were performed in a volume of 30 μ l and contained 50 mM Tris-maleate (pH 6.7), 10 mM MnCl₂, 0.3 nM CMP-[¹⁴C]NeuNAc (Amersham), and 10 μ g of cell extract protein. Acceptor substrate (LacNAc; Sigma) was added to a final concentration of 0.1 mM. Control assays with no added acceptor were performed using the same conditions. Reactions were incubated at 37°C for 2 h. Assays were terminated by the addition of 30 μ l of ethanol, followed by the addition of 540 μ l of distilled water. The terminated assays were desalted over Dowex AG 1 (AC-) and Dowex AG 50 (H+). Neutral saccharides were eluted with 4 ml of water and charged saccharides with 14 ml of 0.5 M acetic acid (45). An aliquot of each reaction was subjected to scintillation counting. Another aliquot was used for high-performance liquid chromatography confirmation of product structure (37, 45).

RNA Isolation. Total cellular RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. Poly(A)⁺ RNA was prepared using oligo(dT)-cellulose chromatography (42).

Northern Blot Analysis. Poly(A)⁺ RNA (15 μ g, divided into 5 μ g/lane) was denatured and separated with 1.2% formaldehyde agarose gel electrophoresis and transferred to Hybond-N membranes (Amersham). The membrane was cut into separate strips, which were then prehybridized for 4 h at 42°C. Gene-specific probes were constructed as described previously, and hybridization to β -actin message was used as control (41-43). Probes were labeled with [³²P]dCTP by random priming and purified from unincorporated isotope at a specific activity of 1×10^9 cpm/ μ g or higher (42). Hybridization was carried out at 42°C for 16-20 h. The final wash was carried out at 65°C with $0.5 \times$ SSC and 0.2% SDS for 30 min. Autoradiograms were scanned, and the images were sized, grouped, and labeled using Adobe Photoshop 5.0 (Mountain View, CA).

RT-PCR Analysis. First-strand cDNA was prepared using 200 ng of poly(A)⁺ RNA. Synthesis of cDNA was carried out in a 50- μ l reaction volume with 12 units of Moloney murine leukemia virus reverse transcriptase and 125 ng of lower strand cDNA primers (42). PCR amplifications were performed using the lower strand cDNA primers and previously described *FUT3-FUT7* gene-specific upper strand primers and amplification profiles (40, 42). To control for genomic DNA contamination, parallel amplifications of all samples with no reverse transcriptase were performed (data not shown). RT-PCR of human cytoplasmic β -actin was performed with previously reported primers to verify the quality/quantity of RNA and further exclude the possibility of genomic contamination (42, 43).

Construction of *FUT3* Antisense, Sense, and Control Plasmids for Stable Transfection of HT-29LMM. The plasmid pcDNA3 (InVitrogen, Carlsbad, CA) was chosen for cloning and selection of *FUT3* antisense, sense, and control constructs in HT-29LMM due to preliminary data showing high level expression of CAT in stable transfection experiments of parental HT-29 cells (data not shown). The CAT coding region (Pharmacia, Piscataway, NJ) was cloned in the sense orientation into the *Hind*III site of pcDNA-3 and served as control throughout expression studies. The plasmid pcDNA3-FUT3S was created by digestion of pFUT3 (52) with *Xho*I and *Xba*I and directional cloning into pcDNA3. Likewise, pFUT3 was also digested with *Xho*I and *Hind*III, and the resulting fragment was cloned in antisense orientation to the CMV promoter in pcDNA3, yielding the expression vector pcDNA3-FUT3AS. Finally, a truncated coding region antisense construct was created by amplification of *FUT3* bp 733-1004 (33) with the *Hind*III-containing primers *aagct-CTGGCCTTCGAGAACTCCTTGACC* (upper strand, *Hind*III in lowercase italic) and *aagcttAGTGCCAGCTGAAGGAGCGAGGCC* (lower strand). The resulting 272-bp fragment, which corresponds to the putative catalytic

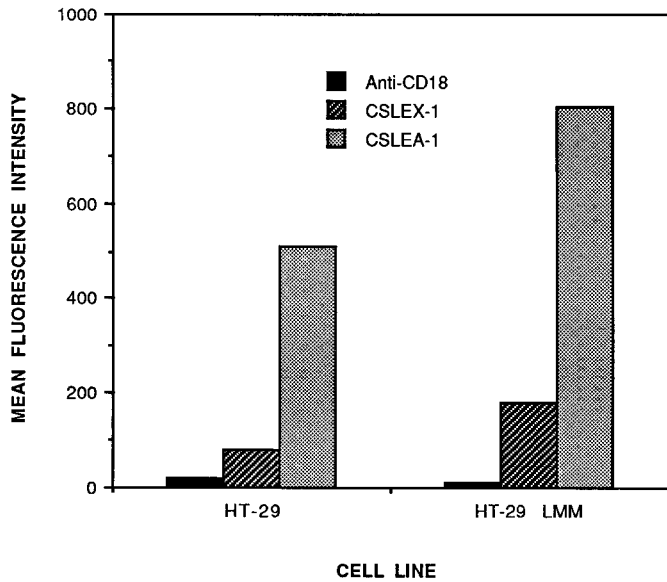


Fig. 1. Flow cytometry analysis of surface antigens on HT-29 and HT-29LMM cells. Cells were harvested, stained with monoclonal antibodies, and subjected to flow cytometric analysis as described in "Materials and Methods." Anti-CD18 results are presented as negative controls. Data presented here are representative of the mean fluorescence intensity values from three separate experiments.

domain of FUT3 (33), was cloned in reverse orientation into the *Hind*III site of pcDNA3 to yield the vector pcDNA3-FUT3CDAS (Fig. 5).

Stable Transfection, Selection, and Cloning of FUT3 Antisense and Sense HT-29LMM Cell Lines. Approximately 24 h before transfection, HT-29LMM cells were plated at a density of $1.30-1.50 \times 10^6$ cells per 60-mm dish in 5 ml of RPMI 1640 with 10% fetal bovine serum. In separate tubes, 8 μ g of the *Pvu*I-linearized plasmids pcDNA3-CAT, pcDNA3-FUT3S, pcDNA3-FUT3AS, or pcDNA3-FUT3CDAS (above and Fig. 5) were mixed with 25 μ l of Lipofectamine reagent (Life Technologies, Inc., Gaithersburg, MD) in 200 μ l of serum-free media and incubated for 45 min at room temperature to allow complex formation. The plates were washed twice with serum-free media, the DNA-liposome complexes were diluted with 800 μ l of additional serum-free media, and the dilute complexes were added to each plate. The plates were incubated for 24 h at 37°C in 5% CO₂. The following day, the serum-free media and unincorporated liposomal complexes were replaced with 5 ml of RPMI 1640 with 10% fetal bovine serum. Forty-eight h after transfection, G-418 sulfate (Life Technologies) was added to the medium at a concentration of 1 mg/ml.

After 3-4 weeks in G-418, distinct colonies were observed on each plate and selected with 3 mm cloning cylinders (Fisher, Pittsburgh, PA). The colonies were allowed to grow, removed with trypsin, and cloned by limiting dilution in 96 well plates with continued G-418 selection. To assure a homogeneous population, the resulting clones were again subjected to limiting dilution under G-418 selection. After expansion, cells from each well were subjected to the same expression studies described above. Five clonal sense, five clonal antisense, and three clonal CAT control cell lines were established. Representative clones of each were further characterized for glycan, enzyme, and transcript expression. The two sense clones chosen for study were designated as LMM/S1 and LMM/S2. The two antisense clones selected were LMM/AS1 (transfected with pcDNA3-FUT3AS) and LMM/AS2 (transfected with pcDNA3-FUT3CDAS).

HUVEC Adhesion Assays. HUVEC, at passage 2, were plated in 30-mm plates and allowed to grow to confluence 2 days before use in cell adhesion assays. Half of the HUVEC plates were incubated with TNF- α (20 ng/ml) in medium 199 with 10% fetal bovine serum for 7 h, whereas the remaining half were not treated with cytokine. HT-29, HT-29LMM, and transfected cells were washed, fixed, and added to HUVEC monolayers as described (53). After settling, the cells were incubated on the monolayers for 45 min at 6°C. The cells were then removed by gentle aspiration, followed by three media washes as described (53). The number of cells recovered from each plate was determined by counting the pooled washes; the number of bound cells represents the

difference between the number of cells applied to each plate and the number recovered from each plate. For blocking with anti-E-selectin antibody, HUVEC were preincubated with 20 μ g/ml antibody for 30 min at 37°C.

Establishment of Hepatic Metastases. NCR *nu/nu* athymic nude mice were obtained from Taconic (Germantown, NY). They were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care under specific pathogen-free conditions and fed sterile food and water. Seven- to 8-week-old female nude mice weighing 20-22 g were anesthetized with i.p. injections of nembutal. Animals were placed in the supine position and prepared using aseptic technique. Under sterile conditions,

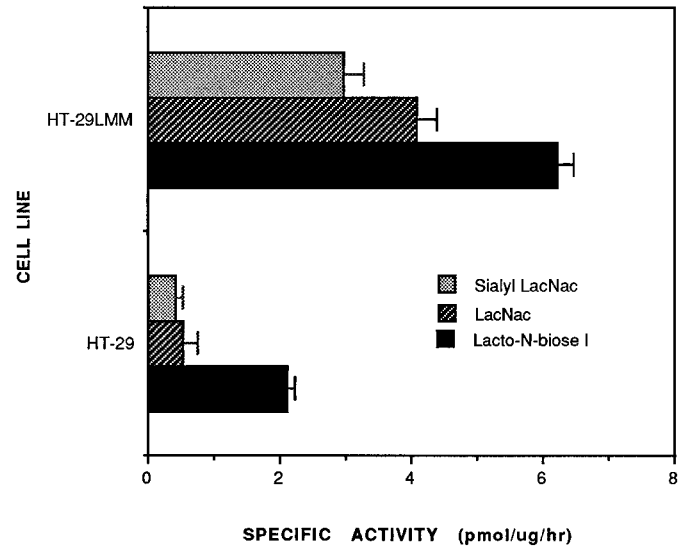


Fig. 2. Fucosyltransferase specific activity of HT-29 and HT-29LMM cellular extracts. As detailed in "Materials and Methods," Triton X-100 detergent extracts prepared from each cell line were assayed for their ability to transfer radiolabeled fucose to low molecular weight carbohydrate acceptors listed in the *inset*. Fucosyltransferase-specific activity is expressed as mean pmol of fucose transferred per μ g extract protein per hour from four separate determinations; bars, SE.

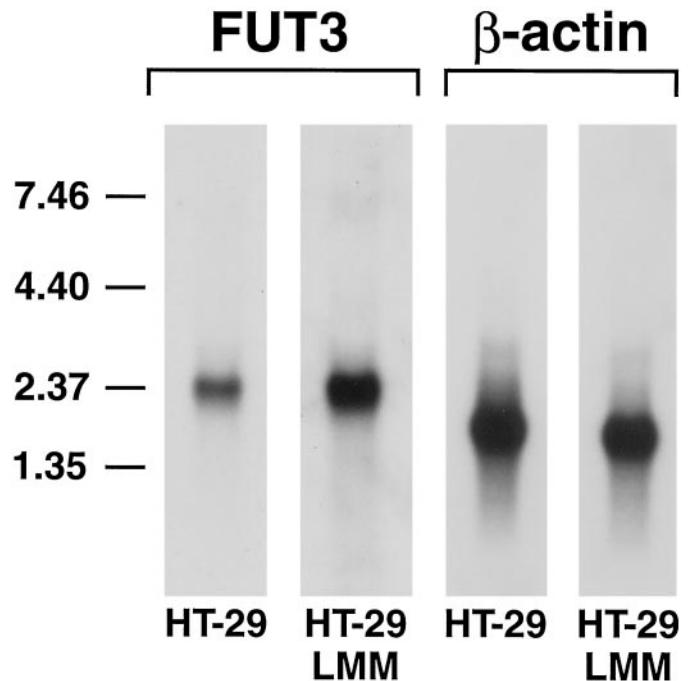


Fig. 3. Northern blot analyses of HT-29 and HT-29LMM cells. Ten μ g of poly(A)⁺ RNA was prepared as described in "Materials and Methods," divided into 5 μ g/lane, electrophoresed, and transferred to a single membrane. The blot was then subdivided into strips and hybridized with ³²P-labeled probes specific for *FUT3* and β -actin.

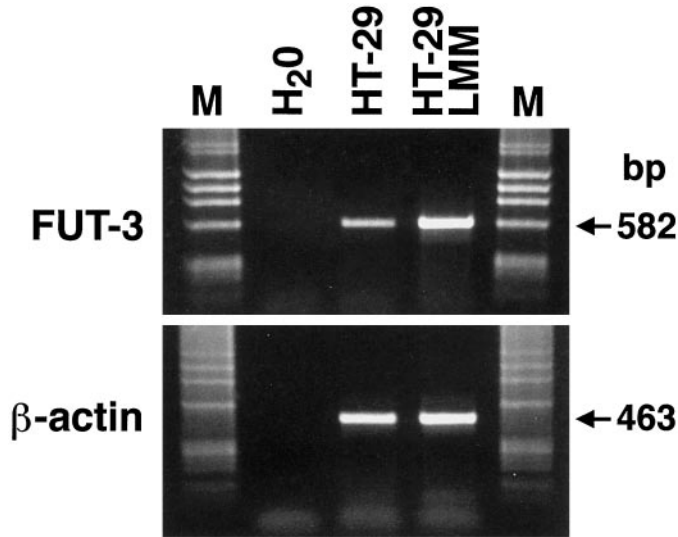


Fig. 4. RT-PCR analysis of HT-29 and HT-29LMM cells. Two hundred ng of poly(A)⁺ RNA were reverse-transcribed and amplified as described in "Materials and Methods." Control amplification of samples with no reverse transcriptase added are not shown. PCR products were electrophoresed in 1% agarose gels and stained with ethidium bromide. Expected fragment sizes (582 and 463 bp) are noted. As noted previously, the primers for β -actin span intronic sequence (42, 43).

a flank incision was made. The spleen was visualized and injected with 1×10^7 tumor cells in 100 μ l of PBS. The contents were returned to the abdominal cavity, and surgical clips were used to close the incision. The mice were allowed to recover, and surgical clips were removed from the animals upon complete healing of the laparotomy incision. Liver metastases were grossly evident by 5–7 weeks. Animals were then sacrificed, and liver harvested, examined, and weighed. Livers were harvested from euthanized animals, placed in 10% formalin, and subsequently embedded in paraffin.

RESULTS

HT-29LMM Cells Express Higher Levels of sLex, sLea, and $\alpha(1,3)$ Fucosyltransferase Activity Than Parental HT-29 Cells. Results of flow cytometry analyses of surface antigens on HT-29 and HT-29LMM cells are summarized in Fig. 1. HT-29 and HT-29LMM cells do not express high levels of the nonsialylated antigen, Lewis x, or the internally fucosylated antigen, VIM-2 (data not shown). Higher surface levels of the fucosylated and sialylated selectin ligands sLea and sLex are seen on the derived cell line HT-29LMM. Previous reports have documented high

expression of dimeric sLex in HT-29LMM (23). To assess whether this high level of surface glycan expression was due to corresponding increases in $\alpha(1,3/1,4)$ fucosyltransferase activity, enzyme assays with low molecular weight carbohydrate acceptors were performed. As shown in Fig. 2, cellular extracts from HT-29LMM transferred fucose more efficiently to both type I and type II acceptors (33) when compared with parental cells. No difference was seen in sialyltransferase activity with HT-29LMM (data not shown). This antigenic and enzymatic profile is consistent with increased expression of Fuc-TIII (33, 37) in HT-29LMM.

HT-29LMM Cells Express Higher Levels of *FUT3* Transcript Than Parental HT-29 Cells. To assess whether surface expression of fucosylated ligands is specifically associated with increased expression of *FUT3* in HT-29LMM, fucosyltransferase transcript analyses were performed. Fig. 3 shows the results of Northern blot analyses of HT-29 and HT-29LMM cells, with higher levels of *FUT3* transcript seen in the derived cell line. RT-PCR analysis of HT-29 and HT-29LMM cells confirms this increase in *FUT3* expression (Fig. 4). Using similar amplification methods (40, 42, 43), expression of other human fucosyltransferase transcripts is low (*FUT4*, *FUT6*, and *FUT7*) or not detectable (*FUT5*) in both cell lines (data not shown). *FUT4*–*FUT7* transcripts were not detectable by hybridization (data not shown). *FUT3* was therefore chosen as the primary target for antisense inhibition studies.

Characterization of HT-29LMM Antisense, Sense, and Control Cell Lines. Constructs used for stable transfection of HT-29LMM cells are shown in Fig. 5. The plasmid pcDNA3-CAT was used throughout the following experiments to monitor expression levels over time by CAT assay (data not shown). The plasmid pcDNA3-FUT3S was transfected to allow quantitative comparison of fucosyltransferase activity in cell lines (Table 1) and to serve as an additional control for high level expression over time.

Enzyme assay results in Table 1 show that transfection of the control plasmid pcDNA3-CAT did not affect fucosyltransferase activities. Stable expression of pcDNA3-FUT3S sense constructs in clones LMM/S1 and LMM/S2 enhanced fucosyltransferase activity. In contrast, the cloned antisense cell lines LMM/AS1 and LMM/AS2 had <2% specific activity with sialyl LacNAc, the trisaccharide precursor for sLex, when compared with untransfected HT-29LMM cells. Furthermore, extracts from LMM/AS1 and LMM/AS2 showed marked reduction in the ability to fucosylate the type I acceptor Lacto-*N*-biose I. No difference was seen in sialyltransferase activity for any of the cell lines or controls (data not shown). This profile is most consistent with reduced activity of

Fig. 5. Constructs used for stable transfection of HT-29LMM cells. The expression plasmid pcDNA3 contains a multicloning site downstream of the CMV promoter (*CMV pr*) and upstream of the neomycin resistance sequences (*Neo*). Constructs were assembled as described in "Materials and Methods" and linearized with *PvuI* before transfection.

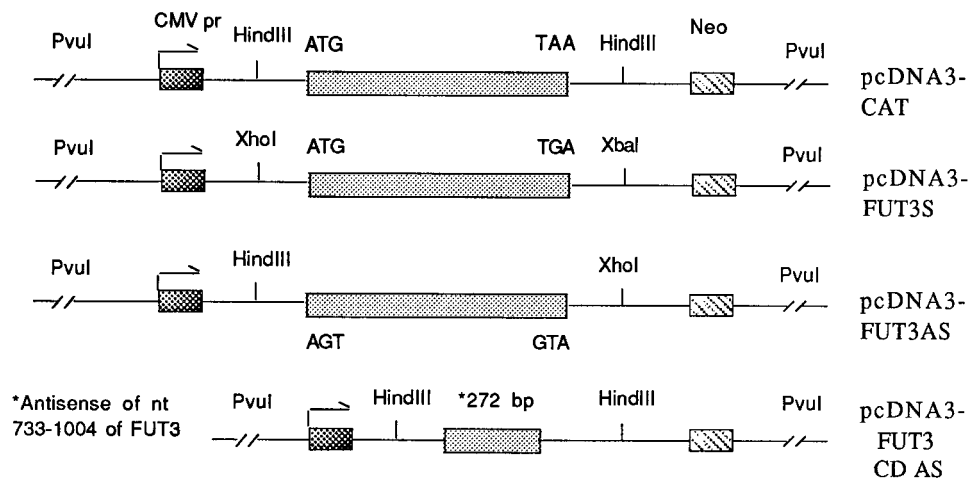


Table 1 $\alpha(1,3)$ Fucosyltransferase activity in cellular extracts

HT-29LMM cells were transfected with constructs and methods described in "Materials and Methods." Fucosyltransferase assays were performed on extracts from transfected cells 28, 56, and 84 days after G-418 selection, cloning, and expansion.

Cells/Constructs	Acceptor	Activity (\pm SD) (pmol/mg/h)
HT-29LMM	LacNAc	4070 \pm 292 ^a
	Sialyl-LacNAc	2980 \pm 311
	Lacto-N-biose I	6230 \pm 324
HT-29LMM/pcDNA3-CAT ^b	LacNAc	4150 \pm 388
	Sialyl-LacNAc	3300 \pm 275
	Lacto-N-biose I	5920 \pm 453
LMM/AS1	LacNAc	152 \pm 20
	Sialyl-LacNAc	34 \pm 13
	Lacto-N-biose I	85 \pm 41
LMM/AS2	LacNAc	141 \pm 17
	Sialyl-LacNAc	46 \pm 32
	Lacto-N-biose I	73 \pm 44
LMM/S1	LacNAc	5510 \pm 477
	Sialyl-LacNAc	4690 \pm 450
	Lacto-N-biose I	7160 \pm 606
LMM/S2	LacNAc	5025 \pm 221
	Sialyl-LacNAc	4270 \pm 340
	Lacto-N-biose I	6900 \pm 598

^a Untransfected HT-29LMM results are from the same assays reported in Fig. 2 (included here for comparison).

^b CAT assays performed at the same time points show stable expression of plasmid pcDNA3-CAT (data not shown).

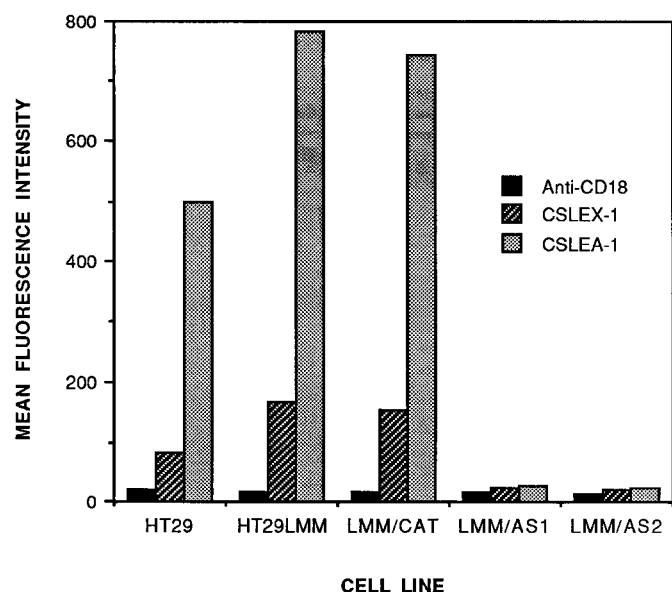


Fig. 6. Flow cytometry analysis of surface antigens on HT-29, HT-29LMM, HT-29LMM/CAT, and stable antisense transfectants LMM/AS1 and LMM/AS2. Cells were harvested, stained with monoclonal antibodies listed in the inset, and subjected to flow cytometric analysis as described (37, 38). Anti-CD18 results are presented as negative controls. Data presented here are representative of the mean fluorescence intensity values from two experiments.

Fuc-TIII in the antisense lines (33, 37). Flow cytometry analyses of surface antigens on HT-29LMM and stable antisense transfectants (Fig. 6) confirm that the synthesis of selectin ligands sLex and sLea is inhibited in LMM/AS1 and LMM/AS2 cells. Northern blot and RT-PCR analyses show that *FUT3* transcript levels are markedly reduced in both stable antisense transfectant lines (Table 2).

Stable Transfection of HT-29LMM with *FUT3* Antisense Constructs Inhibits Selectin-mediated Cell Adhesion. To assess whether inhibition of sLex and sLea expression on LMM/AS1 and LMM/AS2 was of functional significance, HT29, HT-29LMM, and antisense transfectants were assayed for adhesion to HUVEC (Fig. 7). Each cell line was tested for adhesion to TNF- α -stimulated HUVEC or untreated HUVEC as detailed in "Materials and Methods." Block-

ing with anti-E-selectin monoclonal antibody was performed as described (53, 54). The number of bound cells was calculated by subtracting the number of cells recovered (per plate, in pooled washes) from the number of cells applied to each plate. As summarized in Fig. 7, HT-29LMM cells bound avidly to cytokine-stimulated HUVEC; this binding was specifically inhibited by pretreatment of HUVEC with anti-E-selectin antibody. The control cell line HT-29LMM/pcDNA3-CAT bound HUVEC with similar affinity (data not shown). Neither antisense cell line was capable of adhering to TNF- α -stimulated HUVEC *in vitro*. These cell lines were chosen for *in vivo* metastasis studies below.

Stable Antisense Transfectants of HT-29LMM Do Not Metastasize to Liver. Splenic injections of nude mice with HT-29LMM and the antisense transfectant cell lines LMM/AS1 and LMM/AS2 were performed as described in "Materials and Methods." Splenic tumors and production of experimental liver metastases were evaluated after 7 weeks. All injected mice developed tumors in the spleen. As shown in Table 3, all eight mice injected with HT-29LMM developed liver metastases, consistent with results reported previously (23, 46, 47). Average liver replacement by hepatic metastases of HT-29LMM was 48% (range, 23–89%), and liver mass increased by

Table 2 Quantitation of *FUT3* transcript levels in HT-29LMM and antisense clones by Northern blot and RT-PCR

PolyA⁺ RNA from HT-29LMM cells and stable antisense transfectants was analyzed as described in "Materials and Methods." Signal densities were compared using Adobe Photoshop 5.0.

Cell line	Expression level relative to β -actin
HT-29	0.2 ^a
HT-29LMM	1.0 ^a
LMM/AS1	<0.05
LMM/AS2	<0.05

^a Data from Figs. 3 and 4 for comparison.

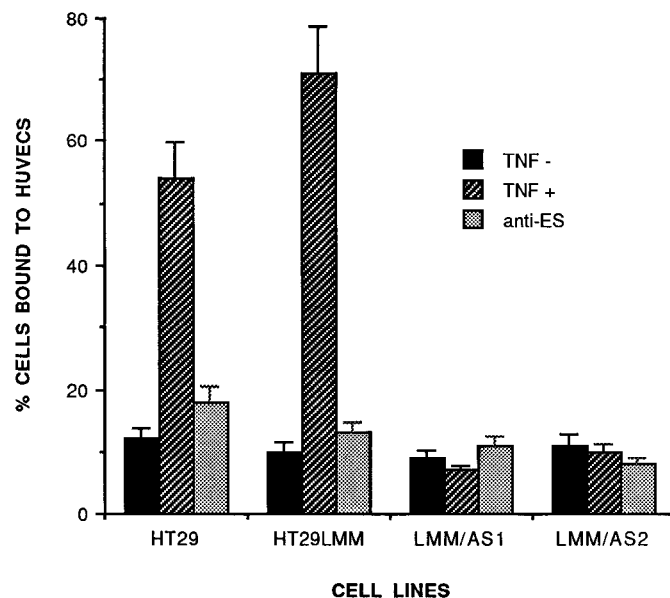


Fig. 7. HT-29LMM and stable antisense transfectant cell line adhesion to human endothelial cells. Each cell line was tested for adhesion to TNF- α -stimulated HUVEC (TNF +, ▨) or untreated HUVEC (TNF -, ■) as detailed in "Materials and Methods." Blocking with anti-E-selectin monoclonal antibody (anti-ES, ▩) was performed as described above. The number of bound cells is calculated by subtracting the number of cells recovered (per plate, in pooled washes) from the number of cells applied to each plate. As reported previously (53), background (nonspecific) adhesion for this assay is ~8–17%; data are the mean values from two separate experiments; bars, SE.

Table 3 Liver metastases in nude mice after splenic injection of HT-29LMM cells and stable antisense transfectant lines

HT-29LMM cells were transfected with constructs, selected, and characterized for fucosylated glycan expression as described above. Tumor cells (10^7) in $100 \mu\text{l}$ of PBS were injected into the spleens of anesthetized nude mice. The animals were sacrificed 7 weeks postoperatively or when liver metastases were easily palpable.

Cells/Constructs	Liver metastases	Liver weights mean (range)
HT-29LMM	8/8	2.45 g (2.00–3.25) ^a
LMM/AS1	0/8	1.26 g (1.14–1.38)
LMM/AS2	0/8	1.33 g (1.15–1.44)

^a $P < 0.01$, HT-29LMM versus LMM/AS1, HT-29LMM versus LMM/AS2.

~2-fold (Table 3). None of the mice injected with LMM/AS1 or LMM/AS2 transfectants developed hepatic metastasis by gross (Fig. 8) or microscopic (Fig. 9) examination.

DISCUSSION

Dissemination of adenocarcinoma cells via the bloodstream significantly affects the diagnosis, treatment, and prognosis of colon cancer, and therapeutic options for metastatic disease are limited. Several lines of experimental and correlative evidence suggest that sLea and sLex are important antigenic determinants of colon carcinoma metastasis to the liver. Our results indicate that the metastatic phenotype of a well-characterized adenocarcinoma cell line can be altered by stable expression of antisense sequences directed at a human $\alpha(1,3/1,4)$ fucosyltransferase gene. These results suggest that Fuc-TIII is an important determinant of sLex and sLea expression in colon cancer, and that *FUT3* is an attractive target for molecular therapy in this disease.

Although sLex and sLea are found on an array of glycoprotein and glycolipid structures on carcinoma cells, it is possible that surface expression of sLex/sLea is not central to the pathogenesis of liver metastasis. Several nonmetastatic carcinoma cell lines, for example, express high levels of sLex/sLea (40, 43). In this hypothesis, increased glycan and/or glycosyltransferase expression could serve as marker(s) for regulation of metastasis-related scaffold molecules (55–60), oncogenes (61, 62), or metastasis-suppressor genes (63, 64). Several glycoprotein scaffolds for sLex have been described in leukocytes that increase affinity of selectin-mediated adhesive interactions (ESL-1 and PSGL-1), but homologues have

not yet been described in human adenocarcinoma (15, 16, 65–67). Surface glycolipids carrying sLea and sLex, however, have also been shown to have roles in tumorigenesis and adhesion (54, 68, 69). In the colon carcinoma cell line HT-29, treatment with *O*-sialoglycoprotease does not affect adherence to E-selectin (44). Glycolipids modified by the addition of sLex/sLea are thus candidates for E-selectin ligands on HT-29 cells, and reducing the expression of these glycans on HT-29LMM is likely the primary mechanism responsible for our results.

HT-29 and HT-29LMM also express high surface levels of the tumor marker CEA. CEA is a homotypic adhesion molecule that is prevalent on colon carcinoma cells (58). This heavily glycosylated protein (57) is cytokine inducible at the level of transcription (55, 70), and its expression correlates with clinical progression (20–22). Transfection of CEA into nonexpressing cell lines has been shown to induce transcription of other cell adhesion molecules (56). The predictive value of serum CEA levels in adenocarcinoma patients can be increased by comeasurement of sLea-containing mucins, and the latter assay is more specific (20–22). A recent histopathological study comparing liver metastasis specimens with paired primary tumors showed that levels of sLex and sLea are higher on metastatic cells, whereas CEA levels remain the same (26). Thus, sLex and sLea are independent predictors of metastatic potential, even when CEA is expressed at high levels, and inhibiting their expression is of primary interest.

To modify the metastatic phenotype, it is important to understand the mechanisms regulating biosynthesis of sLex and sLea in carcinoma cells. Our enzymatic results are consistent with the hypothesis that fucosylation, rather than sialylation (45), is the controlling step for antigen synthesis in HT-29LMM. Other colon carcinoma cell line variants selected for high metastatic potential have been reported to express more sLea than parental cells; this change in phenotype is associated with higher levels of *FUT3* transcript but similar amounts of $\alpha(2,3)$ sialyltransferase message (18). Our results comparing HT-29 and HT-29LMM transcript expression are consistent with fucosylation as the primary mechanism for controlling synthesis and provide target validation for antisense inhibition. The close association of transcript levels to $\alpha(1,3/1,4)$ fucosyltransferase activity also supports our hypothesis

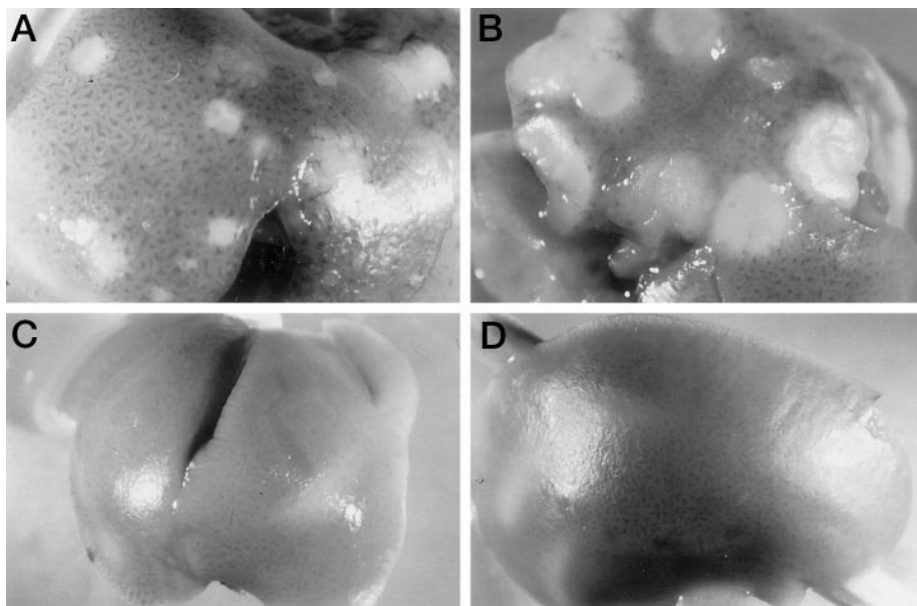
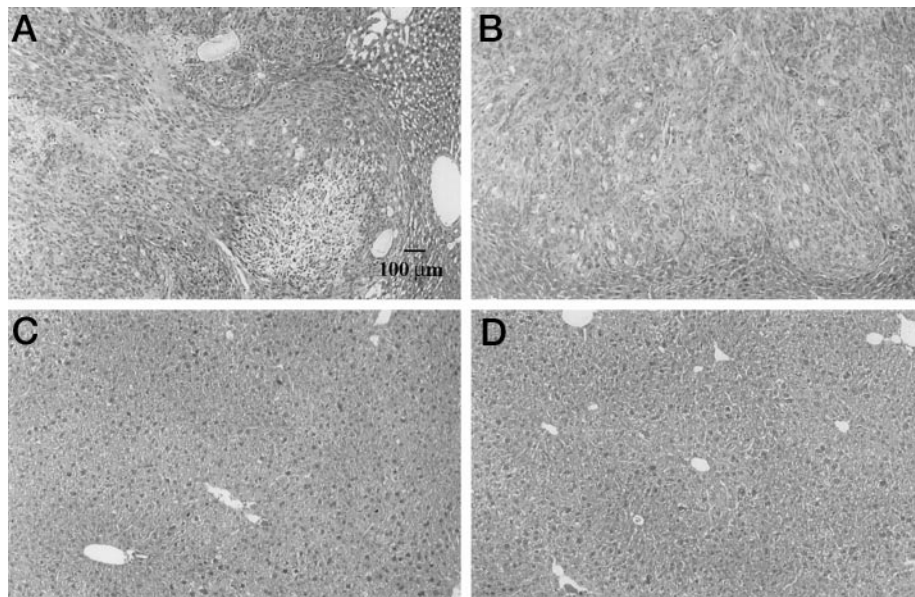


Fig. 8. Gross appearance of livers from nude mice injected with HT-29LMM (A), HT-29LMM/pcDNA3-CAT (B), LMM/AS1 (C), and LMM/AS2 (D) cell lines. Splenic injections were performed as detailed in "Materials and Methods." Macroscopically, the metastases shown in A and B were observed as multiple irregular gray-white nodules of varying size distributed in both lobes of the liver ($n = 8$ specimens for HT-29LMM). No metastases were seen in livers from mice injected with antisense cell lines LMM/AS1 and LMM/AS2 ($n = 8$ mice for each cell line; see Table 3).

Fig. 9. Representative microscopic sections (H&E; magnification scale in A) from livers of nude mice injected with HT-29LMM (A), HT-29LMM/pcDNA3-CAT (B), LMM/AS1 (C), and LMM/AS2 (D) cell lines. Splenic injections were performed as detailed above. Multiple nodules of moderately differentiated colon adenocarcinoma are seen displacing hepatocytes in A and B. No micrometastases were found in multiple sections of liver from animals injected with antisense cell lines LMM/AS1 and LMM/AS2 (C and D, and data not shown).



that *FUT3* expression is regulated at the level of transcription and/or RNA processing.⁴

A characteristic distinguishing Fuc-TIII from Fuc-TIV–Fuc-TVII is that it possesses an additional fucosyltransferase activity, which transfers fucose in $\alpha(1,4)$ linkage to type I precursors to produce sLea and the red cell antigens Lewis a (Le^a) and Lewis b (Le^b). Serological surveys measuring surface expression of Le^a and Le^b demonstrate that 10–20% of the population is deficient in Fuc-TIII activity (*i.e.*, Lewis negative; Ref. 52). The physiological importance of Fuc-TIII in normal tissue is not known but is most likely minor, because the Lewis-negative phenotype is relatively common, and despite epidemiological associations with minor infection (71) and cardiovascular risk (72), causes no known pathology. Conversely, increased Fuc-TIII activity in tumor tissues, aberrantly producing sLex, dimeric sLex, and sLea, may be central in facilitating metastasis, even in Lewis-negative patients (73). Thus, targeting Fuc-TIII for inactivation with antisense technology is an attractive approach for controlling colon carcinoma metastasis while minimizing toxicity to normal cells.

Human carcinoma cells often contain a mixture of $\alpha(1,3)$ fucosyltransferase activities and transcripts (27–32, 40, 43). HT29-LMM cells were chosen for these preliminary target validation studies due to their metastatic phenotype and high expression levels of sLea and *FUT3* transcripts. Although a small amount of *FUT4*, *FUT6*, and *FUT7* transcript can be amplified in HT-29LMM cells, their expression is low, and the enzymes encoded are not capable of constructing sLea (33–41). *FUT5* is not expressed in HT29-LMM. Thus, loss of *FUT3* transcript in these cells provides a direct method of examining the effects of eliminating most $\alpha(1,3/1,4)$ fucosylation events in a single cell type. Extending this model for application to other cell lines and tumor specimens, however, may require sequence-specific inhibition of other $\alpha(1,3)$ fucosyltransferases. Fuc-TVI [the human plasma $\alpha(1,3)$ fucosyltransferase], which is expressed at high levels in some colon carcinoma cells (40, 43), also determines expression of sLex (38), and it will be a cotarget for antisense inhibition studies described below.

Antisense technology offers the possibility of targeting a specific gene to study its function, inhibit its product, or repair a defect (48–50). Antisense is therefore an appealing therapeutic strategy for

malignancies, because it avoids some of the widely cytotoxic effects of current cancer therapy (49, 74). Clinically, antisense offers an advantage over antibody-based targeting (19) because the constructs are often less immunogenic and have more favorable pharmacokinetics (75). Mechanisms by which antisense inhibition work are not well understood but may include RNase H-dependent destruction of transcripts, disruption of pre-mRNA maturation, or blocking of translation initiation (48). Transfection of either the full-length *FUT3* antisense construct or the truncated catalytic domain antisense construct results in markedly reduced levels of *FUT3* transcript. Our results are therefore more consistent with either RNase-dependent transcript destruction or pre-mRNA maturation inhibition, rather than displacement of *FUT3* translation initiation.

The *FUT3* catalytic domain antisense construct also targets *FUT6*, because its sequence is identical in this region (42), allowing us to extend our studies to other cell types. In fact, RT-PCR analyses show that the small amount of *FUT6* transcript present in HT-29LMM was destroyed by expression of this antisense motif.⁵ The region contained in the truncated construct provides a suitable target for preliminary design of antisense ODNs with modified backbones (75). The major obstacle to effective antisense ODN therapy is delivery of the target sequence to the nuclei of the desired cells (76, 77). This process involves cell entry, stable transport, microsomal release, and nuclear uptake (48). Our plasmid-based results (49, 74) with serum-free liposomal transfections will need to be replicated *in vivo* with ODNs in the serum-containing hepatic vascular microenvironment (75–79). Regardless of the mechanism of target inhibition or the delivery mode chosen, antisense human *FUT* sequences appear to be a promising adjuvant approach for treatment of disseminated colon carcinoma.

ACKNOWLEDGMENTS

We thank Lew Romer for the HUVEC used in these and other experiments, Janet Price for providing original stocks of HT-29LMM cells, and Sarah Sparks for supervision of flow cytometry analyses.

⁴ B. Weston and K. Hiller, unpublished data.

⁵ K. Hiller and B. Weston, unpublished data.

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