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Knockdown of fucosyltransferase III disrupts the adhesion of circulating cancer cells to E-selectin without affecting hematopoietic cell adhesion

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Abstract

Adhesive interactions between selectins and their ligands play an essential role during cancer extravasation. Fucosylation of these proteins by fucosyltransferases, or FUTs, is critical for their functions. Using quantitative RT–PCR, we demonstrated that FUT4 and FUT7 are the predominant FUTs expressed in hematopoietic cell line, while FUT3 is heavily expressed by multiple cancer cell lines including the prostate cancer cell line MDA PCa2b. Knockdown of FUT3 expression in MDA PCa2b cells by small interference RNA (siRNA) significantly reduced FUT3 expression. Cell-surface sialyl Lewis antigens were largely abolished. Cell adhesion and cell rolling on the blood vessel wall were simulated by perfusing cancer cells through microtubes coated with recombinant human E-selectin. At physiological levels of wall shear stress, the number of flowing cancer cells recruited to the microtube surface was dramatically reduced by FUT3 knockdown. Higher rolling velocity was also observed, which is consistent with reduced E-selectin binding activity. Interestingly, FUT3 siRNA treatment also significantly reduced cell growth rate. Combined with the novel siRNA delivery platform recently developed in our laboratory, FUT3 siRNA could be a promising conjunctive therapy aiming at reducing the metastatic virulence of circulating epithelial cancer cells.

Keywords

Selectin; Fucosyltransferase; Metastasis

1 Introduction

Metastasis is the major challenge of current cancer therapy. The mechanism of epithelial cancer metastasis is not fully understood. The current paradigm includes at least three steps: (i) the detachment of cancer cells from the primary tumor and their entry into blood or lymphatic vessels (intravasation); (ii) the survival of circulating cancer cells (CCCs), the transendothelial migration of CCCs into distal tissues (extravasation); and (iii) the invasion and engraftment of CCCs into a remote tissue and initiation of a new tumor. Increasing

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evidence suggests that the extravasation of CCCs resembles leukocyte recruitment toward inflammatory sites in which the interactions between selectins and their ligands are a critical component. Selectins are a group of transmembrane glycoproteins that bind to sugar moieties of their ligands in a calcium-dependent way. The three members of this family: P, E, and L-selectin, share significant structural similarity, while E-selectin seems to be particularly important in the metastasis of epithelial cancer cells. E-selectin is expressed by endothelial cells under inflammatory stimulation, or constitutively expressed in some microvessels such as in bone marrow. Binding between E-selectin protein and its counterreceptors expressed by circulating leukocytes or CCCs leads to tethering and rolling of these cells on the blood vessel wall. This event then initiates a cascade of molecular events, eventually leading to firm adhesion and transendothelial migration [1-4]. The importance of E-selectin has been exemplified by its promotion of the extravasation of metastatic colon cancer cells [5-9].

E-selectin binds to sialylated, fucosylated glycans presented by glycoproteins or glycolipids, the identities of which differ among different cell types [10-14]. Sialyl Lewis^x (NeuAc α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc) and sialyl Lewis^a (NeuAc α 2-3Gal β 1-3(Fuc α 1-4)GlcNAc) antigens are two of the minimum carbohydrate motifs required for selectin binding [15,16]. The fucose modules of these motifs are pivotal for their function [17]. Fucosylation is catalyzed by fucosyltransferase enzymes, or FUTs. At least nine verified FUT genes exist in the human genome. Among these, six α 1,3 FUTs (FUT3, -4, -5, -6, -7 and -9) could potentially synthesize sialyl Lewis^x (sLe^x). FUT3 can also function as an α 1,4 FUT to produce sialyl Lewis^a (sLe^a) [18]. Each FUT has a distinct tissue expression pattern [19,20]. Notably, hematopoietic cells express FUT4 and FUT7 at high levels. Both contribute to selectin-dependent leukocyte adhesion and recruitment [21,22].

The importance of sialyl lewis antigens and FUTs in epithelial cancer metastasis has been supported by several lines of evidence. Firstly, it was observed that high E-selectin binding activity, high sialyl Lewis antigen levels or high FUT expression was correlated with high metastatic potency and poor prognosis of epithelial cancers [23-26]. For example, colon carcinoma variants with high sLe^x levels metastasized to mouse liver more efficiently than the variants with low sLe^x levels [27]. Similarly, malignant prostate cancer cells expressed an increased amount of selectin ligands compared to the relatively benign cancer cells, [28] and the α 1,3 FUTs were shown to be responsible for prostate cancer cell trafficking [29]. Another set of evidence came from several experiments that demonstrated that genetically or chemically manipulating the selectin/ligand axis changed the metastatic behavior of epithelial cancer cells [30-34]. For instance, metastasis of human pancreatic or colon cancer cells was inhibited by stable expression of a FUT antisense RNA [35,36].

Current drug designs, such as anti-selectin antibodies, low-molecular-weight selectin antagonists (sialyl Lewis antigen mimics), and metabolic inhibitor of lactosamine synthesis, do not provide any cell type specificity [1,37]. The idea of targeting selectin ligands on epithelial cancer cells (usually different from those on leukocytes) is tempting, yet the same cancer cell type may have multiple selectin ligands, increasing the difficulty of drug design [11,13]. However, if all these ligands are modified by the same FUT enzyme, which also differs between circulating cancer cells and leukocytes, this unique enzyme could be the ideal drug target.

In the following sections we demonstrate that for some cancers, this unique FUT in fact exists and is distinct from those required by hematopoietic cells. Thus, cancer cells may be therapeutically targeted without compromising immune function. Development of small-molecule inhibitors for α 1,3- and α 1,4- FUTs is challenged by the lack of structural

information and low affinity between FUTs and their substrates [37]. In the present study, we targeted the enzyme within the cytoplasm using siRNA molecules to disrupt adhesion to selectins. By combining FUT siRNA with our newly developed targeted siRNA delivery system [38], it would even be possible to target the FUT in CCCs without disrupting the function of normal epithelial cells. This represents the first study to successfully use FUT siRNA to target the selectin-binding activity of prostate cancer cells under flow.

2 Material and methods

2.1 Cell lines and cell culture

HL-60, KG-1a, Colo205 and MDA PCa 2b (ATCC # CRL-2422) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). For the first three cell types, RPMI medium was used. BRFF-HPC1™ medium (Cat# 0403, AthenaES) was used to culture MDA PCa 2b cells. Media was supplemented with 10% FBS, 100 IU/mL penicillin and 10 µg/mL streptomycin. Cells were cultured at 37 °C in an incubator supplied with 5% CO₂. MDA PCa 2b single-cell suspension was obtained with Accutase (Cat# A 6964, Sigma) treatment for 5 min at 37 °C before experiments or passages.

2.3 siRNA Transfection

siRNA Duplexes were purchased from Invitrogen. The catalog number and/or sequences of siRNAs are: Negative Control siRNA Duplexes (Cat# 12935-200);

FUT3 siRNA Duplexes #1 (Cat# FUT3HSS142099)

5'-GCUGUCUGGCCGCACUGCUAUUUCA-3' (sense)

5'-UGAAAUAGCAGUGCGGCCAGACAGC-3' (anti-sense);

FUT3 siRNA Duplexes #2 (Cat# FUT3HSS142101)

5'-ACUGGGGAUAUCAUGUCCAACCCUAA-3' (sense)

5'-UUAGGGUUGGACAUGAUAUCCCAGU-3' (anti-sense).

To transfect MDA PCa 2b cells, 10⁶ cells were suspended in 150 µL of siPORT siRNA Electroporation Buffer (Cat# AM8990, Ambion) and transferred to a 2-mm Gene Pulser Cuvette (Cat# 165-2086, Bio-Rad). Cells were subjected to electroporation (single pulse, 0.4 ms, 200 V) immediately after mixing with 15 µL of 20 uM siRNA solution. Cells were allowed to recover at 37 °C for 10 min and then transferred to 5 mL of medium in a 25 cm² flask for culture.

2.4 Reverse transcription

Four days after siRNA transfection, total RNA was purified using RNeasy Plus mini Kit (Cat# 74134 Qiagen). The 40-µL reverse transcription reaction system included 5–10 µg of total RNA, 1 µL of M-MuLV Reverse Transcriptase (Cat# M0253L, New England Biolabs), 0.5 µL of RNase Inhibitor (Cat# M0307S, New England Biolabs), 1 µL of Random Primers (Cat# 48190-011, Invitrogen), 2.5 µL of dNTP Mix (Cat# 170-8874, New England Biolabs) and 4 µL of M-MuLV Reverse Transcriptase reaction buffer. The reaction mixture was incubated at 42 °C for 1 h followed by an inactivation step at 95 °C for 10 min.

2.5 Real-time quantitative PCR (Q-PCR)

After reverse transcription, cDNA produced from 10 to 40 ng of total RNA was used in each Q-PCR reaction. The 20 µL of Q-PCR reaction system also included 10 µL of iQ™ SYBR Green Supermix (Bio-Rad), 1 µL of 2 µM forward primer and 1 µL of 2 µM reverse primer. The following primers were used:

Primers for GAPDH Q-PCR (product size 170 bp, T_m 86 °C)

5'-AGAGCACAAGAGGAAGAGAGAGAC-3' (Forward)

5'-AGCACAGGGTACTTTATTGATGGT -3' (Reverse)

Primers for FUT3 Q-PCR (Product size 158 bp, T_m 86 °C)

5'-GCCGACCGCAAGGTGTAC-3' (Forward)

5'-TCCAGGTGCTGGCAGTTAG-3' (Reverse)

Primers for FUT4 Q-PCR (Product size 190 bp, T_m 91.5 °C)

5'-GTTGGACTACGAGGAGGCAG-3' (Forward)

5'-ATAAGGCACAAAGACGTCCG-3' (Reverse)

Primers for FUT5 Q-PCR (Product size 100 bp, T_m 84 °C)

5'-TATGGCAGTGGAACCTGTCA-3'(Forward)

5'-CGTCCACAGCAGGATCAGTA-3' (Reverse)

Primers for FUT6 Q-PCR (Product size 223 bp, T_m 90 °C)

5'-CAAACCCATAGCTCTGCCC-3'(Forward)

5'-TTTCAGCTGCCAGCAGTG-3'(Reverse)

Primers for FUT7 Q-PCR (Product size 81 bp, T_m 83 °C)

5'-GGAACGTTTCTGTGCCATCT-3'(Forward)

5'-TGAAACCAACCCTCAAGGTC-3'(Reverse)

Primers for FUT9 Q-PCR (Product size 78, T_m 81 °C)

5'-TCCCATGCAGTTCTGATCCAT-3'(Forward)

5'-GAAGGGTGGCCTAGCTTGCT-3'(Reverse)

Q-PCR reactions were carried out in 96-well PCR plates (Bio-Rad) using the Bio-Rad MyIQ single color Real-time PCR Detection System. The Q-PCR program include 5 min at 95 °C to activate polymerase and 50 PCR cycles (uncoupling 95 °C 20 s, annealing step 58 °C 20 s, elongation step 72 °C 30 s), followed by a melting temperature analysis that increases temperature incrementally from 55 °C to 95 °C. Reactions were performed in triplicate. The relative expression levels of FUTs were calculated as ratios to expression levels of GAPDH.

2.6 Semi-quantitative PCR

After reverse transcription, cDNA produced from 100 ng of total RNA was used in each semi-quantitative PCR reaction. The 50 µL of semi-quantitative PCR reaction system included 25 µL of IQTM Supermix (Bio-Rad), 1 µL of 10 µM forward primer and 1 µL of 10 µM reverse primer. The following primers were used:

Primers for GAPDH

5'-AAGGTCGGAGTCAACGGATTTGGT-3' (Forward)

5'-AGTGATGGCATGGACTGTGGTCAT-3' (Reverse)

Primers for FUT3

5'-CTGTCCCGCTGTTTCAGAGAT-3' (Forward)

5'-CGAAGGCCAGGTAGAACTTG-3' (Reverse)

Semi-quantitative PCR reactions were performed on a Bio-Rad icycler PCR machine. The PCR program included an activation step at 95 °C for 5 min and 29 or 32 PCR cycles (uncoupling 95 °C 30 s, annealing 55 °C 30 s, elongation 72 °C 1 min).

2.7 Flow cytometry

A total of 1×10^5 cells were incubated with 10 μ L of antibody in 100 μ L of PBS containing 3% BSA for 30 min on ice. Cells then were washed with 4 mL PBS once and re-suspended in 200 μ L of PBS buffer. Flow cytometry data was acquired with an Accuri C6 flow cytometer. The following antibodies were used for analysis: FITC conjugated IgM, κ Isotype control (Cat# 555951, BD Pharmingen); FITC Rat Anti-Human Cutaneous Lymphocyte Antigen (Cat# 555947, BD Pharmingen). The expression of cell surface sialyl Lewis antigen was quantified as the difference between mean fluorescence of cells stained with antibody against Human cutaneous lymphocyte antigen and that of the same cells stained with isotype control antibody.

2.8 Cell adhesion assay

Recombinant human E-selectin/Fc Chimera (Cat# 724-ES, R&D systems) was diluted with PBS to concentrations ranging from 1 to 50 μ g/mL. Each well of a 96-well plate was coated with 40 μ L of E-selectin solution for 2 h at room temperature, and then blocked with 100 μ L PBS containing 3% BSA for 1 h before use. MDA PCa 2b cells were fluorescently labeled with calcein AM solution (C1359–100 μ L, Sigma–Aldrich) at 37 °C for 30 min. A final calcein AM concentration of 5 μ g/mL diluted in HBSS+ buffer (Hanks' Balanced Salt Solution containing 0.5% Human Serum Albumin, 10 mM HEPES and 2 mM Calcium Carbonate, pH 7.4) was used. Cells (10^5 in 100 μ L) were then added to each well of the E-selectin-coated 96-well plate and incubated for 1 h at room temperature. Vacuum aspiration and 3 washes with PBS were used to remove non-adherent cells. Adherent cells were then quantified at 480/520 nm (excitation/emission wavelength) with a Multi-detection Microplate Reader (Synergy HT BioTek Plate reader).

2.9 Cell capture and rolling velocity studies

Four days after siRNA transfection, cells (0.5×10^6 /mL) were perfused through E-selectin-coated microtubes at a wall shear stress of 2.5 dynes/cm² for 10 min. Microtubes were placed on the stage of an IX-71 inverted microscope (Olympus, Inc) coupled to a CCD camera (Hitachi) and a TV monitor/DVD recorder (Sony). Twenty images were taken at random regions of each microtube to quantify the number of adherent cells. For rolling velocity studies, samples were perfused at 2 dynes/cm² or 4 dynes/cm² for more than 2 min before video recording. Tubes were video recorded at three random regions, for at least 1 min at each condition. The calculation of rolling velocity has been described previously [39]. Briefly, cells were classified as stationary if they rolled <2 cell diameters in 1 min. Stationary cells were not included in the analysis. Rolling velocity of non-stationary cells was calculated using a Matlab program, which quantified the change in cell position in a given period of time. For examples of the video recordings for both siRNA-treated and untreated cells, see the Supplementary data section.

2.10 Cell growth rate analysis

After electroporation, cells were plated in 25 cm² cell culture flasks to grow. They were then harvested 1, 3, 4 or 5 days after transfection and treated with Accutase to dissociate cell clumps. Total numbers of viable cells were counted manually using a slide cell counter after staining with Trypan Blue.

2.11 Statistics and graphing

All data are shown as Mean $\pm$ SEM. Graphing and statistics were done using Origin 6.1 software. The unpaired t-test was used in most cases. A value of $P<0.05$ was considered statistically significant.

3 Results

3.1 Differential expression of $\alpha 1,3$ -fucosyltransferase (FUT) between epithelial cancer cell lines and hematopoietic cell lines

We posed the question of whether a gene exists that is critical for metastatic cancer cell adhesion, and is simultaneously dispensable for leukocyte function to avoid off-target side effects. The six $\alpha 1,3$ -FUTs potentially responsible for the synthesis of sialyl Lewis antigens are FUT3, FUT4, FUT5, FUT6, FUT7 and FUT9. We checked the expression of these genes in four cell lines using quantitative RT-PCR (Q-RT-PCR). In Fig. 1A, FUT gene expression was normalized to GAPDH expression. FUT4 and FUT7 were the major FUTs expressed by HL60 and KG1a, two hematopoietic cell lines. Colo205, a colon cancer cell line expressed FUT3, FUT4 as well as FUT6. MDA PCa 2b (MDA in short), a prostate cancer cell line, expressed dominantly FUT3. The expression data were then normalized to those of HL60 cells (Fig. 1B) to allow cross-cell line comparison. These results identified FUT3 as a suitable gene target: FUT3 is the only gene that is over-expressed by both epithelial cell lines and showed little expression in hematopoietic cells. As FUT3 thus represents a singular gene target, we proceeded to work with the MDA cell line to study the effects of FUT3 siRNA on cell-adhesion behavior.

3.2 FUT3 siRNA led to effective knockdown of FUT3 expression

MDA PCa 2b cells were transfected with either FUT3 siRNA or negative control siRNA molecules, as described in Materials and methods, Section 2.3. Three days after transfection, cells were harvested, and RNA was purified. The effectiveness of two distinct sets of FUT3 siRNA molecules was studied. Fig. 2A shows Q-RT-PCR results. SiRNA set #1 and set #2 reduced FUT3 mRNA level by $84\pm 3\%$ and $91\pm 9\%$, respectively. Similar results were demonstrated using semi-quantitative RT-PCR (Fig. 2B).

3.3 FUT3 siRNA inhibits cell-surface sialyl Lewis antigen synthesis

To study the impact of FUT3 siRNA on MDA cell surface sialyl lewis antigens, we used flow cytometry analysis with an anti-CLA (Cutaneous Lymphocyte Antigen) antibody that recognizes both sialyl Lewis x and sialyl Lewis a. In a flow cytometric histogram, MDA cells stained with FITC-labeled anti-CLA antibody (red line) showed a right shift of fluorescence intensity compared to the isotype control sample (black line), confirming the expression of sialyl lewis antigens (Fig. 3A top part). This right shift was eliminated by FUT3 siRNA treatment (Fig. 3A bottom part), indicating that, in MDA cells, FUT3 is the major FUT of sialyl lewis antigens in the synthesis pathway. The expression level of CLA antigen was further quantified as described in Materials and methods, Section 2.3. Both FUT3 siRNA set #1 and set #2 reduced cell surface CLA antigens by about 80% (Fig. 2B). To determine the speed and duration of FUT3 siRNA-mediated cell-surface CLA antigen knockdown, we performed a kinetic study (Fig. 3C). It took less than 24 hours to knock down 50% of total cell-surface CLA antigen. Maximum knockdown ($95.8\pm 2.3\%$) was achieved 3.5 days after siRNA transfection. The recovery phase was slower, starting on Day 4.5. A significant knockdown ($63.2\pm 5.8\%$) was retained 8.5 days after transfection.

3.4 FUT3 siRNA inhibits cell adhesion to E-selectin

The interactions between sialyl lewis antigens of cancer cells and E-selectin of endothelial cells initiate cancer cell extravasation. FUT3 siRNA treated cells were deficient in E-selectin-mediated cell adhesion. Four days after siRNA transfection, MDA cells were subjected to adhesion analysis, as described in Materials and methods, Section 2.8. Fig. 4A shows the representative images of adherent control cells (Top) and FUT3 siRNA-treated cells (Bottom) at the same E-selectin concentration. The number of adherent cells was then quantified by labeling cells with calcein and measuring the fluorescence signal using a plate reader. We performed the analysis over a range of E-selectin concentrations (1–50 $\mu\text{g/mL}$). A set of representative dose response curves is shown in Fig. 4B. The mean fluorescence intensity (MFI) was about five-fold lower in FUT3 siRNA-treated cells (342 ± 14) compared to control cells (1833 ± 32), while EC₅₀ was about five-fold higher in FUT3 siRNA treated cells ($8.7 \pm 0.5 \mu\text{g/mL}$) compared to control cells ($1.8 \pm 1.6 \mu\text{g/mL}$), suggesting lower binding affinity to E-selectin in the treated cell population. The results from three independent dose-response experiments are summarized in Fig. 4C. Significant ($P < 0.05$) reduction of adhesion, 91%, was observed for all E-selectin concentrations tested. The largest reduction of adhesion, 91%, was observed at 5 $\mu\text{g/mL}$, which produces adhesion levels comparable to that measured for these cells on human bone marrow endothelium under shear flow[23].

3.5 FUT3 siRNA reduces cell capture and increases cell-rolling velocity

Cancer cell extravasation is not a static process. E-selectin on the surface of endothelial cells functions as a snare by capturing sialyl lewis antigens expressed by circulating cancer cells. The captured cells roll on the blood vessel wall under the imposed shear stress of flowing blood before they form firm adhesions and commit to the fate of metastasis. This capture and rolling process can be mimicked using immobilized proteins as shown earlier [44,45,50,51]. In our laboratory, we simulated the process using E-selectin-coated microtubes and a computer-controlled syringe pump. MDA cells at $0.5 \times 10^6/\text{mL}$ were perfused through these microtubes at physiological levels of shear stress. Captured cells were quantified as described in Materials and methods, Section 2.9. Microtubes without E-selectin coating captured zero cancer cells (data not shown). In ten minutes, 8618 ± 228 of MDA cells were captured within a 50-cm length of microtube. FUT3 siRNA significantly ($P < 0.001$, $n \geq 148$) reduced the number of captured cells (Fig. 5A, B) by 68%. Furthermore, FUT3 siRNA also increased the rolling velocity of MDA cells (Fig. 5C). At a shear stress of 2 dynes/cm^2 , FUT3 siRNA treated cells rolled at $2.67 \pm 0.12 \mu\text{m/s}$, 58% faster than control cells ($1.69 \pm 0.06 \mu\text{m/s}$). At 4 dynes/cm^2 , control cells rolled at $1.90 \pm 0.08 \mu\text{m/s}$, while FUT3 siRNA-treated cells rolled at $3.87 \pm 0.16 \mu\text{m/s}$, more than twice the velocity of the control cells. These results indicate that FUT3 siRNA reduced the affinity of MDA cells to an E-selectin surface. For examples of the video recordings for both siRNA-treated and untreated cells, see the Supplementary data section.

3.6 FUT3 siRNA-reduced cell growth rate

Interestingly, FUT3 siRNA also seemed to have an effect on cell-growth behavior. As shown in Fig. 6A, B, both FUT3 siRNA set #1 and set #2 led to significant reduction in cell proliferation ($P < 0.01$). The extent of reduction was between 21% and 34%. These results suggest that FUT3 and fucosylation may also function in growth-related signaling pathways. Delivery of FUT3 siRNA to epithelial cancer cells will not only block their metastasis but also slow down their proliferation, an added benefit for anti-metastasis applications.

4 Discussion

Malignant prostate cancer has a tendency to metastasize to bone. Given that bone marrow endothelial cells (BMECs) constitutively express E-selectin[40] and many prostate cancer

cell lines present robust E-selectin binding activity [23], the “seed and soil” theory seems to be plausible for explaining the observed tissue preference [41]. Rolling of prostate cancer cells on BMECs has been inhibited by pretreating BMECs with neutralizing anti-E-selectin antibodies [23]. Aggressive, high-grade prostate tumors stained heavily with anti-sLe^x antibodies, while normal prostate epithelial cells were largely negative [23,42]. In addition, transfer of a FUT3 gene to an sLe^x-negative prostate cell line PC-3 increased cancer growth in a transplant mouse model [42]. Taken together, current knowledge supports that capture of sLe^x-bearing CCCs by E-selectin-expressing BMECs is the prelude of the extravasation story for prostate cancers.

Long term, this knowledge can be utilized to make an effort in drug design to disrupt cancer metastasis. One obvious way to block selectin/ligand interactions is to use antibodies against E-selectin or sLe^x, or to use sLe^x mimics. However, introduction of these reagents to the bloodstream will inevitably disrupt normal functions of leukocytes. Another tempting method, which may potentially reduce the off-target side effects, is to block specific selectin ligands of prostate cancer cells. However, E-selectin ligands on the prostate cell membrane include both sLe^x-bearing glycoproteins and glycolipids [23]. Just in the first category, two potential E-selectin ligands were identified: E-selectin ligand 1 (ESL-1) and P-selectin glycoprotein ligand-1 (PSGL-1) [10]. PSGL-1 is also a critical selectin ligand expressed by leukocytes. Given the complexity of the molecular pool that constitutes E-selectin ligands, it is challenging to devise a simple drug design.

Fucosyltransferase may be a better molecular target. All E-selectin ligands share the sLe^x module, and fucosylation is critical for sLe^x function. The expression of glycosyltransferases in metastatic prostate cancer cells was thoroughly analyzed by Barthel and co-workers using Q-RT PCR [43]. Their results indicate that FUT3, 6 and/or 7 may be important in sLe^x synthesis because these three FUTs were expressed at a higher level by the MDA variant with higher E-selectin binding activity than the variant with low activity. However, the relative expression/importance of these three FUTs was not illustrated. In the present study, we found that among all six α 1-3 FUTs, FUT3 is a major FUT expressed by MDA cells, while FUT4 and FUT7 were the major FUTs expressed by cells with hematopoietic origin. This discovery creates a base for designing cell-specific therapies.

We then used an siRNA approach to target FUT3 in MDA cells. The genes encoding FUT3, 5 and 6 exist in a gene cluster on Chromosome 19 and share high similarity in sequence (90%) [19]. In order to obtain unbiased results, two independent sets of siRNA molecules were designed to target only FUT3. SiRNA treatment reduced FUT3 mRNA expression by about 80%, and a dramatic reduction (95%) of the sLe^x antigen was also achieved. This supports the notion that FUT3 is a major FUT enzyme in sLe^x synthesis pathway of MDA prostate cancer cells. A rapid 50% knockdown of sLe^x was observed in less than 24 hours after transfection, reinforcing the potential of this novel therapeutic approach. Similar to other research groups [43], we did not detect sLe^a antigen expression on MDA cells using monoclonal antibody CA 19-9. In addition, FUT3 siRNA did not affect sLe^x expression in HL60 cells (data not shown), indicating that this enzyme is dispensable for leukocyte function. In Colo205 cells, FUT3 siRNA-reduced sLe^x expression only fractionally, suggesting that FUT3, 4, and 6 may be functionally redundant. In such cases, treating cells with a combination of siRNAs targeting multiple FUTs may be more effective.

Rolling on selectins is dependent on two parameters, the force applied to the cell as a result of fluid flow and the site density of the selectin or the selectin ligand molecule [44,45]. By altering the density of sLe^x molecule we can reduce the interactions of the cancer cells with the endothelial cells. FUT7 has been shown to induce prostate cancer cell trafficking to the bone, and FUT7 is expressed in high amounts in leukocytes [29]. In such cases knocking

down a small proportion of FUT7 might be enough to impair cancer-cell interaction with selectins while maintaining leukocyte function. Work by Hammer and colleagues shows that just a 60% decrease in selectin ligand density can result in an 8-fold decrease in adhesion strength as measured by changes in rolling velocity [52].

The functional impact of FUT3 siRNA on MDA cells was assessed with two methods. In a 96-well plate-based adhesion assay, FUT3 siRNA dramatically impaired the ability of cells to adhere to an E-selectin-coated surface and increased the EC₅₀ of dose response curves. In a flow-based cell capturing experiment, FUT3 siRNA significantly reduced the number of cells captured by E-selectin-coated microtubes and increased cell-rolling velocity. Our results further support the functional importance of FUT3 in E-selectin-mediated CCC recruitment and the feasibility of disrupting CCC metastasis using an siRNA approach. An intriguing observation from our research is the cell growth inhibition by FUT3 siRNA. Inhibition of tumor growth with reduced expression of FUT enzymes has been reported in a few references [46,47]. In MDA cells, FUT3 may be important in the fucosylation and proper functions of proliferation-related protein substrates other than E-selectin ligands. On the other hand, E-selectin ligands are transmembrane molecules, which may translate the extracellular adhesive interactions into various intracellular signals. Such signals, potentially important for cell mobility or cell survival, have not been well characterized. For instance, E-selectin was reported to induce tyrosine phosphorylation in HT-29 colon cancer cells [48].

Our study provides support for using FUT3 siRNA to disrupt CCC metastasis. When delivered systemically, FUT3 siRNA will target epithelial cells without affecting leukocytes. A drug delivery device targeting in the circulatory system would help to ensure the delivery of siRNAs specifically to CCCs, not healthy peripheral epithelial cells. Our group has successfully developed a novel method of delivering siRNA using P-selectin conjugated nanolipids. These nanolipids are immobilized to the inner surface of microtubes, which effectively and specifically deliver siRNA molecules to circulating cells, achieving a knockdown efficiencies of over 90% while flowing in a short duration of two hours[38]. The same method could be used to deliver siRNAs to circulating CCCs. A limitation of this approach would be the inability to target cells that have left the circulation and have entered protected microdomains. However, when combined with conventional chemotherapy this limitation may be overcome while increasing the circulation times for CCCs. Furthermore, a combinational cancer therapy can be achieved by coating microtubules simultaneously with molecules that deliver apoptotic signals to CCCs [49]. Future work will focus on developing such therapeutic approaches that minimize off-target toxicities associated with systemic delivery of FUT3 siRNA molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Differential expression of α 1,3-FUTs between epithelial cancer cell lines and hematopoietic cell lines

FUT3, 4, 5, 6, 7 and 9 mRNA levels in Colo205, HL60, KG1a and MDA Pca 2b (MDA) cell lines were measured by quantitative RT-PCR. A, mRNA level was normalized to GAPDH; B, mRNA level was further normalized to the expression of corresponding genes in HL60 cells. Cell line Colo205 and MDA both have significantly ($P < 0.001$, $n = 3$) higher FUT3 expression compared to HL60 and KG1a cells.

Fig. 2. FUT3 siRNA led to effective knockdown of FUT3 expression

A, The knockdown of FUT3 mRNA by two sets of distinct siRNA molecules as demonstrated by quantitative RT-PCR (* represents $P < 0.05$, $n = 3$). B, FUT3 mRNA expression as demonstrated with semi-Q-PCR. Both GAPDH and FUT3 PCR products are around 500 bp.

Fig. 3. FUT3 siRNA inhibits cell-surface sialyl Lewis antigen synthesis

A. Flow cytometry analysis with anti-CLA antibody that recognizes both sLe^x and sLe^a. Top and bottom graphs are the representative results from cells transfected with control siRNA or FUT3 siRNA, respectively. Black histograms indicate isotype control staining and red histograms indicate anti-CLA staining. B. Quantification of sialyl Lewis antigen expression. These data were recorded 3 days after transfection (* represents $P < 0.05$, $n = 3$). C. The kinetics of sialyl Lewis antigen knockdown. siRNA set #2 was used in this experiment, $n = 3$ to 5 for each time point.

Fig. 4. FUT3 siRNA inhibits E-selectin-mediated cell adhesion

A. Images of MDA cells adherent to E-selectin-coated 96-well plates. The top and bottom images represent cells transfected with control siRNA or FUT3 siRNA, respectively (Day 4 after transfection). The scale bar represents 100 μ M in length. B. A representative dose response analysis result ($n = 4$ for each point). C. The effects of FUT3 siRNA on cell adhesion at different E-selectin concentrations. At 1, 5, 10 and 20 μ g/mL E-selectin concentration, FUT3 siRNA treatment led to $67 \pm 15\%$, $91 \pm 1\%$, $83 \pm 1\%$ and $79 \pm 1\%$ reduction of adhesion. * represents $p < 0.05$ and ** represents $P < 0.001$.

Fig. 5. FUT3 siRNA reduced cell capture from flow and increased cell rolling velocity

A. Images of MDA cells captured from flow by E-selectin-coated microtubes. The top and bottom images represent cells transfected with control siRNA or FUT3 siRNA, respectively (Day 4 after transfection). The scale bar represents 100 μ m in length. B. The numbers of cells captured within a 50-cm length of tubing in 10 min. * represents $P < 0.001$. C. The rolling velocity of cells under two distinct shear stresses. Open and filled boxes represent cells transfected with control and FUT3 siRNAs, respectively. * represents $P < 0.001$, $n = 151-165$.

Fig. 6. FUT3 siRNA reduced MDA cell growth rate

A. Growth curves for cells transfected with FUT3 siRNA set 1 and control siRNA; B. Growth curves for cells transfected with FUT3 siRNA set 2 and control siRNA. Results from 3 independent experiments were combined and are shown in each graph. * represents $P < 0.01$