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Analysis of glycosyltransferase expression in metastatic prostate cancer cells capable of rolling activity on microvascular endothelial (E)-selectin

Steven R Barthel[2,3], Jacyln D Gavino[2], Georg K Wiese[2], Jennifer M Jaynes[2], Javed Siddiqui[4], and Charles J Dimitroff[1,2,3]

[2]Department of Dermatology, Brigham and Women's Hospital, Harvard Skin Disease Research Center; [3]Harvard Medical School, Boston, MA 02115; and [4]Department of Urology, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48109, USA

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Prostate cancer (PCa) cell tethering and rolling on microvascular endothelium has been proposed to promote the extravasation of PCa cells. We have shown that these adhesive events are mediated through binding interactions between endothelial (E)-selectin and Lewis carbohydrates on PCa cells. Prior data indicate that E-selectin-mediated rolling of bone-metastatic PCa MDA PCa 2b (MDA) cells is dependent on sialyl Lewis X (sLe^X)-bearing glycoproteins. To explore the molecular basis of sLe^X synthesis and E-selectin ligand (ESL) activity on PCa cells, we compared and contrasted the expression level of glycosyltransferases, characteristically involved in sLe^X and ESL synthesis, in ESL$^+$ MDA cells among other ESL$^-$ metastatic PCa cell lines. We also created and examined ESL^{hi} and ESL^{lo} variants of MDA cells to provide a direct comparison of the glycosyltransferase expression level. We found that normal prostate tissue and all metastatic PCa cell lines expressed glycosyltransferases required for sialo-lactosamine synthesis, including *N*-acetylglucosaminyl-, galactosyl-, and sialyltransferases. However, compared with expression in normal prostate tissue, ESL$^+$ MDA cells expressed a 31- and 10-fold higher level of α1,3 fucosyltransferases (FT) 3 and 6, respectively. Moreover, FT3 and FT6 were expressed at 2- to 354-fold lower levels in ESL$^-$ PCa cell lines. Consistent with these findings, ESL^{hi} MDA cells expressed a 131- and 51-fold higher level of FT3 and FT6, respectively, compared with expression in ESL^{lo} MDA cells. We also noted that α1,3 FT7 was expressed at a 5-fold greater level in ESL^{hi} MDA cells. Furthermore, ESL^{lo} MDA cells did not display sLe^X on glycoproteins capable of bearing sLe^X, notably P-selectin glycoprotein ligand-1. These results implicate the importance of α1,3 FT3, FT6, and/or FT7 in sLe^X and ESL synthesis on metastatic PCa cells.

Keywords: E-selectin/fucosyltransferase/metastasis/prostate cancer/sialyl Lewis X

[1]To whom correspondence should be addressed: Tel: +1-617-525-5693; Fax: +1-617-525-5571; e-mail: cdimitroff@rics.bwh.harvard.edu

Introduction

Metastatic prostate cancer (PCa) is characterized by movement of PCa cells out of the prostate gland into the circulation and subsequent trafficking to and colonization of distant tissues. Nearly 90% of all PCa metastases home to the bone marrow (BM), especially to spinal lumbar via the venous drainage system (Benjamin 2002). The mechanism that directs such osteotropism is unclear and of profound importance for the development of antimetastatic therapeutics. Recent findings by our lab and others indicate that bone metastasis may be orchestrated by specific binding events between ligands displayed on the PCa cell surface and receptors expressed on BM endothelial cells (BMEC). In support, bone-metastatic PCa cells have been shown to attach more avidly to BMEC compared with endothelial linings from other organs, including umbilical vein (Lehr and Pienta 1998; Scott et al. 2001; Simpson et al. 2001), lung (Scott et al. 2001), aorta or skin (Cooper et al. 2000). Consistent with these findings, we have reported previously that bone-metastatic prostate tumor MDA PCa 2b (MDA) cells (Navone et al. 1997) roll and form stable adhesive contacts under hydrodynamic shear flow on endothelial (E)-selectin expressed by BMEC (Dimitroff et al. 2004). Rolling was supported principally by sialyl Lewis X (sLe^X)-bearing glycoproteins, namely P-selectin glycoprotein ligand-1 (PSGL-1), displayed on the MDA cell surface. We hypothesize that recognition of BMEC by circulating PCa cells mimics and exploits identical E-selectin–E-selectin ligand interactions utilized by hematopoietic progenitor cells (HPC) in transit to BM (Hidalgo et al. 2002; Katayama et al. 2003). That is, like bone-tropic HPC (Dimitroff et al. 2001), MDA cells also express the sLe^X-bearing glycoform of PSGL-1 (Dimitroff et al. 2005). Further, the amount of PSGL-1 and sLe^X on PCa tissue is associated with PCa grade or metastasis (Jorgensen et al. 1995; Martensson et al. 1995; Idikio 1997; Dimitroff et al. 2004, 2005). Metastatic PCa cells express functional CXCR4 chemokine receptor (Taichman et al. 2002; Sun et al. 2003; Singh et al. 2004; Engl et al. 2006), collagenases (Singh et al. 2004), ADAM (a disintegrin and metalloproteinase) (Najy et al. 2008), as well as several active integrin heterodimers (Cress et al. 1995; Fornaro et al. 2001; Cooper et al. 2002; Nemeth et al. 2003; Engl et al. 2006). Taken together, these findings support a model whereby PCa cell metastasis to bone obeys the multistep paradigm of HPC recruitment to bone, defined as the "hematopoietic mimicry" hypothesis (Barthel et al. 2007).

Based on the putative role of sLe^X in PCa cell adhesion and metastasis, understanding how sLe^X biosynthesis is regulated is an important yet underappreciated aspect of PCa pathobiology. In the case of HPC and leukocytes, synthesis of sLe^x on E-selectin-binding membrane proteins is well established; it is characterized by a series of catalytic steps in the Golgi compartment by members of the glycosyltransferase gene family

(Lowe 2002, 2003; Lowe and Marth 2003). One of the major E-selectin-binding glycans is a sLe^X moiety on "core 2" branched *O*-linked glycans. Synthesis of this determinant is initiated by core 2 branching enzymes, β1,6 *N*-acetylglucosaminyltransferases (C2GlcNAcT). The sialyllactosamine backbone is completed by sequential catalysis mediated by β1,4 galactosyl (GalT)- and α2,3 sialyltransferases (ST). The terminal step in sLe^X synthesis on the core 2 glycan involves the transfer of fucose to *N*-acetylglucosamine by α1,3 fucosyltransferases (FT) (de Vries et al. 2001). Of the six known α1,3 FTs encoded in the human genome, FT4 and FT7 are expressed in HPC, neutrophils, and T cells and represent key enzymes in selectin ligand-mediated trafficking during routine immunosurveillance and inflammatory conditions (Clarke and Watkins 1996; Knibbs et al. 1996, 1998;Wagers et al. 1996; Le Marer et al. 1997; Naiyer et al. 1999; Huang et al. 2000; Weninger et al. 2000; Homeister et al. 2001; Smithson et al. 2001; Schottelius et al. 2003). In addition, FT4 and FT7 as well as two other α1,3 FTs 3 and 6 are all associated with progression of at least one of several cancers, including leukemia (Le Marer et al. 1997; Kannagi 2001), colon (Majuri et al. 1995; Weston et al. 1999; Hiller et al. 2000), breast (Matsuura et al. 1998; Ding and Zheng 2004), lung (Ogawa et al. 1996; Togayachi et al. 1999), liver (Liu et al. 2000; Wang et al. 2003), gastric (Petretti et al. 1999), or pancreatic (Mas et al. 1998; Aubert et al. 2000). That is, evidence suggests that these enzymes mediate sialyl Lewis carbohydrate synthesis, principally sLe^X and/or sLe^a, which may in turn trigger selectin-binding activity and promote tumor growth and metastasis (Barthel et al. 2007; Miyoshi et al. 2008). In PCa, however, the role of glycosyltransferases, in particular α1,3 FTs, has been largely understudied.

In this report, we present genetic and immunochemical evidence implicating α1,3 FTs as key determinants in the biosynthesis of sLe^X as well as in the generation of E-selectin-binding glycans on metastatic PCa cells. We observed a 31- and 10-fold enhancement in gene expression of FT3 and FT6, respectively, in E-selectin ligand $(ESL)^+$ MDA cells compared with normal prostate tissue, where ESL^- PCa cell lines showed 2- to 354-fold lower levels of FT3 and FT6. To further define the role of glycosyltransferases in ESL synthesis in PCa, we created and then compared glycosyltransferase levels in MDA cell variants expressing low or high ESL levels (ESL^{lo} or ESL^{hi}). In this comparison, high α1,3 FT expression, specifically FT3, FT6, FT7 and FT9, was noted in ESL^{hi} MDA cells and associated with synthesis of sLe^X on PSGL-1. Loss of these FTs from ESL^{lo} MDA cells correlated with the absence of sLe^X on PSGL-1. These results indicate that bone-metastatic PCa cells may share glycosylation machinery with HPC and leukocytes to be exploited as a traffic control mechanism of extravasation. We speculate that bone metastasis in PCa may involve induction of sLe^X on E-selectin-binding glycoproteins via the upregulation of α1,3 FT expression.

Results

α1,3 FTs 3 and 6 are upregulated in ESL^+ metastatic PCa cells

To investigate the role of glycosyltransferases in the synthesis of Lewis carbohydrates and ESL in PCa, we identified first the metastatic PCa cell lines that exhibit robust ESL activity. In binding assays involving immobilized E-selectin, we validated the conspicuous ESL activity of the bone-metastatic PCa cell line, MDA PCa 2b (MDA), which we will refer to henceforth as ESL^+ MDA cells (Dimitroff et al. 2005) (Figure 1A). In contrast, other metastatic PCa cell lines, including PC-3, PC-3M LN4, LNCaP, and DU-145, did not bind E-selectin as previously described (Dimitroff et al. 2005) (Figure 1A). In control binding experiments, HPC (KG1a) cells also possessed robust ESL activity as reported previously (Dimitroff et al. 2001, 2004), while normal prostate epithelial cells did not. In experiments (not shown) neither normal prostate nor metastatic PCa cells, including MDA cells, bound P-selectin, in contrast to KG1a cells, which bound P-selectin avidly. Pretreating KG1a cells and MDA cells with neuraminidase (Figure 1B) or EDTA (not shown) blocked E-selectin-binding completely. Removal of the surface protein by preincubation with bromelain blocked KG1a and MDA E-selectin-binding by 20% and 50%, respectively (not shown), indicating that glycoproteins terminated in sialic acid were major contributors of the E-selectin-binding phenotype (Figure 1B). Residual ESL activity on these cells was likely due to glycolipid ligands as previously described

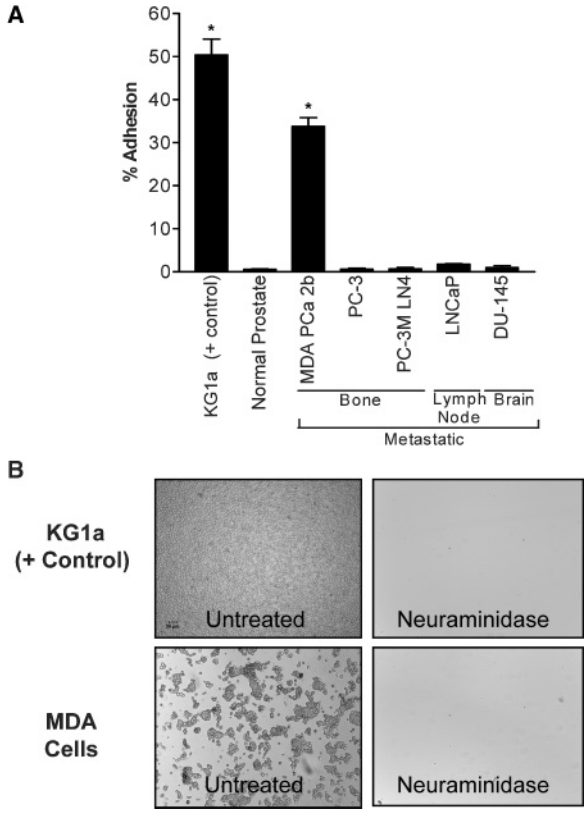

Fig. 1. ESL activity of metastatic PCa MDA cells is dependent on sialylated membrane structures. Metastatic PCa cell lines, MDA, PC-3, PC-3M LN4, LNCaP, and DU-145, normal prostate epithelia, and control ESL^+ KG1a cells were examined for ESL activity in a microtiter 96-well E-selectin-Ig-binding assay. (**A**) KG1a and MDA cells exhibited ESL activity, while all other cells had no activity. Negative control wells, consisting of cells plated on human IgG-adsorbed plastic or cells incubated in an EDTA-containing assay medium, had no ESL activity (not shown). (**B**) Pretreatment of MDA and KG1a cells with *Vibrio cholerae* neuraminidase completely ablated ESL activity. Experiments were performed in triplicate wells on at least three separate occasions. $^*P < 0.01$, Statistical significant difference compared with adhesion to human IgG control; one-way ANOVA with Dunnett's post-test.

Table I. Primers used in real-time PCR analysis of glycosyltransferase expression

Gene	Primer sequences 5′–3′	Product size (BP)	*E*-value	Reference
C2GlcNAcT-I	F: GACGTTGCTGCGAAGG	131	8.0×10^{-1}	Kikuchi, Jiro et al. 2005
	R: CCAAGTGTCTGACACTTACA		7.0×10^{-3}	
C2GlcNAcT-II	F: GCGAAAGAACCCTCAATCAG	96	1.5×10^{-2}	Primer 3 software
	R: GCTGCAGTTTCCCTTCAGTC		1.5×10^{-2}	
C2GlcNAcT-III	F: CCCACTGAGCAAAGAAGAGG	100	1.5×10^{-2}	Primer 3 software
	R: GGCATACACAGCTCGCAGTA		1.5×10^{-2}	
β1,4GalT-I	F: TCACAAGGTGGCCATCATCA	83	7.0×10^{-3}	Sasaki, Norihiko et al. 2005
	R: GCAGGACTGGGTGCAAATAATAT		2.0×10^{-4}	
β1,4GalT-III	F: CCATGTTGCCGTTGCTATGA	76	1.5×10^{-2}	Sasaki, Norihiko et al. 2005
	R: AGTGCTGAGACTCCTCCGAAGT		1.0×10^{-3}	
ST3Gal-I	F: TCGGCCTGGTTCGATGA	63	6.1×10^{-1}	Primer 3 software
	R: CGCGTTCTGGGCGGTCA		6.1×10^{-1}	
ST3Gal-III	F: GGTGGCAGTCGCAGGATTT	76	3.9×10^{-2}	Higai, Koji et al. 2006
	R: CATGCGAACGGTCTCATAGTAGTG		1.0×10^{-4}	
ST3Gal-IV	F: CGGGTGCGAAAGGGTTT	72	6.1×10^{-1}	Higai, Koji et al. 2006
	R: GAGAATCCGAATCTGTTTAGGATTG		4.0×10^{-5}	
FT1	F: TGAGGGATCACTGCCAAAATG	92	5.0×10^{-3}	Higai, Koji et al. 2006
	R: TCTTGGCAGTTTATGAGCTTTAAAAA		1.0×10^{-5}	
FT2	F: GCTCGCTACAGCTCCCTCAT	79	1.5×10^{-2}	Higai, Koji et al. 2006
	R: CGTGGGAGGTGTCAATGTTCT		5.0×10^{-3}	
FT3	F: GCCGACCGCAAGGTGTAC	75	1.5×10^{-1}	Higai, Koji et al. 2006
	R: TGACTTAGGGTTGGACATGATATCC		4.0×10^{-5}	
FT4	F: AAGCCGTTGAGGCGGTTT	88	1.5×10^{-1}	Higai, Koji et al. 2006
	R: ACAGTTGTGTATGAGATTTGGAAGCT		1.0×10^{-5}	
FT5	F: TATGGCAGTGGAACCTGTCA	100	1.5×10^{-2}	Primer 3 software
	R: CGTCCACAGCAGGATCAGTA		1.5×10^{-2}	
FT6	F: CAAAGCCACATCGCATTGAA	93	1.5×10^{-2}	Higai, Koji et al. 2006
	R: ATCCCCGTTGCAGAACCA		1.5×10^{-1}	
FT7	F: TCCGCGTGCGACTGTTC	65	6.1×10^{-1}	Higai, Koji et al. 2006
	R: GTGTGGGTAGCGGTCACAGA		1.5×10^{-2}	
FT8	F: TTGCCATTTATGCTCACCAA	78	1.5×10^{-2}	Primer 3 software
	R: TTCCAGCCACACCAATGATA		1.5×10^{-2}	
FT9	F: TCCCATGCAGTTCTGATCCAT	78	5.0×10^{-3}	Higai, Koji et al. 2006
	R: GAAGGGTGGCCTAGCTTGCT		1.5×10^{-2}	
GAPDH	F: CAGCCTCAAGATCATCAGCA	138	1.5×10^{-2}	Higai, Koji et al. 2006
	R: ACAGTCTTCTGGGTGGCAGT		1.5×10^{-2}	

(Dimitroff et al. 2005). These results strengthen the hypothesis that bone-metastatic PCa cells mimic HPC cells in recognition of E-selectin through the upregulation and synthesis of sialic acid-bearing glycans.

Sialic acid-bearing glycans are synthesized in HPCs by well-established biosynthetic pathways involving glycosyltransferases (Lowe 2002, 2003; Lowe and Marth 2003). Thus, we first determined whether glycosyltransferases involved in generating E-selectin-binding determinants on HPC were also involved in the synthesis of ESL on MDA cells. Using real-time PCR and ESL^+ KG1a (HPC) cells as a positive control for FT4 and FT7 expression, we analyzed the expression of glycosyltransferase genes in ESL^+ MDA cells and in ESL^- metastatic PCa cell lines PC-3, PC-3M LN4, LNCaP, and DU-145. As shown in Table I, we used primer sets specific for core 2 β1,6 *N*-acetylglucosaminyl- (C2GlcNAcT), β1,4 galactosyl- (GalT), α2,3 sialyl- (ST), and α1,3 FTs. RNA was purified from cells and reverse transcribed, and cDNA was used as a template for PCR in real-time. Expression of glycosyltransferase mRNA was normalized to GAPDH mRNA levels, compared with levels of mRNA in normal prostate tissue, and then expressed as mRNA fold difference. The mean (standard error of the mean, SEM) was calculated from a minimum of three separate experiments. Of note, we used normal prostate tissue and not normal human prostate epithelial cell cultures as a comparison tissue. Two of the major supplements in a human prostate epithelia growth medium are retinoic acid and hydrocortisone. Retinoic acid and hydrocortisone are potent negative and positive regulators, respectively, of glycosyltransferases involved in sLe^X and ESL synthesis (Liepkalns et al. 1995; Biol-N'garagba et al. 2003; Yamanaka et al. 2008). Since we were interested in studying the role of glycosyltransferases in ESL expression in PCa, we considered that normal prostate tissue may express a more native and accurate glycosyltransferase expression pattern than expressed in cultured prostate epithelia. Thus, we avoided the down- or upregulation of glycosyltransferase levels that might be associated with normal prostate epithelia grown in culture and that could erroneously augment differences when comparing expression with metastatic PCa cells.

In control experiments, ESL^+ KG1a cells expressed a 54- and 88-fold elevation of FT4 and FT7, respectively, compared with normal prostate tissue (Figure 2). High expression of FT4 and FT7 is in agreement with a prior report establishing these two enzymes as key regulators in the synthesis of sialic acid-bearing glycans on KG1a cells (Clarke and Watkins 1997). ESL^+ MDA cells also showed elevated α1,3 FT expression, suggesting that ESL^+ MDA cells may harness the same family of enzymes as KG1a cells for the synthesis of E-selectin-binding glycans (Figure 2). Whereas FT4 and FT7 were elevated in KG1a cells, MDA cells exhibited a 31- and 10-fold elevation of FT3 and FT6, respectively. Furthermore, MDA cells expressed the complete set of all other glycosyltransferases required for the synthesis

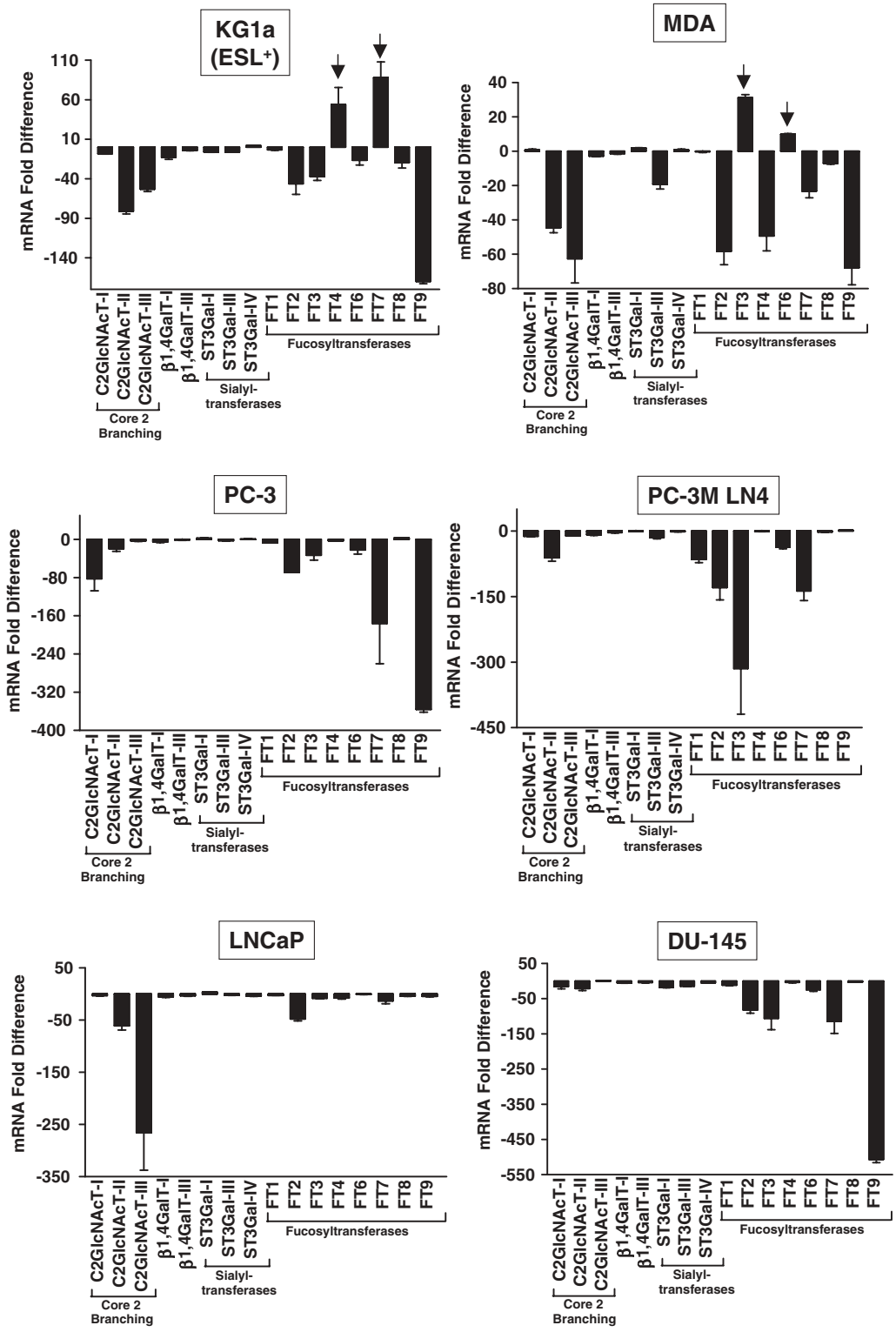

Fig. 2. Elevated α1,3 fucosyltransferase expression is related to ESL activity on metastatic PCa MDA cells. Real-time PCR analysis of putative sLe^X-synthesizing glycosyltransferases, including *N*-acetylglucosaminyl-, galactosyl-, sialyl-, and fucosyltransferases, was performed on cDNA isolated from control ESL^+ KG1a cells, metastatic PCa cell lines, MDA, PC-3, PC-3M LN4, LNCaP, and DU-145, and normal prostate tissue. Expression is reported as the mean (SEM) mRNA fold difference after normalizing to expression level in fresh normal prostate epithelial tissue. Arrows denote elevation of FT3 and FT6 expression in ESL^+ MDA cells and FT4 and FT7 in ESL^+ KG1a cells. Experiments were performed a minimum of three times.

of sialyllactosamine E-selectin-binding structures, including C2GlcNAcTI-III, β1,4 GalTI and III, and α2,3 STs, ST3GalI, III and IV. Likewise, all ESL$^-$ metastatic PCa cell lines expressed C2GlcNAcTs, GalTs, STs, as well as eight of nine FTs; however, none displayed elevated FT3 or FT6 (Figure 2). In fact, ESL$^-$ PCa cell lines exhibited a 2-to 354-fold reduction in FT3 and FT6 expression. FT5 was not detected in any of the PCa cells or in normal prostate tissue using two different primer sets. Interestingly, with the exception of FT5, normal prostate tissue expressed all glycosyltransferases tested, indicating that prostate epithelia contain the prerequisite glycosylation machinery for ESL synthesis but lack the upregulation of α1,3 FTs that may correspond to completion of ESL synthesis and PCa progression and metastasis. These results indicated that elevated FT3 and FT6 may be directly associated with ESL activity on MDA cells.

Sialyl Le^X is the E-selectin-binding determinant on ESL$^+$ metastatic PCa cells

FT3 and FT6 are potential synthesizers of sLe^X (de Vries et al. 2001). In addition, FT3 can also potentially generate sLe^a by virtue of its α1,4 FT catalytic activity (de Vries et al. 2001). Since both sLe^X and sLe^a can bind E-selectin (Phillips et al. 1990; Iwai et al. 1993; Weitz-Schmidt et al. 1996; Inoue et al. 1997), we assayed whether one or both of the sLe^X or sLe^a antigens may be responsible for the FT3- and FT6-mediated ESL activity on MDA cells. We performed flow cytometric analysis with several mAbs that recognize different structural variants of sLe^X and also analyzed sLe^a. Graphical representations of mAb specificities for Lewis carbohydrate epitopes are depicted in Figure 3. In control experiments, ESL$^+$ KG1a cells reacted with all five anti-sLe^X mAbs, including HECA-452, CSLEX-1, FH-6, KM-93, and CHO-131 (Figure 4A). Robust synthesis of sLe^X in KG1a cells is consistent with the high expression of FT4 and FT7 in Figure 2 and in agreement with previous reports (Clarke and Watkins 1997; Dimitroff et al. 2001). ESL$^+$ MDA cells also reacted with anti-sLe^X mAbs, including HECA-452, CSLEX-1, FH-6, and KM-93. MDA cells, however, were not reactive to mAb CHO-131, an indicator of sLe^X on core 2 *O*-glycans and a positive predictor of P-selectin-binding (Walcheck et al. 2002; Ni et al. 2006) (Figure 4A). Lack of CHO-131 reactivity is, therefore, in accord with cell binding results in which MDA cells bound E- but not P-selectin. Furthermore, neither MDA nor KG1a cells expressed Le^X or sLe^a (Figure 4B). The absence of Le^X expression on MDA cells is consistent with 49- and 68-fold decreases in FT4 and FT9 (Figure 2), respectively, which fucosylate nonsialylated lactosamines (de Vries et al. 2001; Nakayama et al. 2001), and is in agreement with previous reports of minimal Le^X staining in formalin-fixed benign and PCa tissue (Martensson et al. 1995). In contrast to MDA cells, HT-29 colon carcinoma cells were positive for Le^X and for sLe^a as reported previously (Mack et al. 1998; Hasegawa et al. 2005) (data not shown). These findings indicated that glycosyltransferase activity in ESL$^+$ KG1a and ESL$^+$ MDA cells may be skewed toward α1,3 FT and not α1,4 FT activity, as evidenced by sLe^X and not sLe^a expression.

To further analyze α1,3 FT and α1,4 FT activity in ESL$^+$ MDA cells, we assayed the expression of two other Lewis carbohydrates, Le^y and Le^b, which are dependent on either α1,3 or α1,4 FT activity, respectively. We found that MDA and control

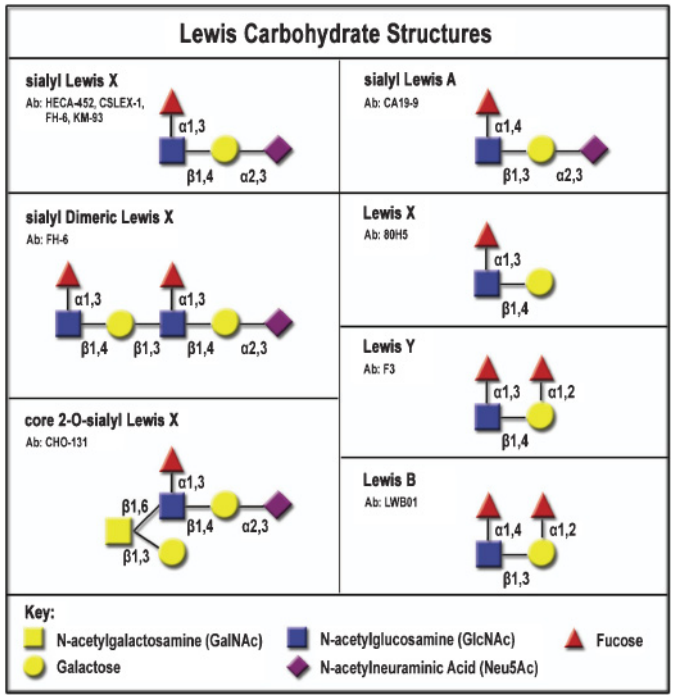

Fig. 3. Structures of Lewis carbohydrates. Schematic of Lewis carbohydrate determinants recognized by respective mAbs in flow cytometric assays. Epitopes recognized by HECA-452, CSLEX-1, FH-6, KM-93, CA19-9, and F3 (Kannagi and Hakomori 2001) as well as CHO-131 (Walcheck et al. 2002), 80H5 (Benharroch et al. 2000), and LWB01 (Torrado et al. 1992) have been reported.

KG1a cells expressed only a minimal amount of Le^b (Figure 4B), while SW948 and LS174T colon carcinoma cells exhibited robust Le^b expression (not shown). Moreover, Le^y was expressed on MDA cells at a 1000-fold greater fluorescence intensity than Le^b (Figure 4B), which is supported by other findings showing high Le^y expression on 26 of 30 PCa specimens (Martensson et al. 1995). These results suggest that α1,3 FT activity, presumably contributed by FT3 and FT6, may control ESL activity on circulating PCa cells and that sLe^a, though a critical carbohydrate feature of ESL on colorectal and pancreatic cancer cells (Iwai et al. 1993; Inoue et al. 1997; Yoshida et al. 1999) may not be a key Lewis carbohydrate structure for eliciting ESL activity on metastatic PCa cells.

Since sLe^X was expressed on ESL$^+$ MDA cells, we assayed whether sLe^X was also expressed on ESL$^-$ metastatic PCa PC-3 cells. There was minimal expression of sLe^X on ESL$^-$ PC-3 cells as assayed with HECA-452, CSLEX-1, FH-6, KM-93, and CHO-131 (Figure 4A). Moreover, PC-3 cells expressed Le^y, but not sLe^a, Le^X, or Le^b (Figure 4B). Another ESL$^-$ cell line, LNCaP, also expressed only minimal sLe^X, whereas PC-3M LN4 and DU-145 cell lines expressed a high amount based on HECA-452 mAb reactivity (data not shown). However, PC-3M LN4 and DU-145 cell lines do not contain anti-sLe^X mAb-reactive glycoproteins (Dimitroff et al. 2005). Collectively, these results suggested that sLe^a, Le^y, Le^X, or Le^b expression does not correspond to ESL activity, whereas sLe^X expression, particularly on glycoproteins, is a direct correlate with ESL activity on metastatic PCa cells.

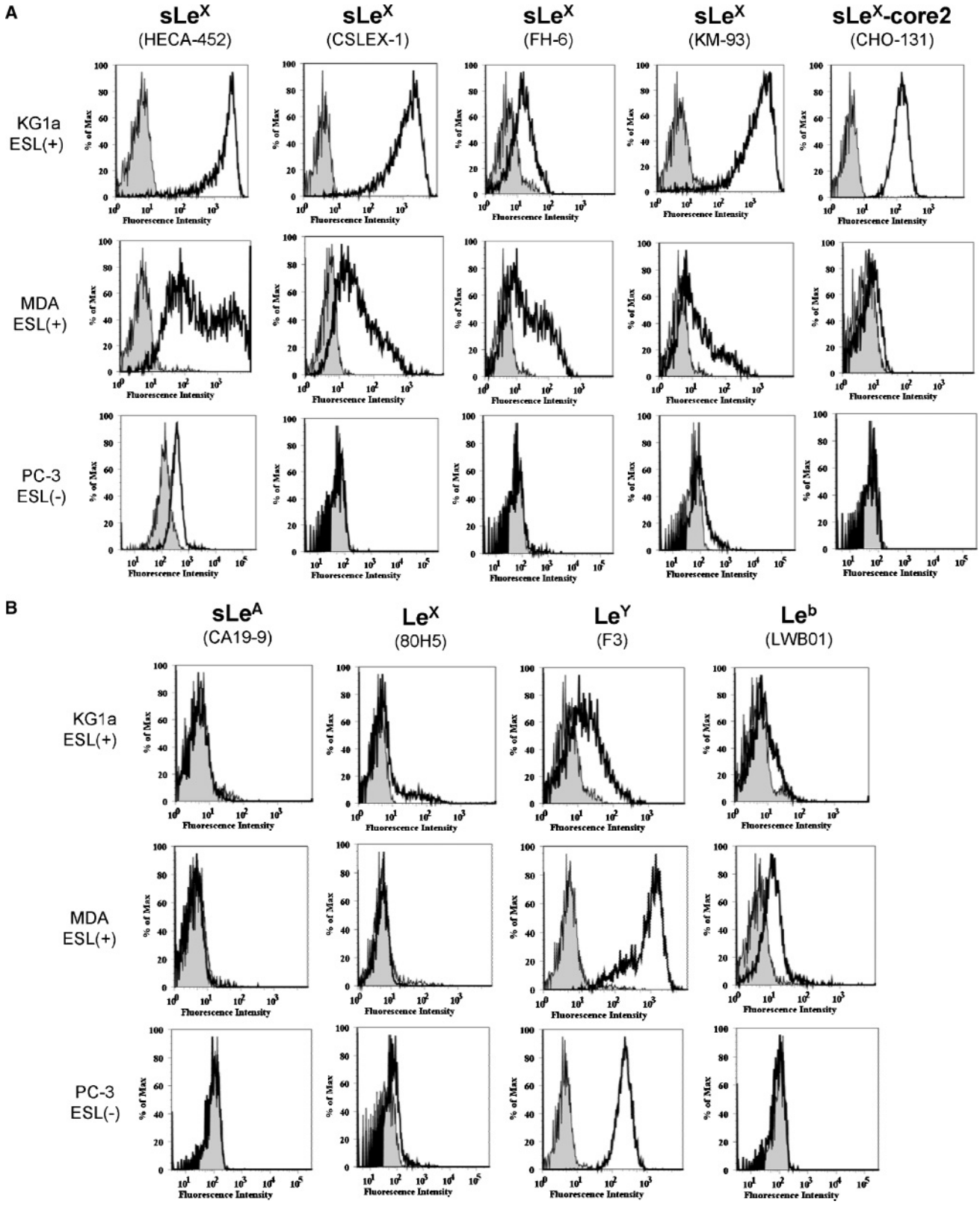

Fig. 4. SLe^X and not sLe^a moieties are elevated on ESL^+ metastatic PCa MDA cells. (**A**) Flow cytometric analysis of sLe^X moieties and (**B**) sLe^a, Le^X, Le^y, and Le^b was performed on control ESL^+ KG1a cells as well as on ESL^+ MDA and ESL^- PC-3 cells. Open histograms: staining with anti-Lewis carbohydrate mAb; shaded histograms: staining with isotype control mAb. ESL^+ cells were recognized by anti-sLe^X mAbs, HECA-452, CSLEX-1, FH-6, KM-93, whereas ESL^- PC-3 cells were negative for sLe^X. ESL^+ and ESL^- PCa cells were negative for sLe^a and positive for Le^Y. These experiments were performed a minimum of three times.

Expression of sLe^X-reactive PSGL-1, ST3Gal-III, and FT3, FT6, FT7, and FT9 corresponds to ESL$^+$ metastatic MDA PCa cells

Since four of five metastatic cell lines were ESL$^-$ and showed a decline in FT3 and FT6 (Figures 1A and Figure 2), we hypothesized that the ESL$^-$ activity might be the result of a decrease in native ESL expression, i.e., through prolonged culturing of PCa cells in vitro. In fact, we found that long-term culturing of MDA cells in the media recommended by the manufacturer led to reduced E-selectin-binding. Such loss of ESL activity might be due to diminution in α1,3 FT expression. We, therefore, took advantage of this drift in ESL phenotype and determined whether loss of E-selectin-binding was associated with a corresponding loss in expression of FT3 and FT6. As shown in photomicrographs, we created ESL^{hi} and ESL^{lo} MDA cell variants, based on ability to bind E-selectin (5A and B). Expression of sLe^X was reduced on ESL^{lo} MDA cells compared to ESL^{hi}, indicating that the loss in ESL activity could be the result of diminished sLe^X synthesis (Figure 5C). In Western blot assays probed with anti-sLe^X mAb HECA-452, ESL^{hi} MDA variants expressed sLe^X on the 120 kDa membrane glycoprotein, identified previously as PSGL-1 (Dimitroff et al. 2005) (Figure 5E). In ligand blotting assays, PSGL-1, along with another glycoprotein, identified before as ESL-1 (Dimitroff et al. 2005), bound E-selectin-Ig chimera (Figure 5E). These blots confirmed prior findings, indicating that PSGL-1 and not ESL-1 functions as the principal E-selectin glycoprotein ligand on MDA cells (Dimitroff et al. 2005). On the other hand, ESL^{lo} MDA cells neither expressed sLe^X nor E-selectin-binding determinants on membrane glycoprotein (Figure 5E), consistent with their inability to recognize E-selectin in cell binding assays and with their reduced expression of sLe^X (Figure 5A, B, and C). Of note, flow cytometric analysis of PSGL-1 showed that both ESL^{lo} and ESL^{hi} MDA variants expressed a similar amount of PSGL-1 protein (Figure 5D). These results suggested that sLe^X-synthesizing enzymes were upregulated in ESL^{hi} compared with ESL^{lo} MDA variants.

To identify glycosyltransferases that directly relate to ESL activity on ESL^{hi} MDA cells, we analyzed mRNA expression of C2GlcNAcTs, GalTs, STs, and FTs by real-time PCR. We found that ST3Gal-III and FT3, FT6, FT7, and FT9 were upregulated by 11-, 131-, 51-, 5-, and 61-fold, respectively, in ESL^{hi} MDA cells compared with expression levels in ESL^{lo} MDA cells (Figure 6). These data were consistent with the difference in the mRNA expression level between ESL$^+$ MDA and ESL$^-$ PCa cells (Figure 2) and reinforced the importance of FT3 and FT6, and potentially FT7, in creating sLe^X and in generating the ESL activity of metastatic PCa cells. Indeed, transfection of FT3 into ESL$^-$ PC-3 cells, for example, induces sLe^X expression and also enhances E-selectin-binding (Inaba et al. 2003).

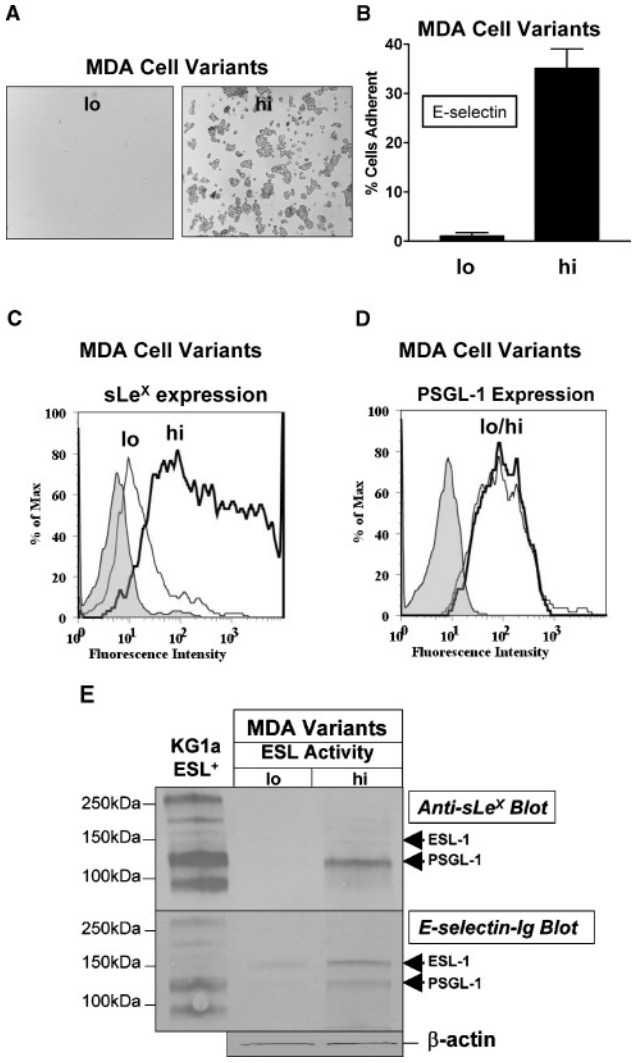

Fig. 5. ESL^{hi} metastatic PCa MDA cell variants express sLe^X- and E-selectin-Ig-reactive PSGL-1. (**A** and **B**) MDA cell variants were separated into ESL^{lo} and ESL^{hi} based on their E-selectin-binding characteristics. (**C** and **D**) Flow cytometric analysis of expression of sLe^X and PSGL-1 on ESL^{lo} (thin-lined histogram) and ESL^{hi} (thick-lined histogram) MDA cell variants. Expression of sLe^X was assayed with mAb HECA-452 and control rat IgM isotype (shaded histogram). Expression of PSGL-1 was assayed with mAb PL2 and control mouse IgG1 isotype (shaded histogram). Histograms are representative of experiments performed on at least five different MDA cell variants. (**E**) Western blot analysis of sLe^X and ESL was performed on ESL^{lo} and ESL^{hi} MDA cell variants. Lysates from control ESL$^+$ HPC (KG1a) cells and ESL^{lo} and ESL^{hi} variants were subjected to SDS–PAGE on 4–20% reducing gels, transferred to immunoblot membrane, and blotted with anti-sLe^X mAb HECA-452 or with E-selectin-Ig chimera. There were no glycoproteins bearing sLe^X on ESL^{lo} MDA cell variants, while sLe^X and E-selectin-binding determinants were expressed by PSGL-1 on ESL^{hi} MDA cell variants. Blotting with secondary rat IgM alone or with E-selectin-Ig in 5 mM EDTA did not result in any staining activity. These experiments were performed a minimum of five times.

Discussion

In this study, we uncover a potential mechanistic role for glycosyltransferases, and in particular of α1,3 FTs, in the regulation and synthesis of sLe^X on E-selectin-binding glycoproteins on metastatic PCa cells. The most prominent carbohydrate moiety implicated in PCa progression and metastasis is sLe^X (Jorgensen et al. 1995; Idikio 1997; Dimitroff et al. 2004, 2005). Elevation of sLe^X expression indicates an alteration in glycosyltransferase activity in PCa cells. However, information on which glycosyltransferase(s) is responsible for the elevated synthesis of sLe^X and resultant ESL activity on metastatic PCa cells has been heretofore unknown. Here, we implicate α1,3 FT3, FT6, and FT7 in the metastatic potential of PCa cells. Specifically, we show that FT3 and FT6 are upregulated 31- and 10-fold, respectively, in bone-metastatic MDA PCa

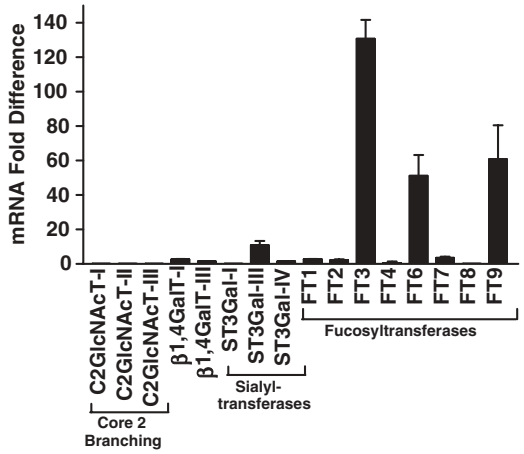

Fig. 6. α2,3 Sialyltransferase III and α1,3 FT3, FT6, FT7, and FT9 are elevated in ESL^{hi} metastatic PCa MDA cell variants. Real-time PCR analysis of *N*-acetylglucosaminyl-, galactosyl-, sialyl-, and fucosyltransferases was performed on cDNA isolated from ESL^{lo} and ESL^{hi} metastatic PCa MDA cell variants. Expression in ESL^{hi} MDA cells is reported as the mean (SEM) fold difference after normalizing to expression levels in ESL^{lo} MDA cells. ST3Gal-III and FT3, FT6, FT7, and FT9 were elevated in ESL^{hi} MDA cells. Experiments were performed a minimum of three times.

cells, compared with expression in fresh normal prostate tissue. Elevation of FT3, FT6, and FT7 was associated with induction of the sLe^X-bearing glycoform of PSGL-1 on the MDA cell surface as well as with binding to immobilized E-selectin. The binding was blocked by incubation with neuraminidase, EDTA, or bromelain, strengthening a role for sLe^X as well as of FT3, FT6, and FT7 in the regulation of E-selectin glycoprotein ligands on PCa cells. Indeed, loss of FT3, FT6, and FT7 in ESL^{lo} MDA cell variants was associated with loss of sLe^X on PSGL-1 and abrogation of E-selectin-binding. ESL^- metastatic PCa cell lines did not express elevated levels of FT3, FT6, or FT7. In fact, they showed a 2- to 354-fold lower expression compared with normal prostate epithelial tissue. Interestingly, both ESL^+ MDA cells and ESL^- cell lines expressed the complete set of glycosyltransferases required for sialyllactosamine chain synthesis on a core 2 *O*-glycan, including core 2 *N*-acetylglucosaminyl-, β1,4 galactosyl-, and α2,3 sialyltransferases. Our results, therefore, highlight the important role of α1,3 FTs in generating sLe^X-bearing adhesive determinants on metastatic PCa cells. Such results strengthen the hypothesis that PCa metastasis may be managed by traffic control mechanisms shared by HPC.

Our findings, implicating that sLe^X synthesis and ESL activity are regulated by FT3, FT6, and/or FT7 in metastatic PCa cells, are strikingly similar to the role of α1,3 FTs observed in several other cancers. In the case of carcinomas, elevation or involvement of FT3, FT6, and/or FT7 has been correlated with the progression of cancer of the colon (Majuri et al. 1995; Weston et al. 1999; Hiller et al. 2000), breast (Matsuura et al. 1998; Ding and Zheng 2004), lung (Ogawa et al. 1996), liver (Liu et al. 2000; Wang et al. 2003), and pancreas (Mas et al. 1998; Aubert et al. 2000). Stable transfection of anti-sense sequences directed at FT3 into human colon carcinoma cells inhibits expression of sLe^X or sLe^a as well as blocks tumor cell adhesion, proliferation, and colonization of the liver (Weston et al. 1999; Hiller et al. 2000). Transfection of FT7 in lung adenocarcinoma cells induces colonization of the lung (Martin-Satue et al. 1999). Over-expression of FT3 in PCa as well as FT6 in breast cancer cells leads to synthesis of sLe^X, enhanced adhesion, and tumor growth (Matsuura et al. 1998; Inaba et al. 2003).

The causal relationship between specific FT expression and organ-specific metastasis may potentially explain the inherent differences in the pattern of PCa metastasis between human and mouse. For example, the genes encoding FT3, FT5, and FT6 are arranged as a gene cluster on the short arm of human chromosome 19 (McCurley et al. 1995). This cluster is absent in mouse. That is, phylogenetic studies indicate that the gene duplication event leading to the emergence of the FT3–FT5–FT6 gene cluster occurred before the separation of man and chimpanzee 10 million years ago but after the mammalian radiation 80 million years ago as well as after the evolution of the mouse (Costache et al. 1997). Interestingly, 90% of all metastatic PCa is found in the BM of humans but only very rarely are there bone metastases found in transgenic mouse models of PCa (Gingrich et al. 1996; Benjamin 2002). In fact, these TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate) mice exhibit PCa metastasis mostly to the lymph nodes and lung (Gingrich et al. 1996). Thus, our results attributing a role for FT3 and FT6 specifically in the bone-homing behavior of PCa could potentially explain why PCa cells metastasize preferentially to bone in humans but only occasionally in mouse. Indeed, FT3 and FT6, as well as FT7, have all been implicated in the osteotropism of breast cancer metastasis (Matsuura et al. 1998; Ding and Zheng 2004). In fact, FT6 and FT3 expression levels have been associated with amount of sLe^X in breast cancer tissue (Matsuura et al. 1998). FT5 has not been detected in breast cancer tissue or in normal epithelial tissue of the kidney, lung, stomach, bladder, uterus, ovary, or salivary gland, mirroring our results for PCa cells and for normal prostate epithelial tissue (Cameron et al. 1995; Matsuura et al. 1998). These findings implicate a critical role for FT3, FT6, and FT7 in the metastasis of PCa.

Additionally, that elevation in FT expression may underlie neoplastic progression of PCa raises the mechanistic question as to why FT3 and FT6 are elevated in PCa. In gastric cancer, for example, FT3 upregulation may be triggered through aberrant transcriptional control due to the hypomethylated state of its promoter (Serpa et al. 2006). There are at least 13 putative sites in the FT3 promoter for binding of several different transcription factors, suggesting that FT3 may be particularly susceptible to upregulation (Dabrowska et al. 2005). FT6, on the other hand, may be regulated posttranscriptionally through aberrant RNA processing, as two different FT6 transcript sizes 2.5 and 3.5 kb have been identified in kidney, liver, or colon (Cameron et al. 1995). The primer set used in our assays anneals to the 5′-untranslated region and, therefore, recognizes both FT6 splice variants. It, therefore, remains to be determined whether only one and/or both of the two splice variants are expressed in PCa cells.

We speculate that upregulation of α1,3 FT3, FT6, and/or FT7 triggers sLe^X synthesis and ESL activity on metastatic PCa cells. How ESL activity promotes PCa metastasis in vivo, however, is still undefined. Regulation of PCa metastasis is likely controlled by a number of factors inherent to a PCa cell and distinct from ESL activity, including steroid receptors, growth/chemotactic factors, integrins, receptor/cytoplasmic kinases,

and transcriptional activators. It is clear that host microenvironmental factors in the prostate gland and in distant tissues, notably bone, also facilitate metastasis, by nurturing PCa cell exiting from the primary tumor and homing and colonization in metastatic sites. In this regard, we and others have shown that BMEC provide a specific repertoire of adhesion molecules, including galectins and CD44, as well as E-selectin, that engage metastatic PCa cells and mediate heterotypic and homotypic binding interactions under static or hydrodynamic shear flow conditions (Clausse et al. 1999; Glinsky et al. 2003; Draffin et al. 2004). These carbohydrate-dependent adhesive processes are critical based on their putative role in initiating the extravasation of circulating PCa cells into tissues, including bone. This mechanism has been formally elucidated in the leukocyte homing paradigm, which has served as a roadmap for our investigations on how PCa cells adhere to BMEC in blood flow. Our findings presented here and previously indicate that rolling of bone-metastatic MDA PCa cells on BMEC mimics HPC rolling behavior on BMEC via E-selectin – sLe^X-bearing glycoconjugates (Dimitroff et al. 2004, 2005). PSGL-1 on MDA cells, in contrast to HPC, did not confer P-selectin ligand activity or express sLe^X-core 2 *O*-glycans recognized by mAb CHO-131. In addition, ESL^+ MDA cells did not synthesize sLe^a despite the upregulation of FT3. An obvious question then is whether metastatic PCa cells in vivo are also devoid of sLe^a synthesis and unable to recognize P-selectin or whether circulating PCa cells acquire this capacity, i.e., through induction of *O*-linked core 2 glycans and sulfation of tyrosine residues on PSGL-1. In any case, our results suggest that loss of glycosyltransferase gene expression in metastatic PCa cells through continuous passaging in vitro may bias studies, and, therefore, strongly underrepresent the native role of selectin/selectin ligands in PCa progression and metastasis. In closing, identifying and understanding how sLe^X and ESL are synthesized in metastatic PCa cell lines as well as in tumor tissue in vivo could be invaluable in providing mechanistic insight into how PCa cell extravasation through BMEC is regulated.

Materials and methods

Cell lines and tissues

Culture conditions for human metastatic prostate tumor cell lines, MDA PCa 2b, PC-3, PC-3M LN4 derived from bone metastases, LNCaP from a lymph node metastasis, and DU-145 from a brain metastasis (American Type Culture Collection (ATCC); Manassas, VA) have been described previously (Dimitroff et al. 2005). Normal prostate epithelial cells were grown in a prostate epithelial basal medium as suggested by the manufacturer (Lonza, Inc., Walkersville, MD). Human HPC KG1a cell line (ATCC), expressing all glycosyltransferases necessary for ESL synthesis, was maintained as described previously (Dimitroff et al. 2005). Fresh frozen normal human prostate tissue was obtained from the Tissue Core facility affiliated with the SPORE in Prostate Cancer Program (University of Michigan Comprehensive Cancer Center).

Antibodies

The following mAbs were employed to study the expression of sLe^X (CD15s): mAb HECA-452 (rat IgM) (BD Biosciences, San Jose, CA); mAb CSLEX-1 (mouse IgM) (BD Biosciences); mAb FH-6 (mouse IgM) (GlycoTech, Gaithersburg, MD); mAb KM-93 (mouse IgM) (Calbiochem, Gibbstown, NJ), and mAb CHO-131 (mouse IgM) (a kind gift from Dr. Bruce Walcheck, University of Minnesota). Expression of other Lewis carbohydrate antigens was assayed with the following mAbs: anti-sLe^a mAb 1116-NS-19-9 (CA19-9) (mouse IgG1) (GeneTex, San Antonio, TX); anti-Le^X mAb 80H5 (mouse IgM) (Beckman Coulter, Fullerton, CA); anti-Le^y mAb F3 (mouse IgM) (Abcam, Cambridge, MA); anti-Le^b mAb LWB01 (mouse IgG1) (Biomeda, Foster City, CA). Anti-PSGL-1 (CD162) mAb PL2 was from Fisher-Scientific (Suwanee, GA). Anti-β-actin mAb AC-15 was from Novus Biologicals (Littleton, CO). The following mAbs purchased from BD Biosciences were used as isotype controls: mouse IgG1; rat IgM; and mouse IgM. Secondary mAbs used in flow cytometric analyses purchased from BD Biosciences included: APC-conjugated goat anti-mouse IgG; FITC-conjugated mouse anti-rat IgM; and FITC-conjugated rat anti-mouse IgM. FITC-conjugated goat anti-mouse IgG was from SouthernBiotech (Birmingham, AL). Secondary mAbs used in Western blotting were alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG and AP-conjugated rabbit anti-rat IgM (Zymed Laboratories, San Francisco, CA).

Real-time PCR analysis of glycosyltransferase gene expression

Cells/tissues were lysed in 1 mL Trizol® reagent (Invitrogen, Carlsbad, CA). Chloroform (0.2 mL) was added; tubes were capped and shaken vigorously for 15 s, incubated at 22°C for 3 min, and then centrifuged at 12,000 RPM for 15 min at 4°C. The upper, colorless phase was transferred to a clean eppendorf tube, mixed with 0.5 mL isopropanol, incubated at 22°C for 10 min, and then centrifuged at 12,000 RPM for 15 min at 4°C. The supernatant was aspirated, the RNA pellet rinsed with 1 mL of 75% ethanol in DEPC-treated water (Invitrogen), vortexed, centrifuged at 7500 RPM for 5 min at 4°C, resuspended in 50 μL DEPC-treated water, and then incubated for 10 min at 37°C to dissolve fully the RNA pellet. Genomic DNA contamination was removed from the preparation of total RNA with the RNeasy® Mini Kit, which included a DNAse I treatment step as per manufacturer's instructions (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 0.5 μg total RNA using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the following conditions: 5 min at room temperature, 30 min at 42°C, and 5 min at 85°C. Quantitation of human glycosyltransferase transcript expression by real-time PCR was performed on the iCycler iQ multicolor real-time PCR detection system (Bio-Rad) with iQ™ SYBR® Green Supermix (Bio-Rad) using 1 μL cDNA. The PCR conditions included 1 cycle at 95°C for 2 min followed by 45 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. The amount of glycosyltransferase transcript was normalized to the amount of GAPDH transcript in the same cDNA sample. Relative fold differences in transcript expression were approximated using the Comparative C_T method. Product specificity was verified by melt curve analysis according to the following conditions: 1 cycle at 95°C for 1 min, 1 cycle at 55°C for 1 min, and 80 cycles of 0.5°C incremental increases each of 10 s beginning at 55°C. The absence of genomic DNA contamination was confirmed by performing real-time

PCR with 1 μL of RNA that had not been subjected to reverse transcription. Primer sequences used for expression analysis of glycosyltransferases as well as of GAPDH by real-time PCR are shown in Table I.

Flow cytometry

Cells from suspension cultures or from adherent prostate tumor cell cultures were harvested by 1 mM EDTA, washed, and suspended at $1–5 \times 10^5$ cells in 100 μL cold PBS/2% FBS. The mAbs recognizing Lewis carbohydrate antigens along with appropriate isotype-matched control mAbs (2 μg/test) were incubated with cells for 30–60 min at 4°C. Following one wash in PBS/2% FBS, cells were incubated with either FITC- or APC-conjugated secondary mAb for 30 min at 4°C. After secondary mAb incubation, cells were pelleted, washed, and resuspended in 500 μL PBS. Fluorescence measurements were collected within 1 h on a BD FACSCanto™ apparatus (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR). Cells were gated based on forward and side scatter.

Selectin-binding assay

To examine E- and P-selectin-binding of metastatic PCa cells, we developed a cell adhesion assay using polystyrene 96-well tissue culture plates with black sides and clear bottoms (Corning, Acton, MA). This system allowed for qualitative and quantitative analysis of limited numbers of PCa cells. Wells were coated first in triplicate with 10 μg/mL recombinant human E- or P-selectin-Ig (R&D Systems, Minneapolis, MN) or negative control human IgG for 24 h at 4°C. Wells were incubated with 1% BSA/PBS for 15 min at 4°C to block nonspecific cell binding. Cells were prepared for adhesion by fluorescent labeling with 5 μg/mL calcein AM (Molecular Probes, Invitrogen) in adhesion assay buffer (RPMI 1640/0.2% BSA) for 30 min at 22°C. Cells were added to wells in 100 μL of 1×10^5 cells per well and incubated for 1 h at 4°C. Wells were washed three times with PBS. Fluorescence (485 nm excitation; 535 nm emission) was measured on a Wallac Victor^2 plate reader (Perkin Elmer, Waltham MA) at 0.1 s intervals at the Institute of Chemistry and Cell Biology Screening Facility of Harvard Medical School (Boston, MA). Values represent cell binding as a percentage of total input cells.

Western blot analysis

Western blotting was performed as described previously (Dimitroff et al. 2004, 2005). Briefly, total protein from HPC KG1a cells or membrane protein from MDA cells was solubilized in a protease inhibitor buffer containing 2% NP-40, and the concentration was determined by Bradford method (Dimitroff et al. 2001). Solubilized protein was diluted in a reducing sample buffer and separated on 4–20% Criterion™ SDS–PAGE gradient gels (Bio-Rad). Resolved proteins were immunoblotted onto Criterion Gel™ polyvinylidene difluoride membranes (Bio-Rad), blocked in FBS for 1 h at 22°C, and then incubated with HECA-452 mAb anti-sLe^X (1 μg/mL), isotype control rat IgM, mouse E-selectin-Ig chimera (1 μg/mL) (R&D Systems), isotype control human IgG (1 μg/mL), or mouse IgG anti-β-actin (1 μg/mL) for 1 h at 37°C. Blots were then washed three times with Tris-buffered saline and 0.1% Tween-20, incubated with alkaline phosphatase (AP)-conjugated rabbit anti-rat IgM, AP-anti-human IgG, or AP-anti-mouse IgG (all at 1:1000; Zymed Laboratories, Inc., San Francisco, CA) for 1 h at 22°C, and washed three times for 15 min each with Tris-buffered saline and 0.1% Tween-20. Blots were developed with a Western Blue® AP substrate (Promega, Madison, WI).

Enzymatic and inhibitor treatments

Where indicated, cells were pretreated in an adhesion assay buffer with 10 mM EDTA or with 0.2 U/mL *Vibrio cholerae* neuraminidase (sialidase) (Roche Applied Sciences, Indianapolis, IN), an enzyme that cleaves terminal sialic acid residues and abrogates selectin/selectin ligand binding, for 1 h at 37°C. Alternatively, cells in an adhesion assay buffer were pretreated for 1 h at 37°C with 0.1% bromelain (Sigma), a protease with broad peptide specificity.

Statistical analysis

Results were analyzed by one-way ANOVA with Dunnett's post-test on GraphPad Prism software® (San Diego, CA).

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Conflict of interest statement

None declared.

Abbreviations

ATCC, American Type Culture Collection; BM, bone marrow; BMEC, bone marrow endothelial cells; C2GlcNAcT, core 2 β1,6 *N*-acetylglucosaminyltransferase; ESL, E-selectin ligand; FT, α1,3 fucosyltransferase; GalT, β1,4 galactosyltransferase; HPC, hematopoietic progenitor cells; Le, Lewis; MDA, MDA PCa 2b cells; mAb, monoclonal antibody; PCa, prostate cancer; PSGL-1, P-selectin glycoprotein ligand-1; SEM, standard error of the mean; sLe^X, sialyl Lewis X; ST, α2,3 sialyltransferase.

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