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ORIGINAL PAPER

Cell Surface Associated Alpha-L-Fucose Moieties Modulate Human Breast Cancer Neoplastic Progression

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Abstract Glycosylation drives critical processes important for mammalian cell–cell and cell–matrix interactions. Alpha-L-fucose (α-L-f) is a key monosaccharide component of oligosaccharides that has been found to be overexpressed during tumor progression. Modification of cell surface fucosylation, we hypothesized, alters tumor cell phenotype and function at the end of the neoplastic progression cascade including tumor invasion. Alpha-L-fucosidase (α-L-fase) is a glycosidase that specifically removes (α-L-f) from oligosaccharide sites. We first verified the effectiveness of the α-L-fase to specifically decrease the level of α-L-f on the cell surface of several human breast cancer cell lines and also examined the recovery time for these cells to repopulate their surfaces. To investigate the potential effect of defucosylation on tumor functions, we studied the proliferation, and invasion in vitro of human breast cancer MDA-MB-231 cells as the representative cell model. We further examined several fucose-associated molecules previously shown to be involved in tumor progression, including CD44 and CD15 (Lewis X antigen). We found that α-L-fase pretreatment significantly decreased the invasive capability of breast cancer cells. Deoxyfuconojirimycin (DFJ), a specific α-L-fase inhibitor, reversed this effect. After fucosidase treatment, the level of both CD15 and CD44 were found to be reduced as measured by flow cytometry. α-L-fase treatment, further, did not affect tumor cell proliferation in vitro under identical experimental conditions. Gelatin zymography of conditioned media from tumor cells treated with α-L-fase demonstrated no change in MMP-2 activity while MMP-9 was significantly reduced.

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In summary, fucose containing glycans were found widely distributed on the cell surface of breast cancer cells and could be effectively removed by α-L-fase treatment. This decreased fucosylation, in turn, was seen to impair the interaction between tumor cells and extracellular matrices, and thus affected key cell functions modulating tumor invasion. Further elucidation of the molecular pathways involved in the inhibition of tumor cell invasion may suggest a rationale for the use of glycobiologic therapeutics to deter tumor progression.

Keywords Breast cancer · Fucose · Glycosylation · Matrix metalloproteases · Neoplastic progression

Abbreviations

α-L-f	alpha-L-fucose
α-L-fase	alpha-L-fucosidase
BSA	bovine serum albumin
FucT	fucosyltransferase
Lotus	*Lotus tetragonolobus purpureaus*
MMP	matrix metalloproteinase
PBS	phosphate-buffered saline
PE	phycoerythrin
sLea	sialyl Lewis A
sLex	sialyl Lewis X
Ulex	*Ulex europaeus* agglutinin I
TBST	Tris-buffered saline with Tween

Glycosylation drives the specific arrangement of oligosaccharides linked to glycoproteins or glycolipids in mammalian systems. It has been well established that glycosylation plays important roles in modulating critical physiologic functions and their alterations both impact and drive pathologic processes. Specifically, changes in the composition and quantity of cell surface glycosylation-associated molecules are common features of malignant transformation and progression. Functionally, aberrant glycosylation facilitates tumor invasion, metastasis, and evasion of host immunosurveillance [1]. Studies by us and others have detected fucosylated species of lipid which appeared detectable by ^{1}H NMR spectroscopy in malignant cells from patient samples and transformed cells in culture but not in their normal or non-transformed counterparts [2].

Alpha-L-fucose (α-L-f) ($C_6H_{12}O_6$), a monosaccharide component of glycosylation, has been found to be overexpressed during tumorigenesis and neoplastic progression [2]. Elevated expression of a-L-f has been reported in thyroid carcinoma [3], ovarian carcinoma [4], colorectal adenocarcinoma [5] and brain metastasis from lung carcinoma [6]. Increased fucose incorporation into serum glycoproteins was also found in patients with breast cancer [7]. Tumor progression involves a cohort of glycoproteins (adhesion molecules, matrix-degrading enzymes, growth factors) and glycans (Lewis antigens and other selectins).

CD44 is a family of multifunctional cell surface adhesion molecules rich in α-L-f and is comprised of more than 20 variants. CD44 is involved in various cell–cell and cell–matrix interactions and is also known to play an important role in the late stages of tumor progression including metastasis [8]. Further, the expression of certain CD44 variants closely correlates with both the progression and prognosis of breast cancer [9]. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which degrade most ECM components. MMP-2 and MMP-9 are critical for tumor invasion of mammary carcinoma through basement membrane as they are the major MMPs which degrade collagen IV, the major collagenous components of these matrices [10].

Breast cancer ranks second in the mortality among malignancies in American women and the prognosis is largely predicated by tumor invasion and distant metastasis. The extent of invasion at initial diagnosis has been shown to be one of the most valuable prognostic factors for patients who present with invasive disease [11], and poor prognosis in breast cancer is closely correlated with biomarkers of progression; i.e. neo-angiogenesis, invasion and metastasis [12]. Tumor metastasis of breast carcinoma is the final event in a complex multistage process which involves the crucial step of tumor invasion of neoplastic cells through the basement membrane acting as a barrier between the epithelium and stroma and between the stroma and the vasculature. Targeting the machinery for processing tumor cell surface oligosaccharides, new drugs have been developed to inhibit these metastasis [13].

As α-L-f-containing molecules are closely correlated with neoplastic progression, a rationale was established to study the potential effects of modification of fucose expression on tumor cells. We hypothesized that decreasing the fucose expression would significantly alter the behavior of breast cancer cells. By removal of α-L-f from oligosaccharide sites on the highly invasive and metastatic human breast cancer cell line MDA-MB-231 as well as others, with alpha-L-fucosidase (α-L-fase), we present a proof-of-principle for the validity of this strategy to inhibit tumor cell invasion. Further, we suggest a pathway for its mechanism of action.

Materials and Methods

Cell Lines

MDA-MB-231, a highly invasive human breast cancer cell line was obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA), and cultured in Leibo-

vitz's (L-15) medium supplemented with 10% fetal bovine serum and antibiotics (both from Media Preparation Shared Facilities, University of Alabama at Birmingham, AL, USA). Cells were maintained in 37°C in a humidified atmosphere without exogenous CO_2 as recommended by ATCC. Other human cells including immortalized MCF-10-A, fully transformed T47D and MCF-7 cells along with HT-1080 human fibrosarcoma cells were maintained under similar standard techniques or recovered and expanded from 150°C freezer stocks.

Antibodies, Lectins and Antagonistic Agents

The following antibodies and antagonists were used: Rabbit anti-human CD44 polyclonal antibody HCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phycoerythrin (PE) labeled monoclonal mouse anti-human CD44 antibody, FITC labeled monoclonal mouse anti-human CD15 and anti-human CD44 antibody, FITC labeled *Ulex europaeus* agglutinin and *Lotus tetragonolobus* lectin were all purchased from Caltag (Burlingame, CA, USA). Deoxyfuconojirimycin was acquired from Industrial Research Ltd. (Wellington, New Zealand). Biotin, peroxidase and FITC labeled *Ulex europaeus* agglutinin I (*Ulex*) were purchased from EY labs (San Mateo, CA, USA). Collagen type I, gelatin, heparin, hyaluronic acid, tunicamycin, swainsonine, benzyl-*N*-acetyl-α-D-galactosamine and alpha-L-fucosidase were all purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Other materials used included: Micron centrifugal filters YM-10 (Millipore, Bedford, MA, USA), a MTT assay kit (Promega, Madison, WI, USA), and BCA (Pierce, Rockford, IL, USA). Streptavidin alkaline phosphatase and Western Blue® stabilized substrate for alkaline phosphatase were purchased from Promega. Transwell plate inserts (6.5 mm diameter, 8.0 μm pore size) were purchased from Costar® (Corning, NY, USA). All other chemicals were obtained from Sigma Chemical Co. if not individually specified.

HuBiogel® Human Extracellular Matrix

HuBiogel® was kindly provided initially by Diversified Scientific Incorporated and subsequently by Vivo Biosciences, Inc. (both of Birmingham, AL, USA). Hubiogel® is a complex biologically-active extracellular matrix derived from normal human amniotic membranes, as modified from reconstitution techniques first developed by Siegal et al. [14]. The major components of Hubiogel are types I and IV collagen, laminin, entactin, tenascin, and heparan sulfate proteoglycan. Non-detectable are the growth factors EGF, $FGF_{1\ and\ 2}$, TGF-alpha, TGF-$beta_{1-3}$ and PDGF [14]. Hubiogel® has been validated as a suitable model of human derived ECM for the study of tumor invasion [15]. HT 1080 cell, a highly invasive human fibrosarcoma cell line, was used as a positive control cell line to further define the effectiveness of the technique.

Alpha-L-fucosidase Treatment

Alpha-L-fucosidase (α-L-fase) was diluted in sterile PBS to a concentration of 1.69 mU/ml (8.8 mU/10^6 cells), as this concentration was found to maximally decrease the fucose expression on MDA-MB-231 cells without significantly affecting cell viability. Briefly, cells were cultured to 70–80% confluence, followed by 0.25% trypsinizing. Cell numbers were counted, 8.8 mU α-L-fase/10^6 cells was mixed together and then incubated at 37°C for 30 min. Cells were finally washed with PBS and centrifuged to remove any residual α-L-fase.

Flow Cytometry

Cells were treated with α-L-fase as described above i.e. 8.8 mU/10^6 cells for 30 min at 37°C. Briefly, the same number of cells were sham treated with PBS as control. Both groups of cells were incubated with *Ulex europaeus* I lectin labeled with FITC, anti-CD15 antibody labeled with FITC and anti-CD44 antibody labeled with phycoerythrin. In parallel, a second negative (unlabeled) control was sham treated with the appropriate isotype control and run in parallel with the untreated autofluorescence control. In a duplicate set of experiments cells were washed with PBS and fixed in 1% paraformaldehyde prior to flow cytometry analysis. Flow cytometry was performed on a B-D FACS Caliber instrument using two-color analysis to produce two-dimensional histograms, as well as one-dimensional histograms of each fluorescence channel. The result were calculated and presented as fluorescence intensity (mean±SEM).

Invasion Assay

Transwell plate inserts (6.5 mm diameter, 8.0 mm pore size) were coated with Hubiogel® (0.64 mg/ml) and left to dry overnight in a biosafety hood. Cells were trypsinized and treated with α-L-fase as described. In parallel, the same number of cells was treated with α-L-fase plus 1 nM deoxyfuconojirimycin hydrochloride (DFJ) simultaneously at 37°C for 30 min. As a further control, cells under identical conditions were sham treated with PBS. From each group, 5×10^4 cells were plated in 400 μl of appropriate media containing 1% FBS. The lower chambers were filled with L-15 medium containing 10% FBS. The invasion assay assemblies were emersed in six-well culture plates with 10% FBS supplemented culture medium. For experiments with glycosylation inhibitors, to the upper chambers were added tunicamycin (10 μg/ml), swainsonine

(0.3 μg/ml), benzyl-*N*-acetyl-α-D-galactosamine (i.e., Benzyl-GalNAc, 1.5 μg/ml) or *Ulex* lectin (5 μg/ml). After 48 h, filters were removed and stained with a dilute solution of crystal violet. The number of invading cells on the whole filter was counted under ×200 magnification with the aid of a reticle-containing eyepiece.

Proliferation Assay

Cells were trypsinized, resuspended in PBS, counted and a subset treated with α-L-fase as described. In 100 ml of medium, 2×10^3 cells/well were then plated onto a 96-well culture plate. At 0, 24, 48 and 72 h post-treatment, an MTT assay was performed in triplicate, as per the manufacturer's instructions. The optic density, after calibration, was read with an ELISA reader at 490 nm. As an alternative method and alternative check, cell proliferation was also determined by counting individual alliquots of cells, in triplicate, using an automatic Beckman Particle counter. In these later experiments, cells in the presence or absence of α-L-fase were grown in 24-well culture plates for the indicated time. At the end of each time point, cells were harvested in trypsin-EDTA. Each time point represented five samples, and each experiment was performed in triplicate. There was no statistical difference between the two methodologies (data not shown).

Zymography

A gelatin substrate zymography method was utilized as reported previously [16]. In summary, cells were trypsinized, washed with PBS twice and treated with α-L-fase, α-L-fase plus deoxyfuconojirimycin (DFJ) or PBS as sham control. Identical numbers of cells (5×10^6) cultured in L-15 complete medium (containing 10% FBS) were used. After allowing 24 hr for attachment, the old medium was replaced with serum-free L-15 medium; cells were then incubated for another 48hs. Conditioned media from cells with or without α-L-fase treatment were collected and concentrated with a Micron Centrifugal Filter YM-10 following the protocol provided by the manufacturer. The protein concentration in the conditioned media was determined by conventional BCA methods. Fifty micrograms samples were loaded onto a Tris–glycine–SDS polyacrylamide gel incorporated with 0.1% gelatin and subjected to electrophoresis under non-reducing conditions. The gel was then rinsed in 2.5% Triton X-100, followed by incubation in Bio-Rad Zymogram Development Buffer (50 mM Tris–HCl [pH 7.5], 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35) overnight at 37°C. The gel was stained with 0.5% Commassie Blue (in 40% methanol, 10% acetic acid), and then destained in 40% methanol and 10% acetic acid until white bands appeared against the blue background, indicating the site(s) of gelatinolytic activity.

Lectin Histochemistry and Morphometry

MDA-MB-231 cells were treated as previously described in the presence or absence of α-L-fase and compared to the same number of PBS sham-treated cells cultured on cover-slips for the indicated times. At the end of the each time point, cells were washed with PBS, and blocked with 5% BSA in PBS, followed by incubation with biotin labeled *Ulex* (5 μg/ml) in 5% BSA in PBS for 2 h, detected by streptavidin-alkaline phosphorylase for 1 h, the color developed by Western Blue® stabilized substrate for Alkaline Phosphatase following the protocol of the manufacturer (Promega). The image was captured with a BX51 conventional brightfield Olympus Microscope (Melville, NY, USA) and fucose expression determined by utilizing Bioquant image analysis software (Bioquant Corp., Nashville, TN, USA). Each experimental group was composed of three cover-slips and for each cover-slip, three random fields were examined and the images captured digitally. An arbitrary cut-off value for the intensity of staining was set and a positive ratio of α-L-f expression was calculated comparing "positivity" (above threshold) to all staining.

Data Analysis

The data were analyzed utilizing Excel® (Microsoft, Seattle, WA, USA) and SPSS® (Chicago, IL, USA) software. The statistical analysis of non-Gaussian distributed data was carried out using standard non-parametric tests (Wilcoxon signed rank test and *U*-Mann–Whitney test). A paired two samples *t* test was also utilized where appropriate. A p value of <0.05 was considered statistically significant.

Results

α-L-Fucose is Widely Expressed on Breast Cancer Cells and is Decreased with α-L-fucosidase Exposure

We have previously demonstrated that virtually all human breast cancer cell lines tested displayed α-L-f on their cell surfaces. To test the effectiveness of defucosylation by α-L-fase, we utilized flow cytometry to quantify the surface change of α-L-f on MDA-MB-231, MCF-7 and T47D cells after exposure to 1.69 mU/μl (8.8 mU/10^6 cells) of α-L-fase for 30 min at 37°C. As is seen in Fig. 1a–b, using the FITC labeled fucose specific lectin *Ulex* I, (Fig. 1a) or FITC labeled *Lotus* lectin (Fig. 1b) α-L-fase was found to reduce α-L-f expression without significantly damaging cell viability (see also Fig. 2). By

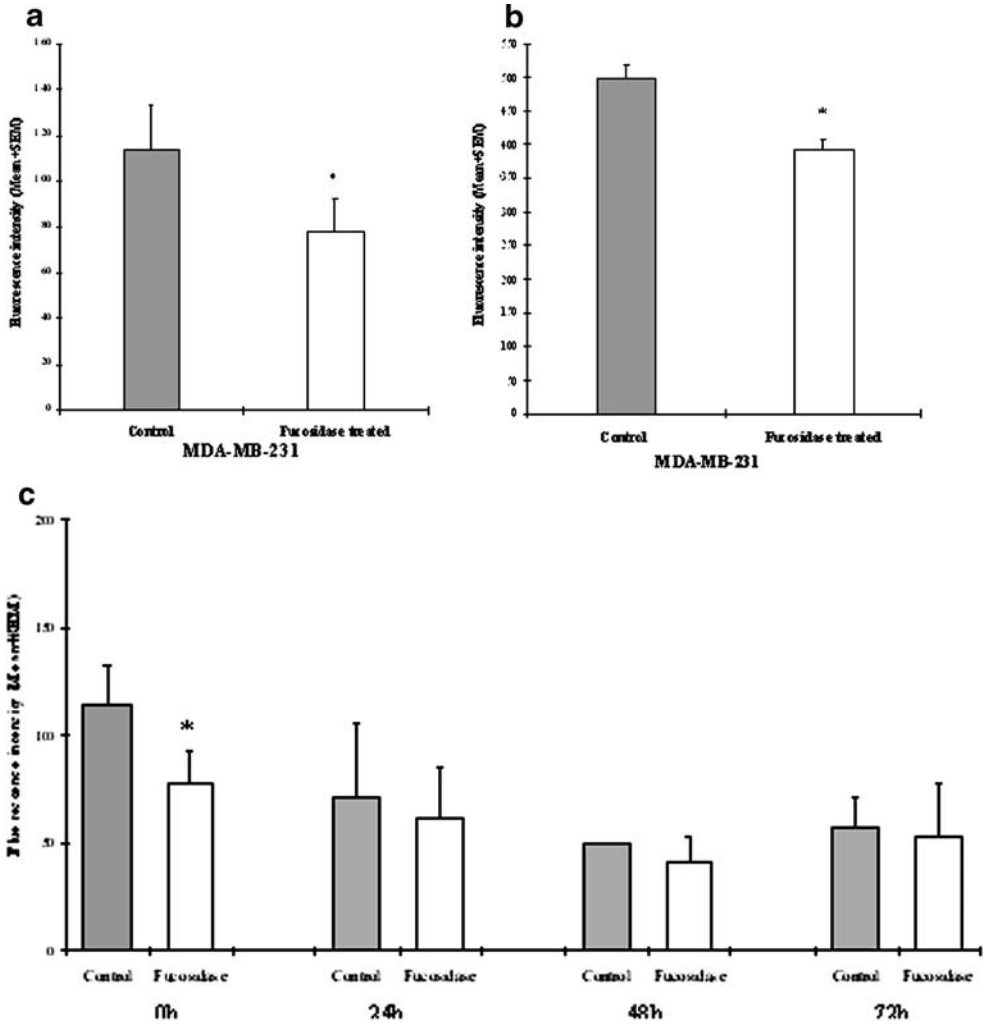


Fig. 1 **a** and 1**b**: α-L-fucosidase treatment decreased the expression of fucose on human beast cancer cells: MDA-MB-231(**a** and **b**), MCF-7, and T47D (not shown) human breast cancer cells were cultured to 70–80% confluence, trypsinized and washed with PBS twice. Cells were mixed with 8.8 mU α-L-fase/10^6 cells and incubated at 37°C for 30 min. Immediately after the treatment period, cells were washed with PBS and centrifuged to remove α-L-fase. Cells were then treated with the α-L-f specific lectins, FITC labeled *Ulex europaeus* 1 (**a**) or *Lotus tetragonolobus* (**b**). In parallel, negative (unlabeled) control samples were sham treated (autofluorescence control). Flow Cytometry was performed and the mean fluorescence intensity ± SEM was recorded from nine independent experiments, respectively. Each pair of experimental groups were analyze by a paired two sample *t* test and found to be significant ($p<0.05$). A Wilcoxon matched pairs signed rank test also revealed a significant difference ($p<0.01$). *Shaded box* untreated control, *empty box* α-L-fase. **c** Flow cytometric analysis of α-L-f expression of MDA-MB-231 cells over time. Cells were treated with α-L-fase as described in the text and except for time "0 h", cells were left to recover for the indicated time periods. FITC labeled *Ulex* flow cytometry was performed at the indicated time points. The bar graph shows the mean±SEM for the fluorescence intensity in three independent experiments. Each pair (control versus α-L-fase treated) were analyzed by paired two sample *t* tests ($p>0.05$), as well as a Wilcoxon matched-pairs signed rank test ($p>0.05$). Only at zero time (0 h) was there a statistically significant difference indicating the short-lived effect of the treatment. *Shaded box* untreated control, *empty box* α-L-fase

flow cytometry we further demonstrated that α-L-f expressed on tumor cells could be significantly decreased immediately by α-L-fase, but that this defucosylation effect was short-lived (Fig. 1c). Lastly, by using peroxidase-labeled *Ulex* lectin blotting as well as biotin-labeled *Ulex* histochemistry, we further demonstrated that α-L-f expression had recovered by 12 h after α-L-fase treatment (Fig. 2).

α-L-Fucosidase Treatment Significantly Decreased the Invasion of MDA-MB-231 Cells Through HuBiogel

To investigate the effect of defucosylation on tumor cell invasion, we performed in vitro invasion assays using Costar® Transwell plate inserts (6.5 mm diameter, 8.0 mm pore size) coated with the complex normal human extracellular matrix, HuBiogel®. We determined that under

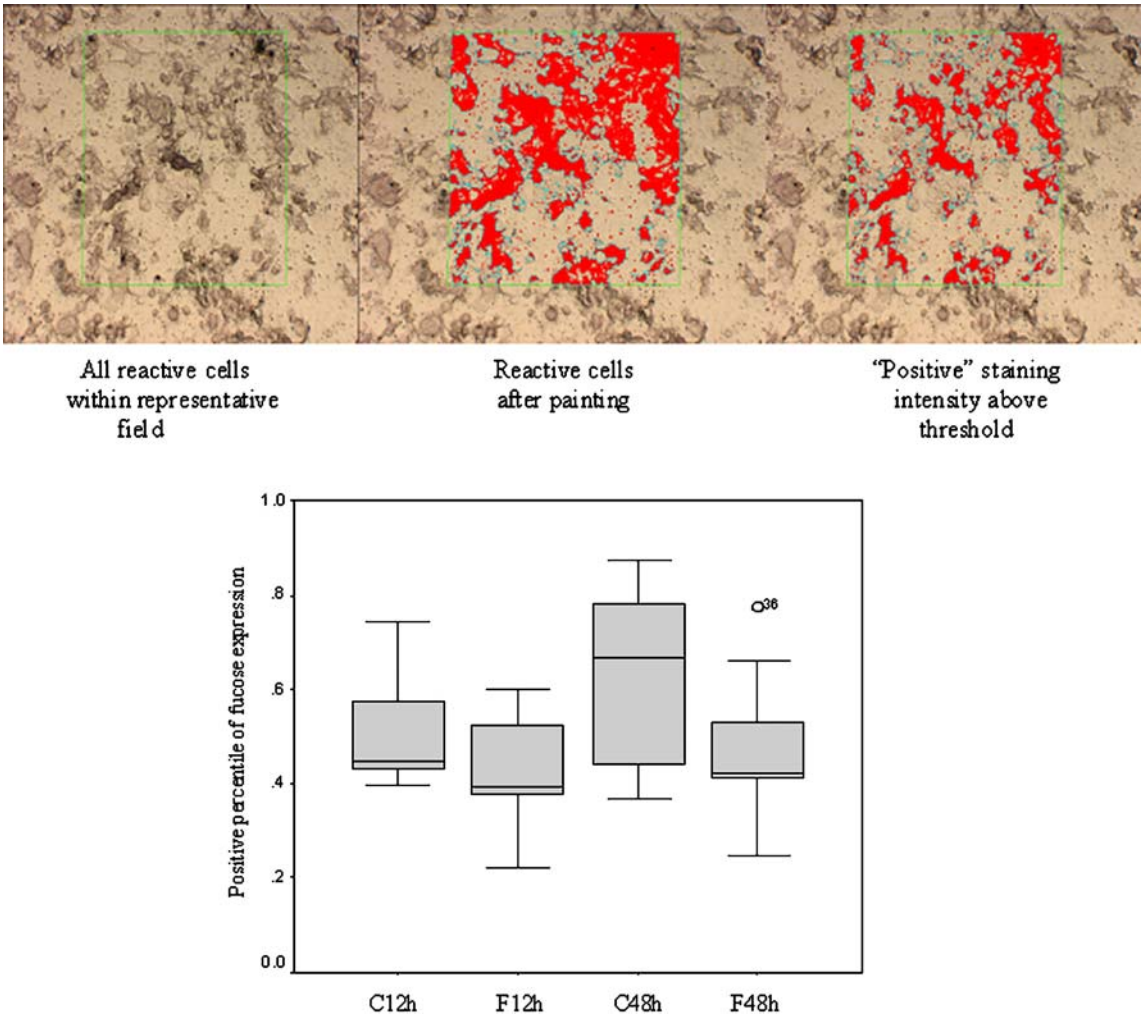
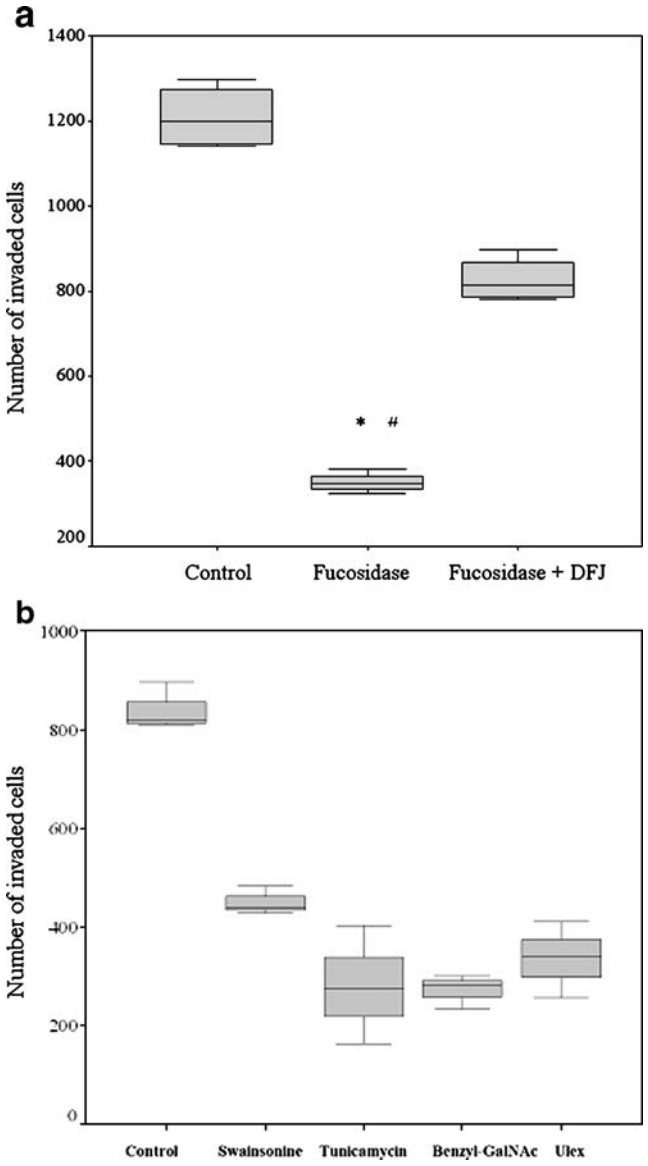


Fig. 2 Top: Lectin histochemistry and morphometry. MDA-MB-231 cells treated with α-L-fase as previously described together with PBS Sham-treated cells were cultured on cover-slips for the indicated times. At the end of the each experiment, the cells on the cover-slips were blocked with 5% BSA in PBS followed by incubation with biotin labeled *Ulex* (5 μg/ml) in 5% BSA/PBS for 2 h, detected by streptavidin-alkaline phosphorylase for 1 h, and the color developed in the presence of Western Blue®. The image was captured by conventional brightfield microscopy and the ratio of α-L-f expression to nonexpression determined morphometrically by Bioquant software. Each experimental group was examined in triplicate, and for any one group, three random areas were measured. The *left panel* demonstrates all 'positive' cells prior to morphometric painting. The *middle panel* is the same image after painting and the *left panel* is representative of the arbitrary cut-off value selected as truly "positive" for the intensity of the staining expression. *Bottom* box-plot of the fucose expression detected by lectin histochemistry. Fucose expression at 12 and 48 h was calculated as described in *top*. $n=9$ for each time point. A Mann–Whitney test was performed to compare the difference between control and α-L-fase treated groups ($p>0.05$). In the *box plot*, the box stretches from the lower hinge (defined as the 25th percentile) to the upper hinge (the 75th percentile); median, minimum and maximum are also displayed. The differences are not significant ($p>0.05$) at either point (see test for details)

the same experimental conditions for decreasing cell surface α-L-f (8.8 mU a-L-fase/10^6 cells for 30 min at 37°C) MDA-MB-231 cell invasion through HuBiogel was significantly decreased. Furthermore, this inhibition could be reversed by deoxyfuconojirimycin, an α-L-fase specific inhibitor (Fig. 3a). We also found that the *N*,*O*-glycosylation inhibitors tunicamycin and swainsonine as well as the *O*-glycosylation inhibitor, benzyl-GalNAc significantly inhibited the invasion of MDA-MB-231 cells through HuBiogel (Fig. 3b). Interestingly, the α-L-f specific lectin, *Ulex europaeus* also caused a statistically significant inhibitory effect on the invasion of the same as breast cancer cells in vitro (Fig. 3b).

The Proliferation and Viability of MDA-MB-231 Cells was Unaffected by α-L-Fucosidase Treatment

To exclude the possibility that the observed decrease in tumor cell invasion was actually a function of the direct inhibition of the cell growth by α-L-fase, we studied the effect of α-L-fase treatment on cell proliferation using two different assays. Our data demonstrate that α-L-fase



◀ **Fig. 3** A α-L-fase treatment significantly decreased the invasion of MDA-MB-231 cells through HuBiogel®. Deoxyfuconojirimycin (DFJ), a specific inhibitor of α-L-f [17], abolished the inhibitory effect of α-L-f on tumor cell invasion. Invasion chambers were separated by inert filters coated with HuBiogel (0.64 mg/ml). Cells were treated with α-L-fase as described above. In *parallel*, the same number of cells were treated with α-L-fase plus 1 nM DFJ simultaneously at 37°C for 30 min. A third group of cells were sham-treated with PBS. 5×10^4 cells from each group were plated in 400 ml of medium containing 1% FBS in the upper chamber. The inserts were then placed in 24-well plates containing complete culture medium (10% FBS). At the end of 48 h, filters were removed and stained with crystal violet. The number of invading cells on the whole filter was counted at ×200 with the aid of a grid eyepiece. Data represent the mean of three independent filters. Differences in invasion between the groups was analyzed by the Mann–Whitney test *$p<0.05$ (α-L-fase vs. control) #$p<0.05$ (α-L-fase vs. α-L-fase + DFJ). **b** *N*,*O*-glycosylation inhibitors and the α-L-f-binding lectin *Ulex* significantly decreased the invasion of MDA-MB-231 cells through HuBiogel®. The number of invading tumor cells on the whole filter was counted as described in Fig. 4. Data represent the mean of three independent filters; each group treated was compared to control cells treated with PBS alone by a Mann–Whitney test. The upper chamber contained swainsonine (0.3 μg/ml), tunicamycin (10 μg/ml), benzyl-*N*-acetyl-α-D-galactosamine (i.e., benzyl-GalNAc, 1.5 μg/ml) or *Ulex* lectin (5 μg/ml). In each case the difference in invasion was significant $p<0.05$

affected neither the viability nor the proliferation of MDA-MB-231 cells as shown by an MTT assay (Fig. 4) nor by automated cell counting (data not shown).

The Mechanism by Which α-L-Fucosidase Treatment Specifically Decreased MDA-MB-231 Breast Cancer Cell Invasion may be Through Modulation of the Activity of Secreted MMP-9

Tumor cells secrete matrix metalloproteinases (MMP) to degrade ECM components to facilitate their invasion and enhance angiogenesis. Gelatin substrate zymography is an established method to indirectly measure the activity of active MMP-2 and MMP-9, which are the known major MMPs secreted by human breast cancer cells responsible for the degradation of basement membrane (mainly type IV collagen). To investigate the effect of α-L-fase treatment on the gelatinolytic ability of MDA-MB-231 tumor cells, we studied the conditioned medium by zymography from untreated cells, α-L-fase treated cells and α-L-fase treated cells in the presence of deoxyfuconojirimycin. The gelatin zymogram revealed a significant decrease in the gelatinolytic activity of MMP-9 after treatment with α-L-fase. Deoxyfuconojirimycin could be seen to reverse this effect (Fig. 5). MMP-2 showed no significant changes under these same experimental conditions.

α-L-Fucosidase Treatment Down-Regulates the Cell Surface Expression of CD44 and CD15 on MDA-MB-231 Cells

The CD44 family of adhesion molecules mediate multiple cellular functions and the standard (H) form is best known as the hyaluronan receptor. It has been linked to tumor invasion and metastasis. These molecules are transcriptionally upregulated by several growth factors and selected cytokines. CD44 and its many isoforms under go extensive post-translational modifications including glycosaminoglycan addition and glycosylation resulting in tumor cells being decorated on their surfaces by α-L-f. We thus, investigated the effect of α-L-fase on the expression of CD44 on the surface of breast cancer cells. MDA-MB-231 cells were treated with α-L-fase as described in the Materials and Methods. We found α-L-fase significantly decreased expression of cell surface CD44 as detected by flow cytometry using phycoerythrin-labeled monoclonal antibody against the predominant form of CD44 (Fig. 6a).

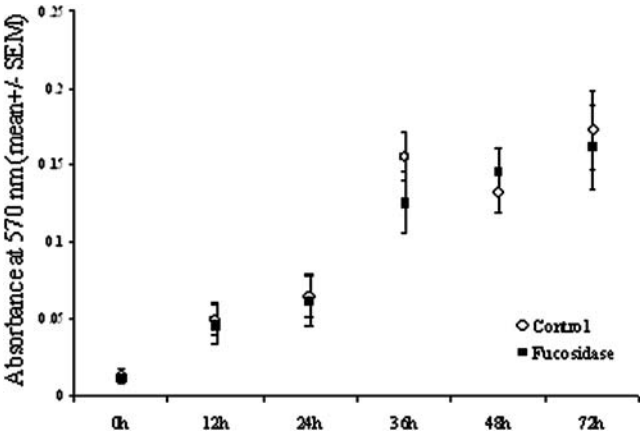


Fig. 4 α-L-fase treatment did not affect the viability of MDA-MB-231 cells. Cells were trypsinized, resuspended in PBS, and treated with or without α-L-fase. 2×10^3 cells per well were then plated in 96-well culture plates. At the end of each reported time point, MTT solution was added to the cells according to the manufacturer's instructions. Each assay was performed in triplicate and the optic density was calibrated following the procedure recommended by the manufacturer. Each time point represents the mean of three samples for both groups. Data represent the absorbance spectra at 570 nm. A Wilcoxon signed rank test and paired two sample *t*-tests were performed to evaluate the difference between α-L-fase treated and control groups at all six time points. There was no statistically significant differences between any group ($p>0.05$)

CD15 (Lewis X), an α-L-f containing tetrasaccharide important in the adhesion of tumor cells to the vascular endothelium, was also found to be abundantly expressed on breast cancer cells as determined by flow cytometry (Fig. 6b). Western blotting following the cells being lysed immediately after exposure to α-L-fase revealed markedly diminished levels which returned to normal over 48 h (data not shown). Essentially identical results were found by flow cytometry.

Discussion

Alterations in the amount, composition and linkage configurations of glycosylation associated molecules are known to correlate with malignant transformation, tumor progression and poor prognosis of cancer patients [18, 19]. A switch in glycosylation patterns has been reported to signal the transformation of breast cancer to a metastatic phenotype [20], and glycosylation is known to play a direct functional role in cancer cell motility and invasiveness [21]. Of direct relevance to this work, Carcel-Trullols et al. [22] found that a bone colonizing variant of MDA-MB-231 cells displayed an altered glycosylation pattern (as compared to the parental cells), differential binding to bone marrow endothelial cells, enhanced ECM binding, and an increased invasive potential, all of which boosted bone metastasis and bone colonization capacities.

Our findings also illustrated general deglycosylation by both *N*-glycosylation inhibitors (Tunicamycin, Swainsonine), and O-glycosylation inhibitors (benzyl-*N*-acetyl-α-D-galactosamine) significantly decreases the invasiveness of MDA-MB-231 human breast cancer cells through complex extracellular matrices in vitro, arguing for the importance of intact glycosylation for functional neoplastic progression. Tunicamycin, an *N*-glycosylation inhibitor, has also previously been recognized to inhibit the proliferation of MCF-7 breast cancer cells [23] and induce apoptosis in MDA-MB-231 cells [24] while inhibition of synthesis of complex type *N*-glycans by swainsonine (a mannosidase II inhibitor) led to a 50% decrease of cell migration in malignant melanoma cells [25]. Thus these effects must be excluded before reaching definitive conclusions about decreased invasion which we have shown in this manuscript.

Best known as a key component of blood group antigens, the major physiological functions of α-L-f include modulating leukocyte-endothelial adhesion, host–microbe interactions, fertilization, ontogenesis and differentiation [26]. Our study revealed high levels of α-L-f expressed on the surface of human breast cancer cells. The increased expression of α-L-f on tumor cells is not only a phenotypic marker of transformation [malignancy], but also known to participate in tumor progression, both directly and indirectly. Specifically, serum α-L-f levels have been found to be significantly increased in patients with breast cancer and associated with their response to therapy, with tumor recurrence and with metastases [27]. Recently, the levels of abnormally-fucosylated haptoglobin and α-1,3 fucosyltransferase were found to increase in breast cancer patients with progressive disease, and to decrease with successful therapy [28–29]. The expression level of fucosyltransferase VII has also been linked to lymph node metastasis and poor prognosis of patients with breast cancer [30].

Various approaches have been proposed to down regulate the expression of a-L-f on tumor cells, which including interfering with fucosyltransferase [31], altering

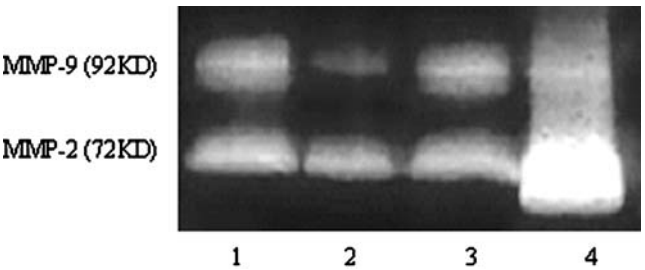


Fig. 5 Gelatin zymography of conditioned medium from both α-L-fase treated and untreated MDA-MB-231 cells. *Lane 1*cells without α-L-fase treatment; *lane 2* cells treated with α-L-fase (8.8 mU/10^6 cells); *lane 3* cells co-treated with α-L-fase (8.8 mU/10^6 cells) and DFJ (1nM); *lane 4* HT 1080 fibrosarcoma cells as MMP positive control. 0.005 mg of purified human MMP-2 and MMP-9 served as the gelatinase zymograph standards (not shown). A significant reduction in MMP-9 is seen and verified by densitometry but not in MMP2 in the presence of α-L-fase

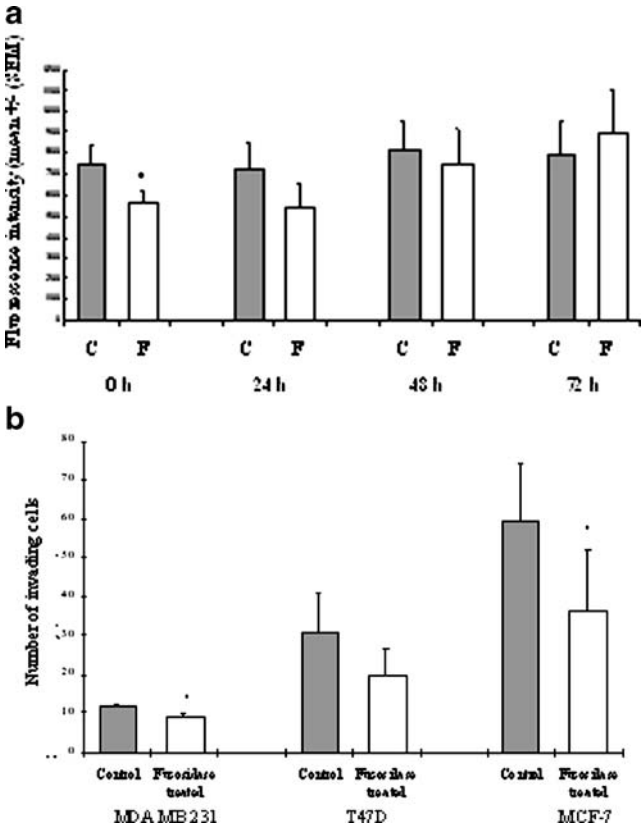


Fig. 6 A: CD44 changes as detected by PE labeled monocolonal antibody in a flow cytometry assay with and without fucosidase treatment. *Shaded box* untreated control, *empty box* α-L-fase MDA-MB-231 cells were treated with α-L-fase as described in "Materials and Methods." Both groups of cells were incubated with the monoclonal PE-labeled anti-CD44 antibody. A Wilcoxon signed rank test and paired two sample *t* test were performed to analyze the difference in the mean fluorescence intensity between control and α-L-fase treated groups. At each time point the histogram represents the mean±SEM for five independent experiments. A significant difference ($p<0.05$) was appreciated only for time "0". Rapid reconstitution resulted in no difference ($p>0.05$) for CD44 at all other times. **b** Effects of α-L-fase treatment on CD15 expression of human breast cancer cells as shown by flow cytometry. CD15 expression was evaluated by FITC labeled *Ulex* flow cytometry at the time points indicated using methods as described in "Materials and Methods." The *bar graphs* demonstrate the mean±SEM of the fluorescence intensity in 5(for MDA-MB-231and T47D) and seven (for MCF-7) independent experiments. Each pair (control vs. fucosidase treated) were analyzed by a paired two sample *t*-test, as well as a Wilcoxon matched-pairs signed rank test (p (0.05), MDA-MB-231 and MCF-7 cells showed a statistically significant difference. However, for T47D, the difference approached significance but did not reach it. *Shaded box* untreated control, *empty box* α-L-fase

the expression of upstream regulators [32] and up-regulating competitive glycoforms [33]. α-L-fase (E.C. 3.2.1.51) is a lysosomal glycosidase, which has the capability to hydrolyze the glycosidic bond linked α-L-f in α-1,2-, α-1,3-, α-1,4- and α-1,6-linkages in glycosylation chains [34]. The effectiveness of our defucyosylation protocol has been confirmed by our flow cytometry experiments. This enzymatic removal of α-L-f that we used in this study has several advantages including speed and convenience to achieve the defucosylation effect. It also suggests a potentially practical clinical route of administration via intravenous and intralumnial application. In separate experiments we tested a range (from one-eighth to eightfold concentration of α-L-fase). As expected there was a concentration dependent effect although it was not linear (data not shown). 8.8 mU/10^6 cells for 30 min was found to be the most ideal concentration when weighing effectiveness against viability. We also used DFJ, a potent fucosidase inhibitor, as an enzyme antagonist [17]. With the elevation of fucosylation known to be present in cancers, that α-L-fase levels were found to rise in parallel is not unexpected and may represent a host-reaction to a high "fucose-burden". Serum α-L-fase activity has been found to be significantly elevated in patients with colorectal cancer and low α-L-fase activity in cancer tissue has been associated with a higher rate of recurrence [35]. Thus, the rationale exists that by reducing the "fucose-burden" by exogenous α-L-fase one may interfere with tumor functions associated with such abnormal high level of fucose.

Several (glycoprotein) targets appear to utilize fucosylation to promote tumor metastasis and invasion, these include the α-L-f containing glycans, Lewis X, sialyl Lewis X and sialyl Lewis A, functioning as E-selectin epitopes, to promote tumor cell interaction with endothelial cells. Fucosylation may also functionally modify adhesive molecules, such as integrins, cadherins and, as noted above, CD44 [36]. CD15 (i.e., Lewis X antigen) is a branched polysaccharide (3-fucosyl-*N*-acetyllactosamine) whose expression is found on the surface of normal human polymorphonuclear neutrophils and several human neoplasms including breast carcinomas [37]. The over expression of Lewis X has been correlated with the grading, clinical stage, lymph node metastasis, recurrence and prognosis of breast cancer [38]. As α-L-f is one of the key component monosaccharides of the Lewis X rigid structure, it was not surprising to find CD15 levels decreased on the breast cancer cells we examined after defucosylation. Lewis X expression in breast cancer was commonly found to localize at the leading edge of invading tumor or at the outer edge of carcinoma in situ, suggesting a potential role for it in invasiveness and metastasis [39]. Other α-L-f containing Lewis family glycans have also been implicated in the progression of breast cancer and my have also been disrupted by the α-L-fase treatment. One example of this is the Lewis Y/B antigens, whose high expression is associated with decreased survival in lymph node negative breast carcinomas [40]. In the metastatic lesions of breast carcinomas, Lewis A/sialyl-Lewis A expression has also been shown to be reduced when compared with primary tumors [41]. The proliferation of human breast cancer cells after α-L-fase treatment was

evaluated by two methods. Both revealed unchanged cell proliferation during treatment. This is consistent with previous findings that mammalian cell lines lacking cell surface fucosylated glycans are still viable with no apparent defect in cell division [42–43]. This may be explained by the knowledge that cell surface fucosylated glycans mainly participate in cell–cell and cell–matrix interactions rather than transcriptional regulation and that our defucosylation treatment was reversible and short lived.

Tumor invasion is the hallmark of epithelial malignancies and is critical to the metastatic cascade which often ends in the death of patients. Accumulating evidence has shown that modification of glycosylation will affect tumor invasiveness [44]. The matrix metalloproteinases family of protein degrading enzymes cleaves selected ECM components and facilitates tumor invasion. Among the MMPs, MMP-2 and MMP-9 attract significant attention because of their known role in degrading type IV collagen, which is the main collagenous component of basement membranes. Altered MMP-9 levels have been found to be associated with lobular breast carcinoma with highly invasive potential [45]. The direct role that MMP-9 plays in the invasive ability of MDA-MB-231 cells to cross basement membranes has been confirmed [46], and decreased MMP-2 and MMP-9 activity have previously been noted to be closely correlated with decreased tumor invasion through HuBiogel [16]. The MDA-MB-231 cell line, which is highly invasive, is known to secrete high levels of MMP-9. Our findings show that defucosylation significantly inhibited the invasion of these cells. This presumably is mediated by α-L-fase induced decreasing gelatinolytic activity of MMP-9. Further, it is recognized that the FBS used in these assays may contain certain interfering factors affecting the invasion assay system. As we provided an untreated control as well as an inhibitor for the target enzyme any observed differences would be minimized by this strategy, lending greater strength to the statistically observed difference.

CD44 is widely distributed among different cells and tissues and plays important roles in many biological and pathological processes such as lymphocyte homing, T-cell activation, wound healing, angiogenesis, and metastatic spread of tumor cells. Fucosylation has previously been in many of these processes associated with CD44, e.g., fucosylated CD44 has been seen to decrease during chemotherapy of canine lymphosarcoma [47]. Additionally, *N*-/*O*-glycosylation or glycosaminoglycan (GAG) additions on CD44 is important for the communication between ECM and intracellular protein components. Changes in *N*- and *O*-glycosylation of CD44s modulate its cleavage [48] and adhesion on hyaluronic acid [49]. Several distinct oligosaccharide structures have been found to affect CD44-mediated HA binding [50]. CD44 is also known to up-regulate invasive and metastatic invasion and migration [51], linking our results to these. The observed decreased levels of CD44 seen in these studies after α-L-fase treatment may come simply from the fact that alteration of glycosylation impairs its recognition by antibodies. Other have shown, in fact, that a combination of neuraminidase and *O*-glycosidase treatment rendered CD44 reactive to antibodies which previously failed to recognize the expression on tumor cells [52].

Our study adds to the growing body of knowledge which asserts the importance of α-L-f in the cancer phenotype of many human malignancies. It has been shown that the α-L-f containing sfialyl Lewis X and sialyl Lewis A antigens confer upon cells a key "prometastatic glycosylation phenotype" [53]. Hakomori and associates have further constructed the glycosynapse model of carbohydrate-bearing membrane microdomains as the controlling functional units in carbohydrate dependent cell adhesion and cell signaling [53]. Fundamental knowledge of α-L-f s function at the molecular level has been advanced by the recent proof of a novel mechanochemical property, the catch-slip bond, which mediates the attachment of important fucose-containing ligands to selectins [54]. There is, furthermore, evidence that α-L-f is critical in ligand-mediated cell signaling and signal processing pathways, quite apart from the known cell adhesion properties [55–56]. Specifically, we have performed soft agar clonogenic assays and found no statistically significant difference in anchorage independent growth in breast cancer cells in the presence or absence of α-L-fase. As these cells are already fully transformed, different results might have been obtained if similar experiments were repeated with untransformed or partially transformed mammary epithelial cells.

Currently, carbohydrate-based drugs are rapidly being utilized by the modern biotechnology and pharmaceutical industries and many carbohydrate-based compounds are in various stages of clinical trials [57]. There is, thus, a growing recognition within the mainstream of glycobiology that cell surface carbohydrates, especially α-L-f containing Lewis antigens, are closely associated with hematogenous metastasis of cancers, and therefore are logical targets for antimetastatic therapies [58].

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Conflict of interest statement Drs. Singh and Siegal hold intellectual property rights for HuBiogel™ (US Patent No. 10/546,506 [Patent Pending], International Application No. PCT/US2004/005255 [Patent Pending] to the UAB Research Foundation. Additionally, Dr. Singh is the CEO of In Vivo Biosciences, Inc., which holds the

exclusive worldwide rights to market this biomaterial. All other authors have declared that they have no other real or potential conflict of interest associated with this manuscript.

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