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# Effects of Defucosylation on Human Breast Cancer Cells

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# EFFECTS OF DEFUCOSYLATION ON HUMAN BREAST CANCER CELLS

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## ABSTRACT

Carbohydrates can be conjugated to protein or lipids and participate in multiple cell-cell and cell-matrix interactions. Altered glycosylation events may be associated with cancer development and progression. Elevated fucose-containing glycans are one of the most prominent examples of malignancy-associated changes. Our work is based on the hypothesis that modification of cell surface fucosylation can alter certain cell functions known to be critical for neoplastic progression. Alpha-L-fucosidase (α-L-fase) is a glycosidase that specifically removes α-L-fucose (α-L-f) from glycosylated proteins and lipids. We found α-L-fase treatment decreased the level of α-l-f on cell surfaces of the highly metastatic human breast cancer MDA-MB-231 and several other human breast cancer cell lines. Important cellular functions including proliferation, adhesion and invasion of these cells were studied to evaluate the potential effects of α-l-fase. Expression of CD44, CD15, sialyl Lewis X and the activity of matrix metalloproteinase 2 and 9, all involved in tumor progression, were also examined after α-L-fase treatment. Fucosidase treatment was found not to affect cell proliferation in vitro, but to dramatically decrease the invasive capability of tumor cells in vitro. Zymography of conditioned media

from these cells revealed a decreased MMP-9 activity after treatment. Deoxyfuconojirimycin, an α-L-fucosidase inhibitor, reversed the inhibition of both cell invasion and MMP-9 activities. Defucosylation impaired cell adhesion to several components of the extracellular matrices, and diminished the detection of sialyl Lewis X, CD15 and CD44. A dys-synchrony of β1 integrin-fucose colocalization was also identified. In a physiologically-simulating flow chamber system, α-l-fase treatment was found to impair the rolling of MDA-MB-231 cells on human umbilical vein endothelial cells and purified adhesion molecules. In summary, fucose-containing glycans were found to be widely expressed on the cell surface of breast cancer cells and could be effectively removed by fucosidase treatment. Defucosylation impaired interactions critical for mammary neoplastic progression between cells and external milieu, and thus, affected their functions related to dissemination. Decreasing cell fucosylation may thus provide a novel approach to deter tumor progression.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α -L-f | Alpha-L-fucose |
| α -L-fase | Alpha-L-fucosidase |
| BSA | Bovine Serum Albumin |
| DFJ | Deoxyfuconojirimycin |
| ECM | Extracellular matrix |
| FucT | Fucosyltransferase |
| GnT-V | N-acetylglucosaminyltransferase V |
| HUVEC | Human Umbilical Vein Endothelial Cell |
| ICAM-1 | Intercellular Adhesion Molecule-1 |
| Lex | Lewis x |
| Lotus | Lotus Tetragonolobus Purpureaus |
| mAb | Monoclonal antibody |
| MMP | Matrix Metalloproteinase |
| PBS | Phosphate-Buffered Saline |
| PE | Phycoerythrin |
| sLea | sialyl Lewis a |
| sLex | sialyl Lewis x |

| | |
|---|---|
| TBST | TRIS-Buffered Saline with Tween |
| Ulex | Ulex Europeus agglutinin I |

# CHAPTER 1

# INTRODUCTION OF GLYCOSYLATION IN CANCER

## Glycosylation Overview

Glycosylation is defined as the conjugation of saccharides to carrier proteins (**Figure 1**) and lipids. Monosaccharides, the smallest carbohydrate units and, thus, the building blocks for glycosylation, interact via multiple linkages to form oligosaccharides and polysaccharides，which are also referred to as saccharides or glycans. The glycosidic linkages are the covalent bonds connecting monosaccharides through hydroxyl groups at different positions in the carbohydrate ring, which can be further divided into α or β linkages, depending on the position of the oxygen to the anomeric carbon. Oligosaccharides are linear or branched chains comprising several monosaccharides, while polysaccharides usually contain large glycans with repeating oligosaccharide motifs. Glycoconjugates are oligosaccharide units covalently linked through glycosidic bonds to protein forming glycoproteins, to lipids forming glycolipids, and to other polysaccharides constituting proteoglycans. With advances

in glycobiology, “the glycoform”, denoting the specific conformation and composition of oligosaccharides within the glycan chain has become the accepted umbrella terminology. In summary, glycosylated structures are both highly complicated and variable due to the innumerable combinations of monosaccharides, linkage variables and positioning of hydroxyl groups, as well as molecular branching patterns, giving these glycoforms their unique structures and functional characteristics.

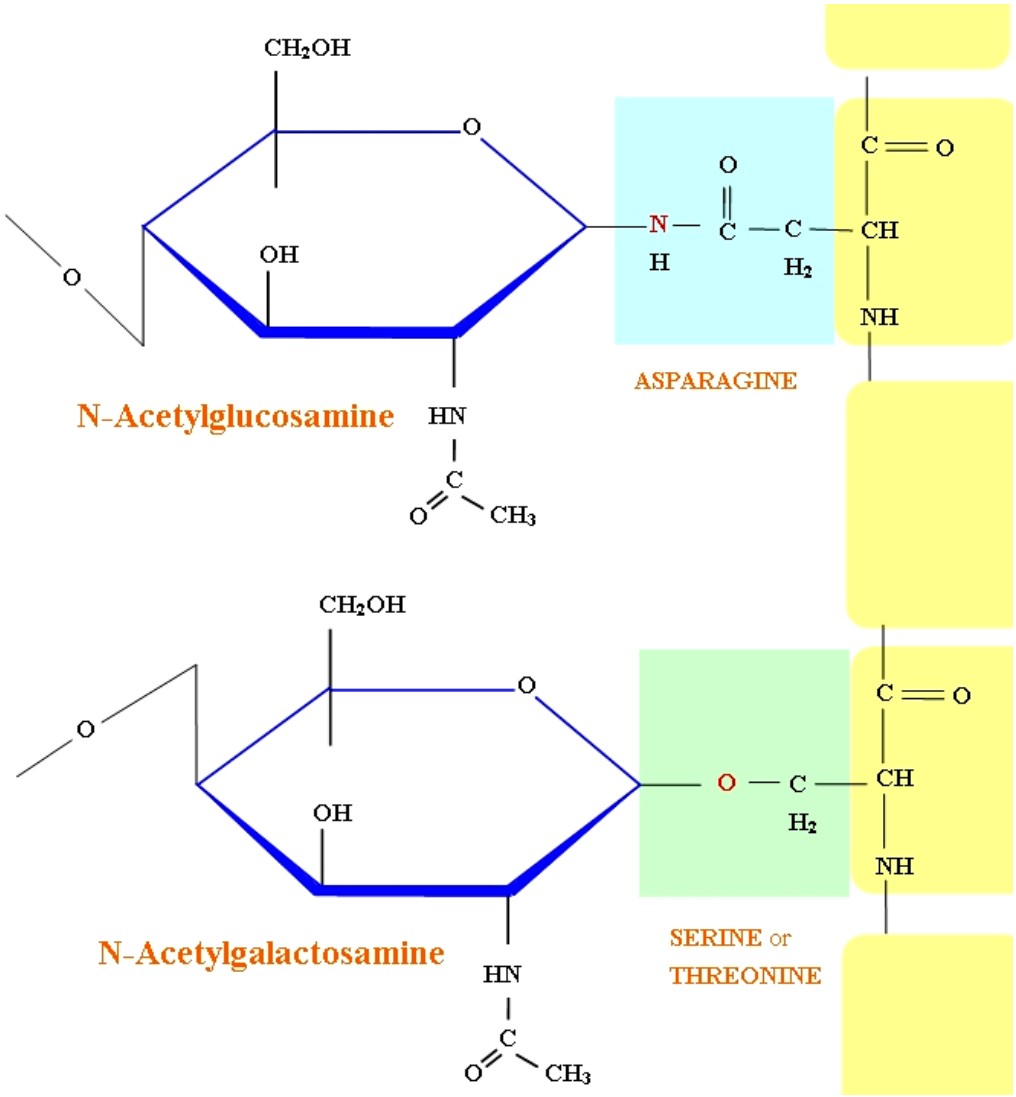



**Figure 1. Illustration of N- (Top) and O-glycosylation (Bottom) on a representative glycoprotein**. Through an -$NH_2$ or -OH group, Asparagine, or Serine (Threonine) in a polypeptide is conjugated to N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) respectively, the latter further linked to other monosaccharides to build the glycosylated chain. The yellow box represents individual amino acids in a polypeptide backbone. The blue and green boxes represent characteristic "R" groups of asparagine, serine or threonine respectively. The N- or O-glycosylations are named according to the atom (highlightened in red) which links the carbohydrate rings to the polypeptide.

## Biological Roles of Glycosylations

Glycosylation is one of the most important post-translational modifications that occur in proteins and carbohydrate conjugations. Glycans are now widely acknowledged to play critical roles in both protein function and molecular lifespan. Glycosylation events attract relatively less attention from biomedical investigators than that afforded proteins and nucleic acids, mainly due to the enormous diversity of glycans, underlying their many versatile functions. The prevalent expression of carbohydrates associated with various types of glycoprotein and glycolipid moieties underlie their participation in various molecular communication events between cells and between the cell and its matrix. It has been previously established that glycosylation of proteins modulate that protein's role in recognition, stabilization and maintenance of the conformational state of that protein [1], which in turn influences cell differentiation, embryogenesis, leukocyte trafficking, immune regulation [2] and many other biological events important for organism development, growth and survival. The characteristics of glycosylation mediated recognition and interactions are alternatively weak, and multiple, yet precise and intricately encoded [3]. The sum of these subtle effects can result in dramatic modification of the final biological functions of these proteins. The functional diversity of glycosylation events arises from their multiple linkages, branching patterns and terminal

modifications. Furthermore, one particular glycan may play different roles depending on the biological module to which it anchors and the specific environment in which the interaction occurs.

The structural and modulating functions that glycans confer are through folding, trafficking, packaging, stabilization, protease protection, and maintenance of quaternary structure [4]. For example, selective glycosylation of specific N-glycosylation sites results in functionally distinct receptor glycoproteins [5]. Additionally, branching arrangements in complex-type tri-antennary N-glycans can regulate ligand-binding properties [6]. Diverse targets of glycosylation modification, in turn, include RNA polymerase, transcription factors, chromatin-associated proteins, proto-oncogenes, tumor suppressors and cytoskeletal components [7]. Further examples include the knowledge that glycosylation of gastric mucins help to maintain its normal structure and the properties of the gastric mucus [8], while the size of carbohydrates bound to the adhesion molecular, ICAM-1, affects its binding to the Mac-1 integrin [9].

In addition, glycosylation events participate in various signal transduction pathways and modulate cell development and differentiation [4]. O-GlcNAc exists as a single monosaccharide residue without further elongation and provides highly dynamic, transient modifying effects. O-GlcNAc modification resembles

phosphorylation and may act as an antagonist to phosphorylation thereby working in concert with it to regulate protein interactions [10] . Glycosylations usually imply incomplete on-off switching and “fine-tuning” effects to modify the functions of the protein to which it is attached. A few examples of these functions include hormone binding [11], intracellular transportation [12], secretion [13] and association of growth factor growth factor receptors [14], along with regulation of cytokines [15] and the secretion of organ/tissue-specific cellular surface molecules such as prostate specific antigen [16].

Thirdly, glycans also constitute ligands for endogenous or exogenous macromolecules by forming recognition epitopes [4]. Interactions involving glycan ligands lead to cell activation, embryonic development, organogenesis, and differentiation as well as transformation and neoplastic progression. With considerable structural specificity, certain glycans act as receptors and/or decoys for a variety of viruses, bacteria, parasites and toxins [4]. Increasing evidence points to the conclusion that, oligosaccharides participate in cell-cell recognition and cell-matrix interactions involving adhesion molecules, e.g., selectins, CD44, cadherins and integrins. Although single site glycan-ligand affinities may be weak, considering the innumerable glycoproteins carrying that specific glycan, it’s not surprising to find

that relatively high-avidity effects can be accumulated at a level sufficient to mediate biologically interactions.

## Glycosylation and Cancer

Alterations of the glycosylation status of mammalian cells are commonly found to be associated with malignant transformation and neoplastic progression [17], and these altered glycans also exert a functional influence on multiple cellular targets. Embryogenesis and cellular activation are generally concomitant with changes in cellular glycosylation profiles; likewise, altered glycosylations are often seen in tumor cells during rapid growth, adhesion to other cells and matrices, invading of surrounding tissues and distant metastases [4]. Certain types of glycan structures are well-known tumor markers useful for clinical diagnosis and predicting prognosis. Either diminished or enhanced expression of certain structures are found in glycosylation states associated with malignancies, other changes include persistence of incomplete or truncated structures, accumulation of precursors, and appearance of novel structures. Examples of these changes include, different glycosylation profiles that have been identified to be cell line specific in regard to MUC1 mucin production in human breast cancer cell lines [18]. Osteolytic

variants of human breast cancer cell lines have further been seen to have prominent differences in the type of glycosylations as compared to non-osteolytic parental cells. Increased expression of certain glycosyltransferase genes have also been noted to be associated with enhanced binding to bone marrow endothelial cells and to the extracellular matrix [19]. Malignancy-related changes have also been revealed in glycolipids. Gangliosides that participate in cell adhesion and subsequent signal transduction alterations have been seen to lead to enhanced motility and invasiveness [20]. Sialyl fucosyl poly-N-acetylgalactosamine gangliosides, without the sialyl Lewis epitopes, lastly have been shown to be able to mediate E-selectin-dependent rolling and tethering under physiologic shear stress conditions [21].

Alterations in the glycoform of tumors are mainly the result of the alteration in the activity of glycosyltransferases. Tissue-specific regulation of glycosyltransferases contributes to its influence on the structural diversity, localization, half-life, and biological activity of glycoproteins. Biosynthesis of carbohydrate structures is tissue-specifically regulated by unique glycosyltransferases including fucosyltransferases, sialyltransferases, and N-acetylglucosaminyltransferases. Multiple glycosyltransferase genes are also up-regulated during neoplastic progression and this increase results in extensive glycosylation of glycoconjugates in cancer cells [22].

During tumorigenesis and tumor progression, prevalent changes occur in the glycosylation of various glycoproteins, including acetylcholinesterase in human breast cancer [23], human chorionic gonadotropin in choriocarcinoma [24], γ-glutamyl transferase in kidney and prostate cancers [25], ribonuclease in pancreatic adenocarcinoma [26], and thyroglobulin in papillary thyroid carcinoma [27]. Some well-established tumor associated antigens also display different glycoforms, in contrast with their normal counterpart. In neoplasms, among these glycosylation changes elevated fucosylation was identified in association with prostate specific antigen (PSA) and α-fetoprotein (AFP) derived from tumor origins [28, 29]. The glycosylation of serum α1 proteinase inhibitor in patients with breast and ovarian cancer has subsequently been found to have decreased branching, an increase in α-2,6-sialic acid and increase in core α-1,6-fucose [30, 31].

Concurrently, haptoglobin has been shown to have increased branching, more branches ending in α-2,3-sialic acid and increased terminal α-1,3-fucose associated with these tumors [32]. Increased fucosylation of serum $\alpha_1$ antitrypsin [33] and transferrin [34] were similarly found in patients with hepatocellular carcinoma. The glycosylation of caricoembryogenic antigen (CEA) on colon epithelial cells from tumor resources has also been seen to be displayed with different patterns, characterized by elevated Lewis x antigen (Lex) and de novo expression of Lewis y

antigen (Ley) on these tumor-associated CEAs. These Lewis antigens have previously been found to mediate the recognition of malignant CEA by dendritic cells [35]. Increased O-glycosylation on mucins has also been reported in T47D breast cancer cells [36]. Abnormal glycosylation of mucin in neoplastic breast tissue has been found to result in the exposure of a highly immunogenic core peptide epitope as well as the normally cryptic core Tn, STn, and TF carbohydrates. O-glycosylation is similarly known to contribute to the conformational and dynamical effects within the immunodominant region of MUC1 [37].

The modifications of antigenic epitopes by glycosylation render significant effects on immunological responses too. Studies have reported that the glycan moiety can affect the carrier glycopeptides' immunogenicity recognized by T cells [38] as well as NK cell mediating immune responses [39]**.** The efficiency of dendritic cells to process mucin and the resulting strength of any CTL activity were similarly see to be inversely correlated with the degree of glycosylation of the antigen [40]. Other studies have shown N-linked glycosylation is required for the effective presentation of mouse mammary tumor virus superantigens to T cells [41]. In other work, all-trans retinoic acid has been seen to decrease the activity of α-1,2-fucosyltransferase in a rat colon carcinoma cell lines variant, resulting in decreased expression of fucosylated glycoconjugates and a significant increase in the

sensitivity to Lymphokine-Activated Killer cell (LAK) cytotoxicity [42]. Removal of fucose from antibodies has been seen to enhance antibody-dependent cellular cytotoxicity (ADCC) by anti-human epidermal growth factor receptor humanized antibody in the immunotherapy of breast cancer patients [43].

Both the clustering and the conformation of carbohydrates displayed are also know to be important in initiating efficient anti-saccharides immune responses [44]. The N-glycosylation of the light chain variable region of antibodies has been reported to affect its antigen-binding activity, and further, with its identification in standard immunologic tests [45]. Along similar lines, the aberrant glycosylation of MUC1 on tumor cells affects the efficiency of monoclonal antibodies to recognize the carrier glycoprotein [46]. Sialic acids on termini of neighboring oligosaccharides significantly limit the recognition of the peptide region of MUC1 by the antibodies HMFG-1/2 and SM-3 [47]. High levels of under-glycosylated MUC1 have been found in benign prostate hypertrophy, as well as in prostate cancer and metastatic prostate cancer tissues, which could not be detected by antibodies specific for MUC1 carbohydrate epitopes [48].

The functional roles that glycans play in tumor metastases are a consequence of aberrant tumor glycosylation, which in turn, translates into a poor prognosis in patients with cancer. Glycans play a fundamental role in defining the molecular

mechanisms for metastases and for promoting the colonization of neoplastic cells in target organs. Further, alterations of glycosylation have been identified during the final transformation of breast cancer to a metastatic phenotype [49] and have been shown to be directly associated with nodal metastases [50]. Specifically, abundant expression of β-1,6-branched oligosaccharides have been associated with breast carcinoma lymph node metastasis and proven to be a predictor of poor outcome in node-negative tumors [51]. Additionally, lymph-node-metastatic mammary carcinomas have been shown to contain more tri- and tetra-antennary structures than those non-metastatic carcinomas [52].

In summary, increased branching of complex carbohydrates, sialylation, and enhanced Lex and sLex antigen expression on heavily glycosylated cell adhesion molecules including integrins, cadherins and immunoglobulin superfamily members, all are known to contribute to the acquisition of the metastatic phenotype [53]. One such example is the finding that N-cadherin from metastatic melanoma cells was found to contain more tri- or tetra-antennary complex type glycans with α-fucose and sialic acid than their non-metastatic parent cells [54]. Changes in the glycosylation pattern of malignant soft tissue tumors are further known to mirror their cellular activity, differentiation, cellular distribution as well as their ability to engage in malignant transformation and tumor progression [55]. The hepatic

metastasis of colorectal cancer are similarly correlated with their surface expression of Lex, sialyl dimeric Lewis x and the binding sites of fucosylated carbohydrate chains [56]**.** Glycosylation events may also yield negative effects on tumor progression. O-linked glycoproteins on SW1990 pancreatic cancer cells have been seen to inhibit their adhesiveness and aggregation. In addition, reducing O-glycosylation has been seen to enhance cell adhesion to Matrigel, laminin and collagen type IV while promoting homotypic cellular aggregation [57].

## Breast Cancer

It is estimated that 32% of all new cancer cases in American women in 2006 were of breast origin and 15% of all cancer deaths in females was directly related to this same group of malignancies, secondary only to lung cancer in frequency [58]. Tremendous efforts have been made regarding the treatment of breast cancer. The combination of chemotherapy, radiation therapy, and surgery has vastly improved the average patient's course. Unfortunately, the majority of deaths from breast cancer is still associated with metastases, notably, to the brain and bone. Decreasing the possibility of breast cancer recurrence, especially distant metastasis, will most

likely produce a survival benefit and a potentially significant improvement in quality of life in women with breast cancer. Thus, early detection and interference of tumor progression (namely, invasion and subsequent metastases) are pivotal in decreasing the death rate of patients with breast cancer.

# CHAPTER 2

# METHODS

## Maintenance of Cell Lines

MDA-MB-231, a highly invasive human breast cancer cell line, was obtained from the ATCC (American Type Culture Collection, Manassas, VA), and cultured in Leibovitz's (L-15) medium supplemented with 10% fetal bovine serum (obtained from the Media Preparation Shared Facilities, University of Alabama at Birmingham, Al). Cells were maintained at 37℃, in a humidified atmosphere without exogenous $CO_2$, as recommended by ATCC. Other human breast cells lines used including T47D and MCF-7 cells along with HT-1080 human fibrosarcoma cells, which were recovered and expanded from -150℃ freezer stocks, were maintained under similar standard techniques. Human Umbilical Vein Endothelial Cells (CRL-1730) were purchased from the ATCC (Manassas, VA), and cultured in Ham's F12K complete growth medium (ATCC 30-2004), supplemented with 10% fetal bovine serum and 0.03 mg/ml endothelial cell growth supplement (Sigma,

Saint Louis, MO). HUVEC cells were cultured on 3.5cm-diameter culture dishes (Costar®, Corning, NY) at 37℃, supplemented with 5% $CO_2$.

**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), phycoerythrin (PE) labeled mouse anti-human CD44 monoclonal antibody, FITC labeled monoclonal mouse anti-human CD15 antibody, mouse anti-human sialyl Lewis x mAb Km93 (Calbiochem, La Jolla, CA), FITC labeled Ulex Europaeus agglutinin & Lotus Tetragonolobus lectin (Caltag, Burlingame, CA). Biotin, peroxidase and FITC labeled Ulex Europeus agglutinin I (Ulex) were purchased from EY labs (San Mateo, CA). Type I collagen, gelatin, heparin, hyaluronic acid, tunicamycin, swainsonine, benzyl-N-acetyl-a-D-galactosamine and α-L-fucosidase (from bovine epididymis) were all purchased from Sigma Chemical Co. (Saint Louis, MO). Deoxyfuconojirimycin was acquired from Industrial Research Ltd., (Wellington, New Zealand). Other materials used included: Micron centrifugal filters YM-10 (Millipore, Bedford, MA), MTT assay kits (Promega, Madison, WI), and BCA agents (Pierce, Rockford, IL). Streptavidin alkaline phosphatase and Western Blue® stabilized substrate for alkaline phosphatase were purchased from Promega (Madison,

MI). Transwell plate inserts (6.5 mm diameter, 8.0 μm pore size) were purchased from Costar® (Corning, NY). CellTrace™ Calcein Red Orange and green, Alexa 647 goat anti-mouse IgG, Alexa 647 anti-mouse IgM, and streptavidin conjugated with Alexa Fluor 488, were purchased from Molecular Probes® (Eugene, OR). All other chemicals were obtained from Sigma® Chemical Co., if not individually specified.

## HuBiogel® a Natural Human Extracellular Matrix

HuBiogel® was kindly provided initially by Diversified Scientific Incorporated and subsequently by In Vivo Biosciences (both of Birmingham, Al). HuBiogel® is a natural complex biologically-active extracellular matrix derived from normal human amniotic membranes, as modified from techniques first developed by Siegal et al. [59]. 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, $FGF_{1,2}$,TGF-alpha, TGF-$\beta_{1-3}$ and PDGF [59]. HuBiogel® has been validated as a suitable model of human derived ECM for the study of tumor invasion [60]. HT 1080, a highly invasive human fibrosarcoma cell line, was used as a positive control cell line to further define the effectiveness of the technique.

**Fucosidase Treatment Protocol**

Fucosidases are the major lysosomal glycosidases that catalyze the removal of fucose residues from glycosylation sites. Alpha-L-fucosidase (E.C. 3.2.1.51), hydrolyzes the α-1,2-, α-1,3-, α-1,4- or α-1,6-L-fucose linkage glycosidic bonds [61]. Exogenous glycosidases have been widely used to modify the composition of cell surface glycoproteins; "Debulking" fucosylation by a direct biochemical approach would be presumably faster, more convenient and more clinically practical compared to available genetic manipulations. Therefore, alpha-L-fucosidase (α-L-fase), was diluted in sterile PBS to a concentration of 1.69 mU/ml (8.8mU/$10^6$ cells). 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% trypsinization. Cell numbers were counted, fucosidase (8.8 mU /$10^6$ cells) was then mixed together with the cells and then incubated, at 37℃, for 30 minutes. Cells were finally washed with PBS and centrifuged to remove any residual fucosidase.

**Invasion Assay of Tumor Cells through HuBiogel®**

Transwell plate inserts (6.5 mm diameter, 8.0 μm 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-fucosidase as described. In parallel, the same number of cells was treated with fucosidase plus 1 nM Deoxyfuconojirimycin hydrochloride (DFJ) simultaneously, at 37℃, for 30 min. As a further control, cells under identical conditions were sham treated with PBS. 5 x $10^4$ cells from each group were plated in 400μl of appropriate media containing 1% FBS. The lower chambers were filled with L-15 medium containing 10% FBS. The invasion assay assemblies were immersed in 6-well culture plates with 10% FBS supplemented culture medium outside the invasion chamber. For experiments using the glycosylation inhibitors, Tunicamycin (10μg/ml), Swainsonine (0.3μg/ml), Benzyl-N-acetyl-a-D-galactosamine (i.e., Benzyl-GalNAc, 1.5μg/ml) or Ulex lectin (5μg/ml) individual agents were added to the upper chambers of the invasion system. After 48 hrs, filters were removed and stained with dilute solution of crystal violet. The number of invading cells on the whole filter was counted under 200 X magnification with the aid of a reticle-containing eyepiece.

**Proliferation Assays measured by MTT and Automatic Cell Counting**

Cells were trypsinized, resuspended in PBS, counted and a subset was treated with fucosidase as described. 2 x $10^3$ cells/well, in 100 ml of medium, were then plated onto a 96-well culture plate. At 0 hr, 24 hrs, 48 hrs and 72 hrs post-treatment, an MTT assay was performed in triplicate, as per the manufacturer's instructions. The optic density was read with an ELISA reader at 490 nm for calibration. As an alternative method, cell proliferation was also determined by counting individual aliquots of cells, in triplicate, using an automatic Beckman Cell counter. In these later experiments, cells in the presence or absence of fucosidase 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 5 samples, and each experiment was performed in triplicate. There was no statistical difference between the 2 methodologies.

**Static Adhesion Assay**

96-wells culture plates were coated with HuBiogel® (0.64 μg/μl), type I collagen (1 mg/ml), hyaluronic acid (1 mg/ml), laminin (5 μg/ml) or fibronectin (1 μg/ml) as noted before [62]. MDA-MB-231 cells were harvested and treated with α-L-

fucosidase or PBS. 10,000 cells under either condition were plated onto these coated wells. The number of adhered cells was determined following the protocol of Rusciano et al. [63]. Cells were let to adhere for different stated times and plated wells were washed with PBS 3 times and fixed with 4% paraformaldehyde for 30 min. Adherent cells were subsequently stained with 0.1% crystal violet for 15 min and washed with distilled water. Crystal violet in the stained cells was then released in 5% Triton X100. The optic density was calibrated with an ELISA plate reader at a wavelength of 570 nm. Data were expressed as the mean of 8 wells $\pm$ SE. Some experiments were measured directly by an Olympus inverted microscope with aid of a grid eyepiece (Fisher). In blocking experiments, hyaluronic Acid and HuBiogel® were coated on 96-well plates and either km93 anti-sLex monoclonal antibody or HCAM anti-CD44 polyclonal antibody were applied to block the specific sLex or CD44 mediated interactions, respectively.

For the static adhesion assay on HUVEC, after fucosidase treatment, the same number ($5 \times 10^3$/ml) of treated and control MDA-MB-231 cells were seeded onto the confluent HUVEC monolayer cultured on 96 well-plates. After the indicated time of co-culture, the wells were then gently washed in PBS and the adhered tumor cells were counted under microscopy with the aid of a grid eyepiece. MDA-MB-231 cells

overlaying HUVECs could be easily identified by their different size and shape under light microscopy. Data were analyzed by the Mann-Whitney Test.

### Zymography to Detect MMPs Activities

The gelatin substrate zymography method was utilized as reported previously [64]. Briefly, cells were trypsinized, washed with PBS twice and treated under three conditions, with fucosidase, with fucosidase plus Deoxyfuconojirimycin (DFJ) or with PBS as a sham control. Identical numbers of cells ($5.0 \times 10^6$) were cultured in L-15 complete medium (containing 10% FBS). After allowing 24 hr for attachment, the old medium was replaced with serum-free L-15 medium; cells were then incubated for another 48 hrs. Conditioned media from cells treated with or without fucosidase were collected and concentrated with a YM-10 Micron Centrifugal Filter following the protocol provided by the manufacturer. The protein concentration in the conditioned media was determined by conventional BCA methods. Fifty microgram 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 (50mM Tris-HCl [pH 7.5], 200mM NaCl, 5mM $CaCl_2$, 0.02% Brij-35) overnight at 37℃. 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 fucosidase and compared to the same number of PBS sham-treated cells cultured on cover-slips for the indicated time points. 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, in 5% BSA (in PBS) for 2 hrs, detected by streptavidin-alkaline phosphorylase for 1 hr, and the color developed by Western Blue® stabilized substrate for alkaline phosphatase following the protocol of the manufacturer (Promega Corp., Madison, WI). The images were captured with a BX51 conventional bright-field Olympus® Microscope (Melville, NY) and the fucose expression intensity was determined by utilizing BIOQUANT® image analysis software (BIOQUANT Corp., Nashville, TN). Each experimental group was

composed of 3 cell surfaced 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 fucose expression was calculated comparing the "positivity" (above threshold) to all staining.

**Flow cytometry**

Cells were treated with fucosidase as described above. Equal number of cells was treated with PBS as control. Both groups of cells were incubated with antibody against Lex (CD15), Ulex Europaeus I lectin labeled with FITC and anti-CD44 antibody labeled with phycoerythrin. In parallel, negative (unlabeled) control samples were sham treated (auto-fluorescence control) or with an appropriate isotope control. The detection of sLex by flow cytometry was reported before [65]. Briefly, detached cells were washed, re-suspended in PBS containing 1% BSA, and then incubated with KM93 monoclonal antibody against sLex (1:50 dilution) for 30 min, at 4℃. After two washes, cells were incubated for 45 min, at 4℃, with a 1:200 dilution of FITC-conjugated goat antibodies against mouse IgM. Cells were again 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 a 2-D histogram, as well as 1-D histograms of each florescence channel. The result were calculated and presented as Fluorescence Intensity (Mean ± SEM).

## Immunofluorescence Analysis

The immunofluorescence expression of Lewis antigens followed the protocol as described before with some modifications [66, 67]. After standard fucosidase treatment, MDA-MB-231 cells were grown on tissue culture-treated glass cover-slips for 24 hrs; cells were fixed in 1% paraformaldehyde/PBS for 20 minutes, and blocked in 2% normal bovine albumin and 0.1% Tween 20 in PBS, for 1 hr, at room temperature. Cells were incubated overnight, at 4℃, with biotin-conjugated lectin Ulex (EY Labs) to bind fucose, diluted monoclonal antibodies against sLex, $\alpha v$ integrin or β1 integrin subunits. After three washes in PBS, secondary antibodies including Alexa 647 goat anti-mouse IgG, Alexa 647 anti-mouse IgM or streptavidin conjugated with Alexa Fluor 488 (Molecular Probes) were diluted in a blocking solution and incubated with the cells, for 1 hour, at room temperature in the dark. Cells were washed 3 times in PBS and mounted, and images were taken by using Olympus® microscope with fluorescence lenses at proper wavelengths.

**Flow Chamber Assay**

An in vitro biophysical model in vitro to study the adhesion of MDA-MB-231 cells to human umbilical vein endothelial cells (HUVEC) under flow conditions was outlined as modified from the report by Gomes et al. [68]. After cell growth reached 80% confluence, MDA-MB-231 cells were trypsinized and counted. For experiments using unlabeled cells, cells were evenly assorted into three groups, two groups were treated with α-L-fucosidase, at 37℃, for 30 and 60 minutes respectively, while the third group, control cells, were incubated with only PBS for 60 minutes. Cells were then diluted in HBSS (Hanks' balanced salt solution) to a final concentration of 1.0 x $10^6$/ml and transferred to 15ml syringe. A GlycoTech (Rockville, MD) flow chamber insert and gasket were inserted into the dish cultured with HUVEC cells reaching 90% confluence, which formed a laminar flow chamber that can be viewed on a microscope. Cells were injected into the flow chamber in Hanks' balanced salt solution at controlled physiological shear rates using a programmable syringe pump (KD Scientific, New Hope, PA). Cells were viewed by an Axiovert 100 microscope (Zeiss Inc., Thornwood, NY) equipped with a CCD camera (Model 300T-RC, Dage-MTI,

Michigan City, IN). For the fluorescence-labeled experiments, fucosidase treated MDA-MB-231 cells were labeled with CellTrace$^{TM}$ Calcein Red Orange (Molecular Probes), while tumor cells in the control group were labeled with Calcein green (Molecular Probes) dye. After incubation with the fluorescence dyes and washing thoroughly, 5ml of each group of cells were mixed well and the mixture of cells with different colors of fluorescence were applied to the flow chambers, followed the procedures described before except under fluorescence microscopy. The image were recorded on sVHS videotape, the number of rolling cells labeled with red color (fucosidase treated) and green color (control) at selected time intervals were counted, respectively.

**Purified Substrate Coatings**

To study the adhesion of cancer cells on individual adhesion molecules under flow conditions, diluted E-selectin, P-selectin and ICAM-1 were pre-coated following published procedures as described before [69]. Briefly, tissue culture dishes (35 mm) were marked with a diamond pen to make a small circle in the center. Then 25μl of 20μg/ml protein A (Sigma) in PBS was placed in the marked area and spread with a pipette tip, incubated at 37℃ for 1 hr, and then washed three times with PBS.

Nonspecific binding was blocked with 2% human serum albumin (HSA, Sigma) in PBS, for 2 hrs, at 4℃ followed by 3 washes with PBS. The marked areas were then coated with 50μl purified substrate (25μg/ml recombinant human ICAM-1/Fc chimera, 5μg/ml recombinant human E-selectin/Fc chimera and 0.5μg/ml recombinant human P-selectin/Fc chimera). All purified coatings were obtained from R&D Systems (Minneapolis, MN).

**Image Analysis of Flow Chamber Assay Results**

For direct manual counting, video was recorded onto sVHS videotapes (SONY Premium grade), and the number of cells undergoing rolling during a specified time interval were counted by two independent observers. For computer-aiding image analysis, selected sequences of 3 minutes in length were digitized to TIF files using the Perception video editing package (Perception PVR-2500, Digital Processing Systems, Markham, Ontario, Canada). Digitized video images were examined frame-by-frame to determine the number of cells rolling on endothelium and number of leukocyte arrests (complete stops). To calculate the flow speed of individual cells, the flow course of each specific cell in one frame was recorded in a

log file by MetaMorph® software (Universal Imaging Corporation, Downingtown, PA). Briefly, when a new object appeared in the image frame (coming into the field of view, either rolling or flowing) they were identified as cells, assigned a number, and tracked. A log file was generated and subsequently processed by programs written by Dr. Dennis Kucik, Department of Pathology in UAB. Processing includes application of exclusion criteria based on area and velocity to discard cell doublets, objects too small to be cells, etc. The remaining cell tracks were processed to produce instantaneous velocity plots and average cell velocity histograms. Results were expressed as means ± SEM and analyzed by a Student's t test using the Excel® software.

**Statistical Analysis**

The data were analyzed utilizing Excel® (Microsoft, Seattle, WA) and SPSS® (Chicago, IL) 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 2 samples t test was also utilized where appropriate. A "p value" less than 0.05 was considered to be statistically significant.

# CHAPTER 3

# MODIFICATION OF CELL SURFACE FUCOSE AFFECTS HUMAN BREAST CANCER CELL FUNCTIONS ASSOCIATED WITH NEOPLASTIC PROGRESSION

## Introduction：Fucose in Cancer

Fucose is a deoxyhexose usually present in the terminal linkage of various N- and O-linked glycoproteins. Fucose can be α-1,2, α-1,3, α-1,4, or α-1,6 linked. Glycans containing fucose play important roles in blood transfusion reactions, selectin-mediated leukocyte-endothelial adhesions, and host-microbe interactions. Increased cell surface fucosylation has been found in various types of malignant neoplasms including thyroid carcinoma [70], prostatic carcinoma [71], urinary malignancies [72] and colon carcinomas [73]. Elevated fucosylation has also been found in both primary lung tumors and their corresponding lymph node metastases [74]. Changes in fucosylation patterns, it has been suggested, could be used for the diagnoses and monitoring of the successes of exogenous therapies [75]. This is due in part to the fact that elevated fucose levels have been identified in the serum of patients with cervical [76], colorectal [73], nasopharyngeal [77], lung [78], prostate [79]

and pancreatic carcinomas [80]. Urinary “free fucose” has also been significantly increased in patients with gallbladder, bile-duct, liver and pancreatic cancer [81], acute nonlymphocytic leukemia and myelodysplastic syndromes [82].

Fucose expression is closely associated with cell differentiation and aggressive behavior in transformed cells. Levels of fucosylation have been shown to be low in normal ovary and benign tumors, while being elevated with advancement of tumor grade in malignant lesions [83]. Increases in the complexity of the fucosylation pattern has also been observed with dedifferentiation and increased tumorigenicity in colorectal carcinoma and adenoma cell lines [84]. Fucosylation of certain glycoproteins has signaled enhanced invasiveness of urothelial tumor cells [85] and the metastatic phenotype of transitional cell carcinoma of the bladder [86], which in turn, was found to be associated with the prognosis of these patients [87]. Fucosylation of haptoglobin was seen to be increased in pancreatic cancer and enhanced with its advanced stage, while diminished after surgical operations [88] . Colorectal cancer cells dissociated from metastasizing tumors have also been shown to have a significantly higher fucosylation level than cells from non-metastasizing tumors [89]. In addition, the expression of fucose in brain metastasis from lung carcinoma was shown to be significantly higher than that of the corresponding primary tumor [90]. The functional significance of elevated fucose expression in malignancies may be partly explained by

the functional roles fucose-containing glycans play in tumor progression. Fucose itself, as a monosaccharide, has been found to be able to inhibit the adhesion of human myeloid leukemia cells to bone marrow stromal cells [91]. L-fucose has also been seen to reduce collagen and non-collagen protein production in cultured cerebral microvessel endothelial cells. Increasing L-fucose in the serum of rats similarly was found to result in decreased collagen production [92]. More importantly, fucose is known to be one of the tetrasaccharides constituting sLea and sLex, which are the ligands for the key selectins that mediate interactions of tumor cells with the endothelium.

The fucosyltransferases, a family of glycosyltransferases that specifically transfer GDP-fucose to the glycosylation chains, have been found to be elevated in a number of tumor types [93]. Biosynthesis of sLex and sLea are mainly controlled by α-1,3/1,4- fucosyltransferases. Mirroring fucose, itself, plasma α-1,3-fucosyltransferase levels have been found to be elevated in patients with hepatocellular carcinoma [94], ovarian cancer [95], esophageal, lung, pancreatic and biliary cancer [96]. Along similar lines, the activity of α-1,3- fucosyltransferase is known to be generally low in the normal gastric mucosa, whereas the activity has been found to be elevated in gastric cancer tissues with enhanced expression of sLex [97]. Fucosyltransferase products from peritoneal tissues contribute to the adhesion of

human gastric and colon cancer cells to the peritoneum [98]. Interestingly, prolonged culture at high cell density are also known to upregulate the expression of fucosyltransferase and sialylated Lewis antigens in human pancreatic cancer cells [99]. Additionally, the highest levels of α-1,3- fucosyltransferases have been found in metastatic liver lesions, in contrast to that found in normal tissues and in primary hepatic carcinoma [100]. The activities of terminal fucosyltransferase have also been seen to be elevated in breast cancer cells [101]. In patients with carcinoma of the ovary or breast and who have subsequently received chemotherapy, levels of α-1,3-fucosyltransferase have increased with disease progression and decreased when responsive to successful therapy [102].

Glycosidases specifically remove certain glycan structures from glycosylation sites, without disturbing intracellular glycosylation synthesis. Fucosidases hydrolyze specific glycosidic bonds between fucose and glycosylated chains. Upregulation of glycosidase may represent a host response to aberrantly elevated expression of certain glycosylation events in malignancies. The activity of α-L-fucosidase has previously been reported to be significantly higher in patients with hepatocellular carcinoma [103] and could be used as a tumor marker for the diagnosis for this type of cancer [104]. In a prospective follow-up study of cirrhotic patients, it was revealed that serum α-L-fucosidase activity was useful in the early detection of hepatocellular carcinoma, and

monitoring of cervical carcinoma [76]. Elevated activities of α-L-fucosidase and other glycosidases have been found in gastric and thyroid tumors [105]. In contrast, decreased activity of α-L-fucosidase may reflect a decompensation state in many other types of malignancies. For example, the activity of α-L-fucosidase has been seen to be significantly lower in patients with advanced malignant epithelial ovarian tumors [106] and colorectal cancer [107] as compared to normal controls, while the recurrence rate of colon cancer was found to be higher when α-L-fucosidase activity significantly decreased in tumor tissues [108].

As fucose-containing macro-molecules are closely correlated with neoplastic progression, a rationale has been established to study the potential effects of modification of fucose expression on tumor cells. The hypothesis proposed by us that by decreasing the fucose expression on cell surfaces, this would exert considerable effect on various phenotypic markers, and in turn, determine biological behaviors of breast cancer cells. By removal of fucose from oligosaccharide sites on the highly invasive and metastatic human breast cancer cell line MDA-MB-231, serving as a surrogate for all breast cancers, with α-L-fucosidase, we provide evidence for the validity of this strategy.

# Results

## *Alpha-L-Fucose is Widely Expressed on Breast Cancer Cells and is Decreased with Alpha-L-fucosidase Treatment*

Lectins are naturally occurring, carbohydrate-binding molecules that can be derived from various diverse biological sources and used to investigate the presence of carbohydrate structures in or on cells, in much the same way as antibodies can be used to probe cells and tissues for the presence of specific antigens. Most current glycobiology technologies depend on lectin-binding to specifically detect the presence of certain monosaccharides or glycans. In this report, fucose-recognizing lectins are used to measure the level of fucose expression.

Previous work in our lab has demonstrated that several human breast cancer cell lines displayed fucose on their cell surfaces. To further prove the effectiveness of defucosylation by α-L-fucosidase, we measured the change of surface expression of fucose by flow cytometry on MDA-MB-231, MCF-7 & T47D cells, after exposing them to fucosidase treatment as described before in Chapter 2.

As is seen in **Figures 2-3**, using the FITC labeled fucose specific lectin Ulex I, or FITC labeled Lotus lectin, fucosidase effectively reduced fucose expression without significantly damaging cell viability. The same results were seen with human T47D cells ( **Figure 4** ) and MCF-7 breast cancer cells( **Figure 5** ).

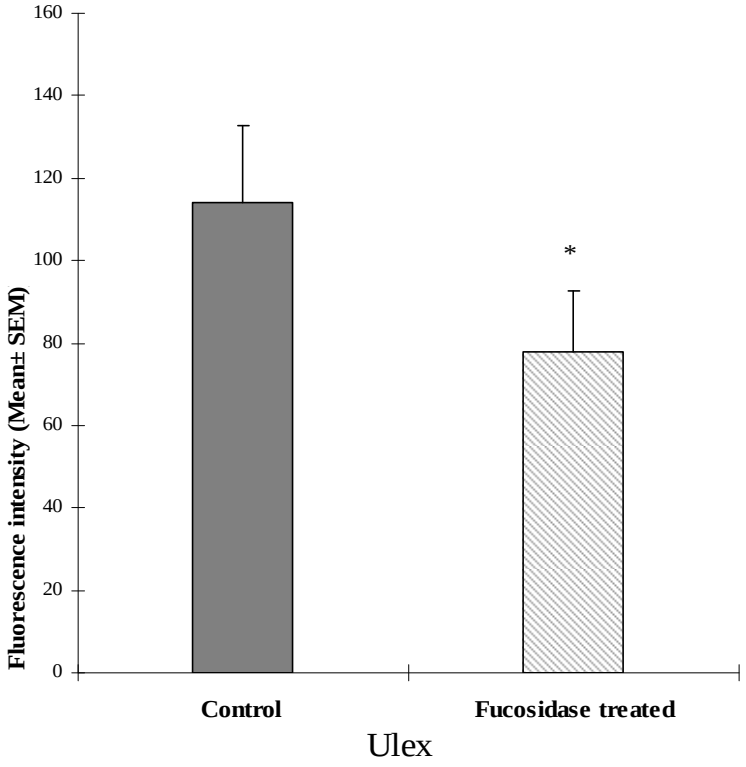



**Figure 2**

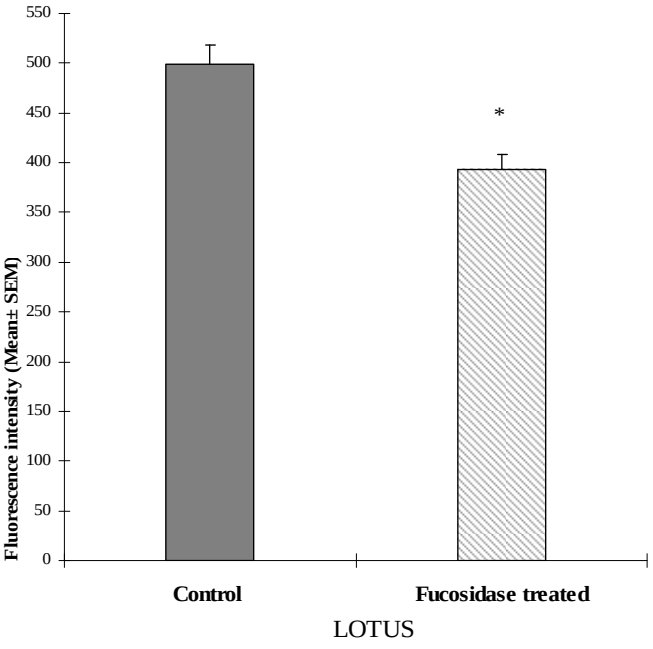



**Figure 3**

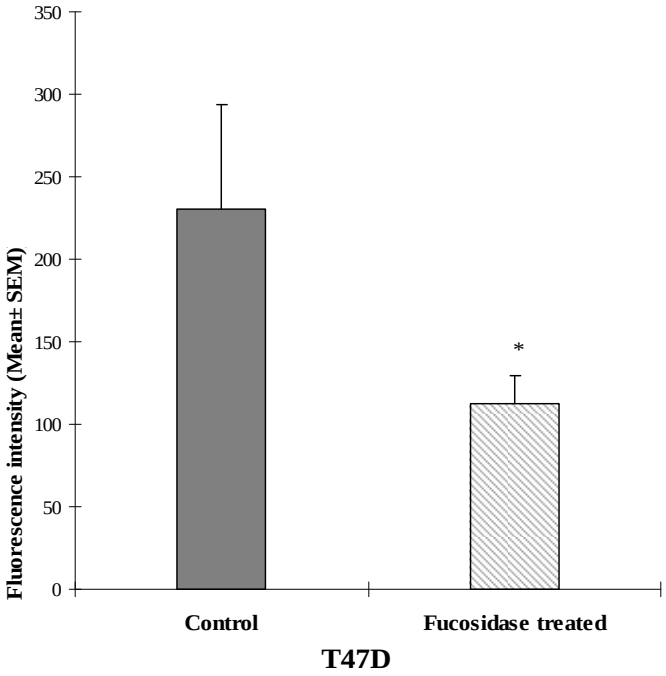



**Figure 4**

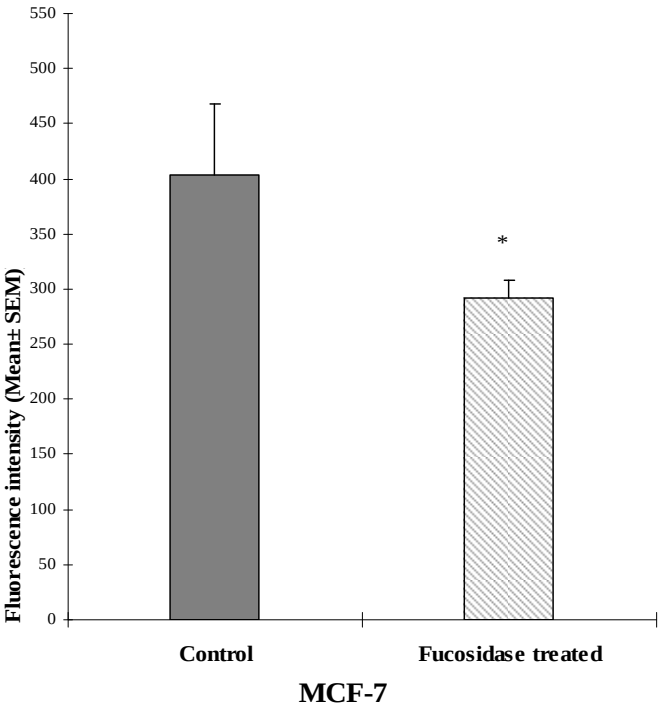



**Figure 5**

**Figure 2-5. α-L-fucosidase treatment decreased the expression of fucose on human beast cancer cells**: MDA-MB-231(**Figure 2&3**), T47D and MCF-7(**Figure 4&5**). Human breast cancer cells were cultured to 70-80% confluence, trypsinized and washed with PBS twice. Cells were mixed with 8.8 mU fucosidase/$10^6$ cells and incubated, at 37°C for 30 minutes. Immediately after the treatment period, cells were washed with PBS and centrifuged to remove fucosidase. Cells were then treated with the fucose specific lectins, FITC labeled Ulex Europeus 1 (**Figure 2,4,5**) or Lotus Tetragonolabus (**Figure 3**). In parallel, negative (unlabeled) control samples were sham treated (autofluorescence control). Flow Cytometry was performed and the mean fluorescence intensity ± SEM was calculated from 9 independent experiments, respectively. Each pair of experimental groups were analyze by a paired 2 sample t test and found to be significant ($p<0.05$). A Wilcoxon matched pairs signed rank test also revealed a significant difference ($p<0.01$) for MDA-MB-231 cells & $p<0.05$ for all others. ■ Untreated control; □ Fucosidase

By flow cytometry we further demonstrated that fucose expressed on tumor cells could be significantly decreased immediately by fucosidase, and this defucosylation effect was reversible **(Figure 6)**.

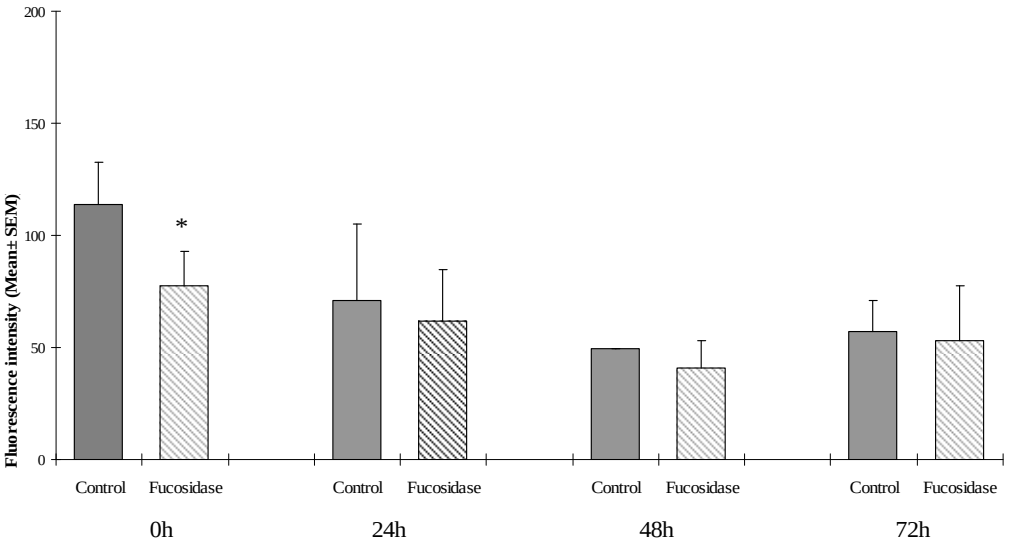
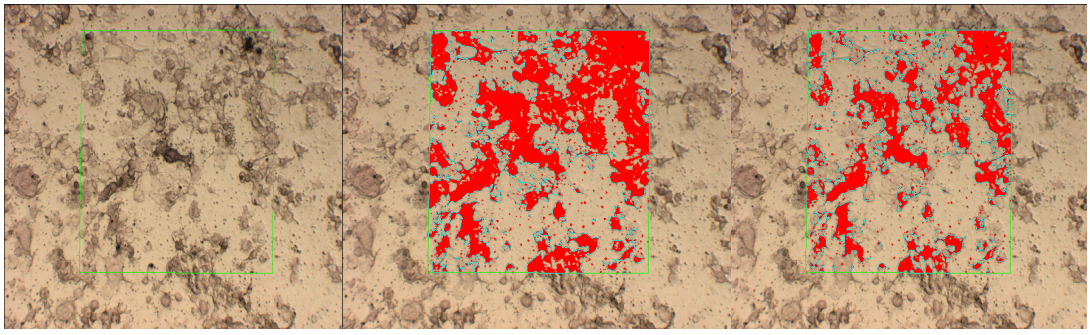


**Figure 6**. **Flow cytometry of fucose expression on MDA-MB-231 cells over time.** Cells were treated with α-L-fucosidase as described in the text and cells were left to recover for the indicated time periods. FITC labeled Ulex flow cytometry was performed at the indicated time points. The bar graph shows the mean ± SEM for the fluorescence intensity in three independent experiments. Each pair (control versus fucosidase treated) were analyzed by paired 2 sample t tests ($p>0.05$), as well as a Wilcoxon matched-pairs signed rank test ($p >0.05$). Only at time "0h" was there a statistically significant difference indicating the short-lived effect of the treatment. ■ untreated control □ fucosidase

Lastly, by using peroxidase-labeled Ulex lectin blotting as well as biotin-labeled Ulex histochemistry, we further demonstrated that fucose expression on breast cancer cell surfaces recovered by 12 hrs after fucosidase treatment as shown in **Figure 7**. The result of quantitative analysis of the data is shown in **Figure 8**.



All reactive cells within representative field

Reactive cells after painting

"Positive" staining intensity above threshold

**Figure 7**. **Lectin histochemistry and the morphometry of fucose expression.** MDA-MB-231 cells treated with fucosidase 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 (5μg/ml) in 5% BSA/PBS for 2

hrs, detected by streptavidin-alkaline phosphorylase for 1 hour, and the color developed in the presence of Western Blue®. The image was captured by conventional bright-field microscopy and the ratio of fucose expression to nonexpression was 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.

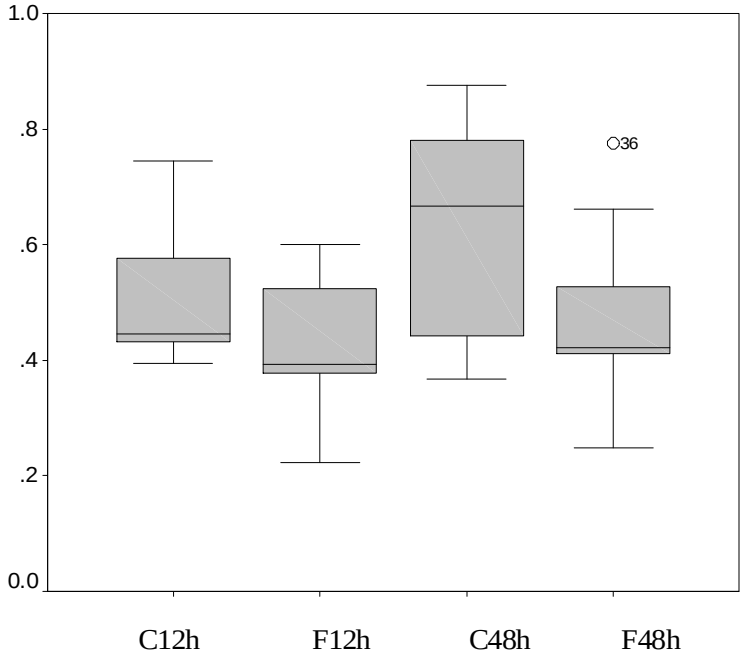



**Figure 8**. **Box-plot of the fucose expression as detected by lectin histochemistry.** Positive ratio of fucose expression at 12 hrs (C12h, F12h) and 48 hrs (C48h, F48h) for each time point (n=9), was calculated as described in **Figure 7**. A Mann-Whitney test was performed to compare the difference between control and fucosidase treated groups ($p>0.05$). In the box plot, y axis represents ratio of positivity, the box stretches from the lower hinge (defined as the 25$^{th}$ percentile) to the upper hinge (the 75$^{th}$ percentile); Median, Minimum and Maximum are also displayed. The differences are not significant ($p>0.05$) at either point.

***Alpha-L-fucosidase Treatment significantly Decreased the Invasion of MDA-MB-231 Cells through HuBiogel®***

To investigate the potential effect of defucosylation on breast cancer cell invasion, we performed in vitro invasion assays using Costar® Transwell plate inserts coated with the complex natural human extracellular matrix, HuBiogel®. Under the same experimental conditions for decreasing cell surface fucose, we found that MDA-MB-231 cell invasion through HuBiogel® was significantly decreased. Furthermore, this inhibition could be reversed by Deoxyfuconojirimycin, a fucosidase specific inhibitor [109] **(Figure 9)**.

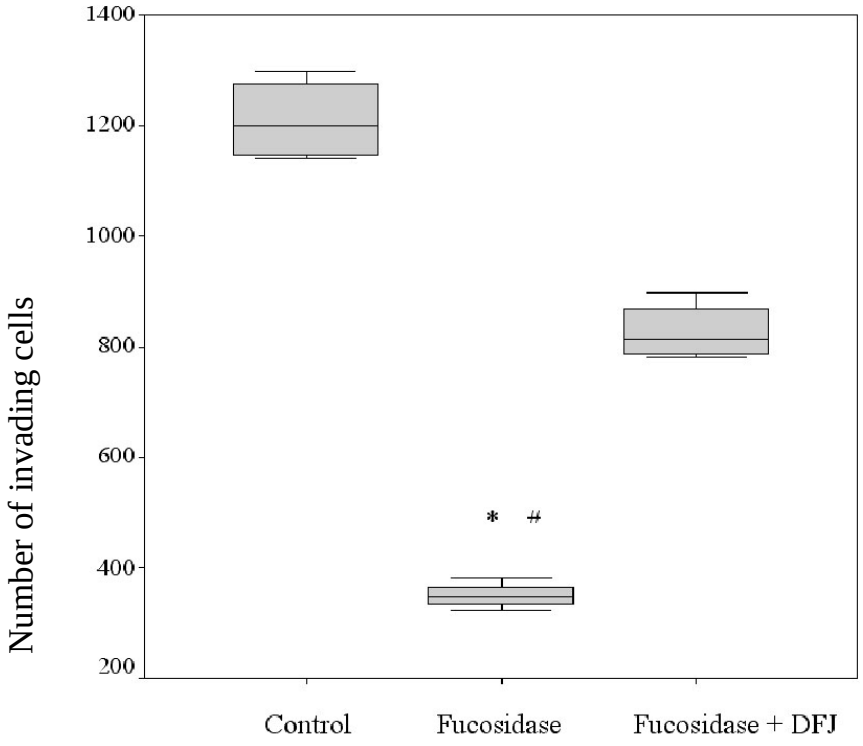


**Figure 9. Fucosidase treatment significantly decreased the invasion of MDA-MB-231 cells through HuBiogel®.** Deoxyfuconojirimycin, a specific inhibitor of fucosidase [109], abolished the inhibitory effect of fucosidase on tumor cell invasion. Invasion chambers were separated by inert filters coated with HuBiogel® (0.64 mg/ml). Cells were treated with fucosidase as described above. In parallel, the same number of cells was treated with fucosidase plus 1 nM Deoxyfuconojirimycin hydrochloride (DFJ) simultaneously, at 37℃, for 30 min. A third group of cells were sham-treated with PBS. 5 x $10^4$ 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 hrs, filters were removed and stained with crystal violet. The number of invading cells on the whole filter was counted at 200 X with the aid of a grid eyepiece. Data represent the mean of three independent filters. Differences in invasion between the groups were analyzed by the Mann-Whitney test. * indicates $p<0.05$ (fucosidase *vs.* control); # indicates $p<0.05$ (fucosidase *vs.* fucosidase + DFJ).

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® **(Figure 10)**. Interestingly, the fucose specific lectin, Ulex also caused a statistically significant inhibitory effect on the invasion of breast cancer cells in vitro **(Figure 10)**.

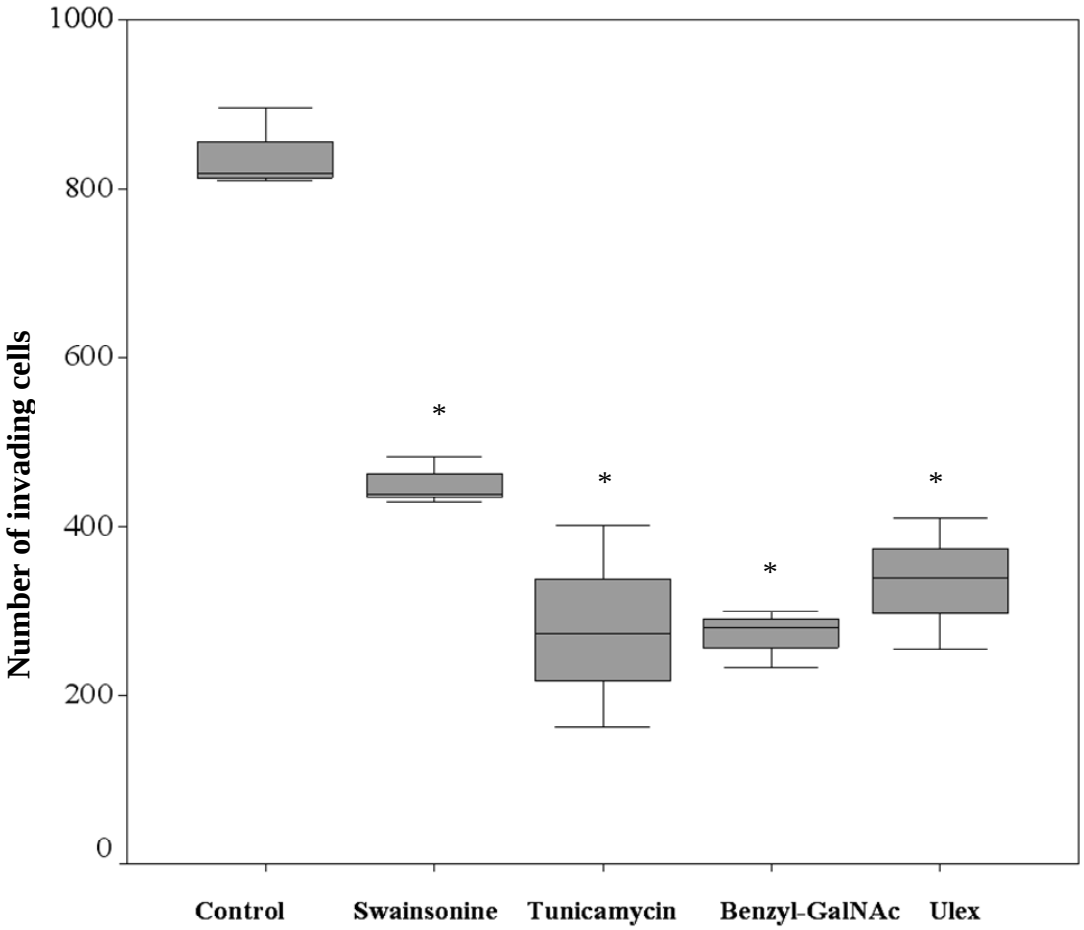



**Figure 10. N, O-glycosylation inhibitors and the fucose-binding lectin Ulex significantly decreased the invasion of MDA-MB-231 cells through HuBiogel®.** The number of invading tumor cells on the whole filter was counted (as described in **Figure 9**). Data represent the mean of three independent filters; each group treated was compared to control cells treated with PBS alone by a Mann-Whitney test. The upper chamber contained Tunicamycin (10µg/ml), Swainsonine (0.3µg/ml), Benzyl-N-acetyl-a-D-galactosamine (i.e., Benzyl-GalNAc, 1.5µg/ml) or Ulex lectin (5µg/ml). In each case, the difference in invasion was significant ($p<0.05$).

***The Proliferation of MDA-MB-231 cells was Unaffected by Fucosidase Treatment***

To explore the possibility that the observed decrease in tumor cell invasion was actually a function of the direct inhibition of the cell growth by fucosidase, we studied the effect of fucosidase treatment on cell proliferation using two different assays. Our data demonstrated that, fucosidase affected neither the viability nor the proliferation of MDA-MB-231 cells as shown by an MTT assay **(Figure 11)** or by automated cell counting **(Figure 12).**

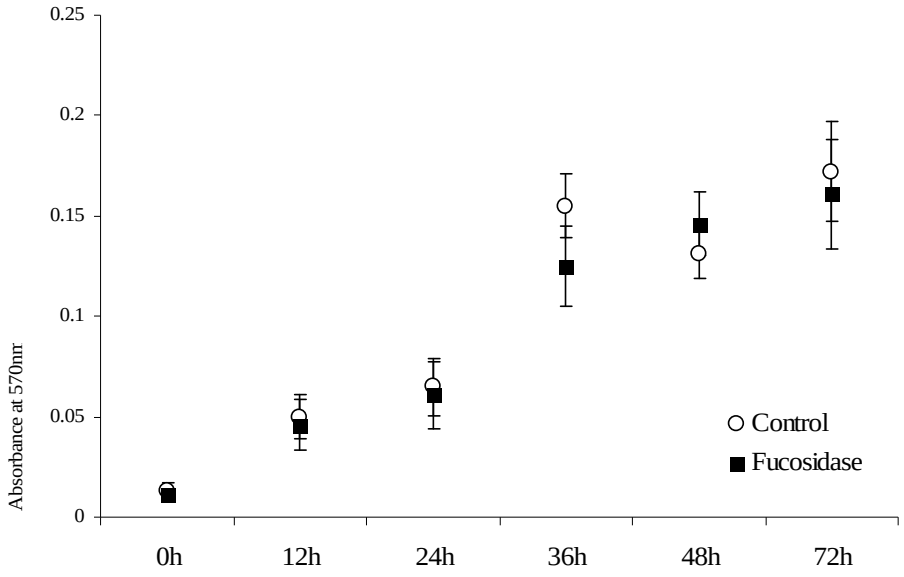



**Figure 11**

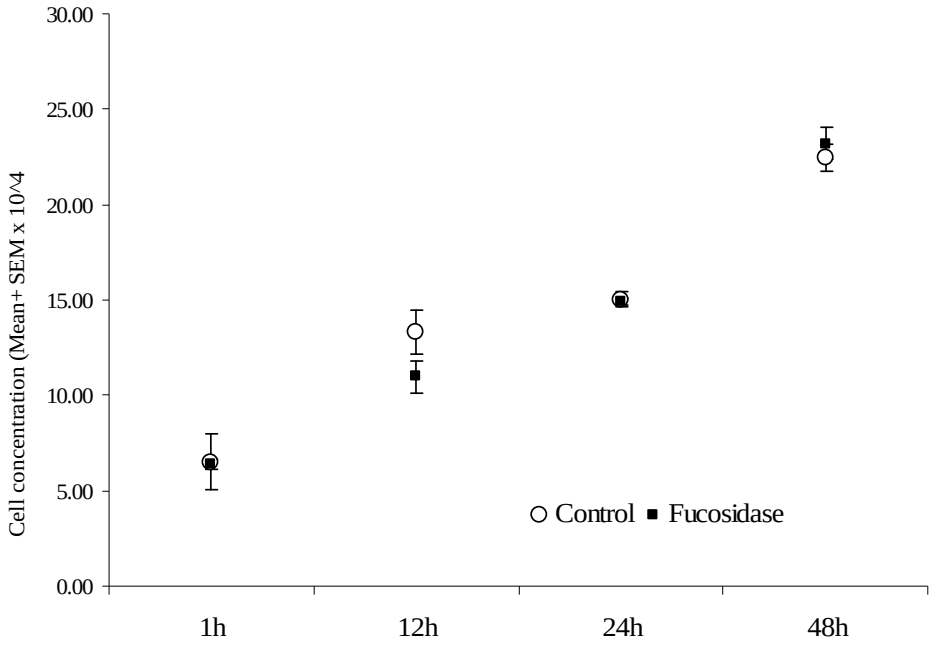



**Figure 12**

**Figure 11-12. Fucosidase treatment did not affect the proliferation of MDA-MB-231 cells as revealed in an MTT assay (Figure 11) nor by Automatic Cell Counting (Figure 12).** Cells were trypsinized, resuspended in PBS, and treated with or without fucosidase. 2 x $10^3$ 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 as recommended by the manufacturer. Each time point represents the mean of 3 samples for both groups. Data represent the absorbance spectra at 570 nm **(Figure 11)**. Similar cell proliferation assays were carried out by an Automatic Cell Counter as described before (**Figure 12).** A Wilcoxon signed rank test and paired two sample t-tests were performed to evaluate the difference between fucosidase treated & control groups at all 6 time points. There was no statistically significant differences between any group ($p>0.05$).

***Fucosidase Treatment Specifically Decreased MDA-MB-231 Breast Cancer Cell Invasion may through Modulation of the Activity of MMP-9***

Matrix Metalloproteinases (MMPs) are zinc-dependent endopeptidases which degrade most ECM components. MMP-2 and MMP-9, also known as gelatinases, are critical for the tumor invasion of mammary carcinoma through basement membranes, through the degradation of collagen type IV, which is the major collagen type of these structures. 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 fucosidase treatment on the gelatinolytic ability of MDA-MB-231 tumor cells, we studied the MMP activities in conditioned medium by zymography from untreated cells, fucosidase treated cells, and fucosidase treated cells in the presence of deoxyfuconojirimycin. The gelatin zymography revealed a significant decrease in the gelatinolytic activity of MMP-9 after treatment with fucosidase. Deoxyfuconojirimycin could be seen to reverse this effect **(Figure 13)**. MMP-2 showed no significant changes under these same experimental conditions.

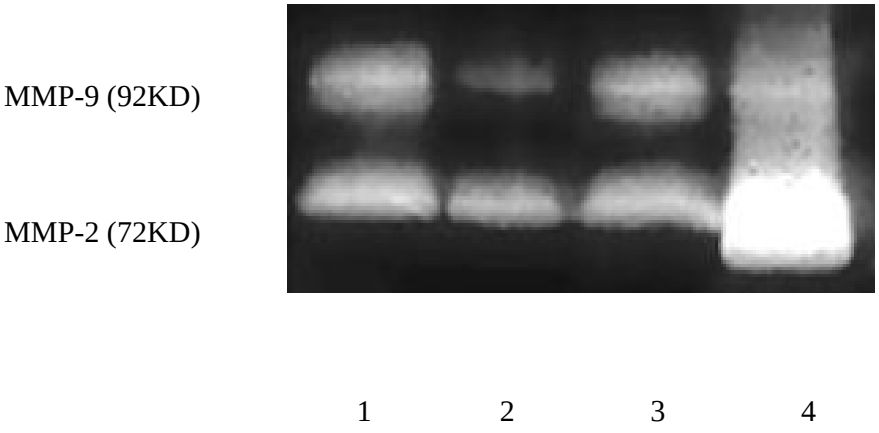



**Figure 13.** Gelatin zymography of conditioned medium from both fucosidase treated and untreated MDA-MB-231 cells. **Lane1**,cells without fucosidase treatment; **Lane 2**, cells treated with fucosidase (8.8 mU/$10^6$ cells); **Lane 3**, cells co-treated with fucosidase (8.8 mU/$10^6$ cells) and DFJ (1nM); **Lane 4**, HT 1080 fibrosarcoma cells as MMP positive control. 5μg of purified human MMP-2 and MMP-9 served as the gelatinase zymograph standards (not shown). A significant reduction in MMP-9 gelatinolytic activity is seen and verified by densitometry but not in that of MMP2 in the presence of fucosidase.

## 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. Glycosylation is known to play a direct functional role in cancer cell motility and invasiveness [110]. Carcel-Trullols et al. 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 increased invasive potential, all of which boosted bone metastasis and bone colonization capacities [19].

Fucose, best known as a key component of blood group antigens, is involved in many critical physiological functions, including leukocyte-endothelial adhesion, host-microbe interactions, fertilization, ontogenesis and differentiation. Our study revealed high levels of fucose expressed on the surface of human breast cancer cells (**Figure 2-5**). Increased expression of fucose on tumor cells is not only a phenotypic marker of malignant transformation, but also facilitates tumor progression, directly and/or indirectly. Serum fucose levels have been found to be significantly increased in patients with breast cancer and associated with therapy responses, tumor recurrence and metastases [111].

Knowing elevated fucosylation are prevalent in cancers, it's not surprising to find that fucosidase level rises in parallel, which may representing a host-reaction to a high "fucose-burden". Thus, the rationale exists that by reducing the "fucose-burden" by exogenous fucosidase one may interfere with tumor functions associated with such abnormal high level of fucose. Various approaches have been proposed to down-regulate the expression of fucose on tumor cells, e.g., modulation of fucosyltransferase [112], altering expression of upstream regulators [65] and up-

regulating competitive glycoforms [113]. The biochemical approach that we use relies on α-L-fucosidase (E.C. 3.2.1.51), a glycosidase which hydrolyzes the glycosidic linked fucose in several different linkages within glycosylation chains [114]. The effectiveness of our defucosylation protocol has been confirmed by flow cytometry experiments (**Figure 2-5**). Enzymatic removal of the fucose that we used in this study has several advantages, including speedy and convenient achievement of the defucosylation effect and the preventing of interfering intrinsic cellular glycosylation branches. It also suggests a potentially practical clinical route of administration via intravenous and intraluminal applications.

In separate experiments, we tested a range (from one-eighth to an eight fold concentration of our current protocol). As expected, there was a concentration dependent effect although it was not linear. When weighing effectiveness against viability, we found 8.8 mU/$10^6$ cells for 30 minutes is the most ideal concentration. The relative specificity of the fucosidase effects could be illustrated by DFJ, a potent fucosidase inhibitor, as an enzyme antagonist [109]. The proliferation of human breast cancer cells after fucosidase treatment was evaluated by two methods. Both revealed unchanged cell proliferation during treatment **(Figure 11-12)**. This is consistent with previous findings that mammalian cell lines lacking cell surface fucosylated glycans are still viable without an apparent defect in cell division [115, 116], and same results are

true for the leukocytes in LAD II patients. This may be partly 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. In addition, we also 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 fucosidase (data not shown).

Breast cancer ranks second in 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 [117], and poor prognosis is closely correlated with advanced stage biomarkers of progression; i.e. neo-angiogenesis, invasion and metastasis [118]. Tumor metastasis of breast carcinoma is the final step in a complex of multistage events which involves the crucial step of tumor invasion of neoplastic cells through the basement membrane, a barrier between the epithelium and stroma and between the stroma and the vasculature. Matrix metalloproteinases (MMPs) cleave selected ECM components and facilitates tumor invasion. Among the MMPs, MMP-2 and MMP-9 attract significant attention because of their established roles in degrading type IV collagen,

which is the major collagenous component of basement membranes. The direct role that MMP-9 plays in the invasive ability of MDA-MB-231 cells to cross basement membranes has been well-proved, and decreased MMP-2 and MMP-9 activity has been closely correlated with stunted tumor invasion through HuBiogel® [64] .

Modification of tumor glycosylation is known to be able to modulate the interaction of cells with the extracellular matrix, which may in turn, regulates the expression and activities of MMPs. For example, selectively knockdown of GnT-V expression in the MDA-MB-231 cells didn't affect epidermal growth factor receptor (EGFR) expression, but lowered the expression of N-linked β-1,6-branching on EGFR, suppressed EGF-mediated downstream FAK and ERK signaling and decreased cell invasiveness [119]. In contrast to our observation that fucose appears to be a positive regulator of MMP in breast cancer, Isnard et al. found that fucose and fucose-rich polysaccharides downregulated MMP-2 and MMP-9 activities in non-transformed human dermal fibroblasts in vitro and in vivo [120]. Fucans, a polysaccharide mainly constituted of sulfated L-fucose, were found to inhibit MDA-MB-231 cell invasion through Matrigel. This was partly mediated through a direct interaction of the fucans with laminin resulting in an inhibition of cell adhesion and invasion [121]. Observed changes may originate from altered glycosylation patterns, different cell types or physiologic state along the neoplastic progression pathway.

Current findings show that defucosylation significantly inhibited the invasion of MDA-MB-231 cells (**Figure 9**). This is presumably at least partially mediated by fucosidase induced reduced gelatinolytic activity of MMP-9. Other alterations of cellular functions caused by defucosylation may also contribute in this process. Further studies will be needed to investigate whether disturbing the normal interactions of tumor cells with ECM matrices by defucosylation alters regulation by damaging the known MMPs autocrine/paracrine loop or other as yet unknown mechanisms directly leading to the decreased activity of MMP-9 or other matrix-degrading proteinases.

Findings in this report also illustrated that 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 both simple and complex extracellular matrices in vitro (**Figure 10**), arguing for the importance of intact glycosylation for functional neoplastic progression. Tunicamycin, an N-glycosylation inhibitor, has also previously been found to inhibit the proliferation of MCF-7 breast cancer cells [122] and induce apoptosis in MDA-MB-231 cells [123]. Tunicamycin was also found to inhibit malignant melanoma and mammary carcinoma cells adhesion to laminin, cellular invasiveness through Matrigel and homotypic aggregation of cells

[124]. Decreased glycosylation on CD147 by tunicamycin, down-regulated Matrixmetallproteinase-11 expression and decreased the adhesion of cells to endothelial cells [125].

Swainsonine, in a similar manner, by blocking Golgi α-mannosidase II and abrogating processing of N-glycans was found to inhibit the migration of malignant melanoma cells [126] and bladder carcinoma cells [127]. Other effects of swainsonine include blocking tumor pulmonary colonization [128] and enhancing lymphocyte efficacy and tumor susceptibility to immune attack [129]. Swainsonine has also been shown to increase the transcription of Tissue Inhibitor of Metalloproteinase (TIMP), through which a decrease in tumor invasion may be realized [130]. Benzyl-N-acetyl-α-D-galactosamine, an inhibitor of O-glycosylation, has been shown to significantly decreased sialyl Lewis antigens expression, metalloproteinase activity in HM7 human colon cancer cells while also inhibiting their invasion through Matrigel. [131].

Different drug concentrations, cell types and other intrinsic factors may all contribute to the observed discrepancy in the effects of glycosylation inhibitors. Various effects of deglycosylation on tumor behavior must also be taken into consideration before accepting the definitive conclusions that deglycosylation inhibited invasion of human breast cancer MDA-MB-231 cells. The finding that the fucose-specific lectin, Ulex also caused a significant inhibitory effect on breast cell

invasion may suggest a general disturbance in fucose mediated interactions with ECM is responsible for the observed changes. Ulex has also been found to induce the activation of MMP-2 in endothelial cells [132], which further increased the complexity of glycan-MMP microinteractions.

Our study adds to the growing body of knowledge which asserts the importance of fucose in the cancer phenotype of many human malignancies. Fundamental knowledge of the functions of fucose at the molecular level will be necessary to fully evaluate any hypothesized anti-cancer effects using a defucosylation approach. Further study of the interactions between tumor cells and ECM (or endothelium) will additionally be indispensable as we explore the effects of defucosylation on neoplastic progression.

# CHAPTER 4

# DEFUCOSYLATION BY ALPHA-L-FUCOSIDASE AFFECTS HUMAN BREAST CANCER CELL INTERACTION WITH EXTRACELLULAR MATRICES AND ENDOTHEIIAL CELLS

## Introduction: Lewis Antigens and Adhesion Molecules

Certain glycosylation patterns expressed by tumor cells are involved in the adhesion of tumor cells to extracellular matrices and to the endothelium. These glycosylated molecules either directly constitute adhesion ligands [133] (Lewis X, sialyl Lewis X and sialyl Lewis A) or indirectly influence the function of adhesion molecules including the integrins and CD44 [134-136].

CD44, a family of structurally and functionally variable adhesion molecules are comprised of more than 20 variants. They participate in diverse biological processes, including angiogenesis, wound healing, inflammation, and cancer metastasis [137, 138]. Several malignancies-related glycan antigens have been previously found to be carried by CD44s. Glycosylation patterns in diverse CD44 isoforms have been found to be critical for oncogenesis and tumor progression [139]. The expression of certain CD44 variants has subsequently been shown to closely correlate with both the progression and prognosis of breast cancer [140].

Integrins are a family of heterodimeric transmembrane receptors mediating cell-cell and cell-extracellular matrix adhesion. Integrins affect various cell functions including growth, adhesion, motility, invasiveness and metastasis [141]. Specifically, α-galactosyl residues are known to contribute to the laminin-binding

determinants of the integrin α subunit and are involved in cell adhesion to laminin [142]. Altered glycosylations of integrin subunits, in turn, have been reported to affect integrin-mediated cell adhesion, spreading and other vital cell functions. Similarly, oligosaccharide processing of the fibronectin receptor is important for its binding function but not for receptor assembly or insertion into the plasma membrane [143].

In addition to decorating adhesion molecules, oligosaccharides can also constitute glycan carbohydrates directly. Lewis antigens are oligosaccharides closely associated with ABH blood group antigens. Through the action of a set of glycosyltransferases, their biosynthesis mainly include stepwise addition of monosaccharide units to minimal disaccharides precursors [4]. Lex or Lea antigens are formed by fucose being added, in α-1, 3 or α-1, 4 linkages, to H Type-2 or H Type-1 structures, respectively. Addition of a second fucose onto Lex or Lea will generate the Ley or Leb antigens, respectively (**Figure 14**). The sLex or sLea antigens are synthesized by the addition of a sialic acid group, in an α-2,3 linkage, to the Lex or Lea [144]. As noted above, type 1 antigens (Lea and Leb) are important histo-blood groups. In contrast, type 2 (Lex and Ley) antigens are only weakly expressed in a limited number of tissues in healthy individuals, but increased in malignancies and, thus, considered “tumor-associated” antigens. Altered expression of Lewis family antigens occurs during malignant transformation, tumorigenesis and progression [145]. Lewis antigens have been widely used in the diagnosis and as biomarkers for the prognosis of patients with tumors.

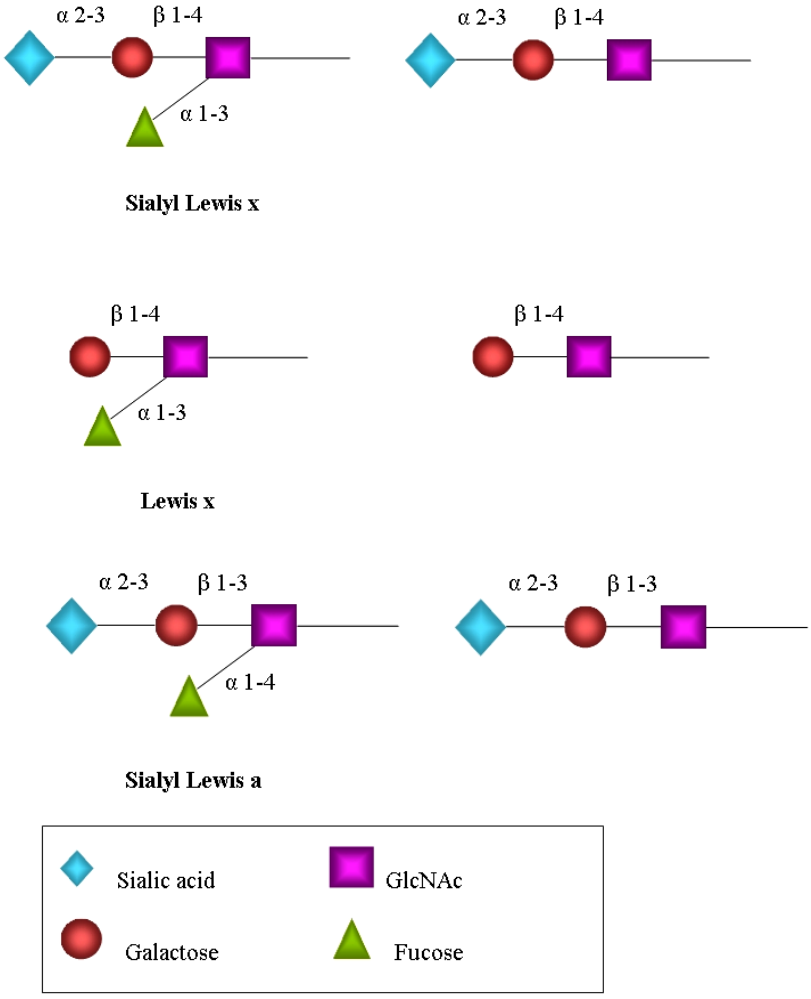



**Figure 14. Representative Lewis antigens (Left) and predicted defucosylated Lewis antigens (Right).** The compositions of three members of the Lewis family are illustrated in terms of their monosaccharide composition and linkage positions. Differences between **sLex** and **sLea** lie in the linkage of the outermost Gal (β1–4 and β1–3, respectively) and in the linkage of the fucose moiety to the internal GlcNAc (α1–3 and α1–4, respectively) **(Left)**. Predicted structure of Lex, sLex and sLea after fucosidase treatment are also outlined (**Right**).

Functional roles of Lewis blood group antigens are mainly illustrated in the process of selectin-dependent leukocyte and tumor cell adhesion where they provide essential contributions to the glycoproteins and glycolipids that function as ligands on these cells [4]. Specifically, higher expression of Ley/b has been associated with both

high grade and poor prognosis of breast cancer [146]. Enhanced expression of Lex has also been found in human breast and colon carcinomas [147], and has been used for screening high-risk populations for urinary bladder carcinoma [148]. Similarly, small-cell lung carcinoma cells express high amount of glycolipids carrying Ley [149], and the expression of Ley has been found to be correlated with the development of colorectal high-grade dysplasia and progression to carcinoma [150], and could be used as a tumor marker for breast cancer [151]. Ley-based immunotherapy has shown promising results in the treatment of breast and colorectal cancers [152]. H type 2 antigens in colon carcinoma cells are also known to contribute to their resistance of these cells to chemotherapy [153], increased tumorigenicity, and even the resistance to natural killer/lymphokine activated killer (NK/LAK) cytotoxicity [154]. Inhibition of fucosylated expression on these tumor cells results in and increased susceptibility to anticancer treatment [155].

The significance of glycosylation to tumor progression (especially as related to metastases) has been recognized for decades. Extravasation of tumor cells, a pivotal step in metastasis, is initiated by interactions of extravasating tumor cells with endothelial cells through selectins and their fucosylated ligands. Sialyl Lewis x (sLex), a fucose-containing tetrasaccharide, has the most active capacity of all of the family of Lewis antigens, to drive neoplastic progression, mainly due to its well-known role as the major ligand for E-selectin, which mediates the rolling of cells on endothelium and is thus intimately associated with leukocytes extravasation and tumor metastases.

The sLex glycan is characteristically expressed in aggressive cancer types and more advanced stages of disease associated with invasion and metastases. Sialyl Lewis antigens are generally not expressed or normal breast tissue, but gradually increase with tumor progression from carcinoma in situ, through invasive carcinomas

to lymph node metastases [156]. Sialyl Lewis antigen-mediated adhesion of MT3 breast cancer cells has been shown to be enhanced when these cells reach confluence, in comparison to growing cells. Interestingly, increased metastatic foci are seen to be present in lungs of mice in tight association with cell density [157]. This, it has been argued, is linked to the theory that tumor cells may gain a selective advantage by presenting pathologic selectin ligands that mediate interactions with endogenous selectins [158].

To explore this line of reasoning, high preoperative serum levels of sLex have been associated with liver metastasis and peritoneal dissemination in patients with gastric cancer [159]. These elevated serum levels are also associated with a lower survival rate in non-small cell lung cancer [160] . The expression of sLex in tumor tissues is closely associated with histopathologic parameters and the prognosis of patients with squamous cell carcinoma of the esophagus [161], renal cell carcinoma [162], colorectal cancer [56, 163], breast cancer [164] and adult T cell leukemia [165]. The accuracy for detecting metastases can reach 89.9% by measuring sLex alone in breast cancer patients [166]. After transfection with human fucosyltransferase VI cDNA, the sLex-low-expresser variant cells have been shown to exhibit increasing α-1,3-fucosyltransferase activity, increasing cell surface sLex, enhanced adhesion to endothelial cells and enhanced liver metastatic potential [167].

Another member of the Lewis antigen family, sialyl Lewis a (sLea), has also been shown to support the adhesion of tumor cells to E-selectin expressed on endothelium and to facilitate hematogenous dissemination [168]. Likewise, the expression of sLea has been shown to be closely correlated with the metastasis potential of colon cancer cell lines [169], and also serve as a biomarker for poor prognosis in patients with colon cancer [170] and gastric cancer [171]. The synthesis of

sLex is under strict regulation of fucosyltransferase and sialyltransferase, which are themselves, known to be elevated in metastatic human colon carcinoma [172]. Lastly, the activities of sLex and α-1,3-fucosyltransferase VI (FT-VI) and FT-VII have been previously correlated with the potential of bladder carcinoma to both invade and metastasize [173] .

Tumor metastasis via the hematogenous route is a complex multistage process starting from tumor invasion into the matrix, accessing of the circulation, and subsequent arrest on a distant endothelium and extravasation at metastatic sites. At the center of this process are circulating tumor cells which eventually adhere to the vascular endothelium. The selectin-dependent events involve tumor cell tethering to platelets, leukocytes, and vascular endothelium, all of which occur under shear stress conditions secondary to blood flow. The initial steps of leukocyte and tumor cell adhesion involve selectin receptor/ligand (mainly sLex) interactions, followed by extravasation [4]. Work by others has shown that decreased sLex/a contributes to a lack of inflammatory breast carcinoma tumor cell binding to endothelial cells and lack of electrostatic repulsions between tumor cells [174].

Selectins are a class of membrane-bound C-type lectins, expressed on vascular endothelium and circulating leukocytes. Selectins are involved in leukocyte-leukocyte interactions (L-selectin) and leukocyte-endothelial cell interactions (L-, E-, and P-selectin). Their functions occur relatively rapidly via on/off kinetics of binding under vascular shear flow. All of the selectins display modest specificity and affinity for sLex. Ligands for L-selectin (GlyCAM-1) contain Gal-6-sulfated and/or GlcNAc-6-sulfated sLex antigens, while the P-selectin glycoprotein ligand (PSGL-1) also has O-glycans containing sLex glycan. Selectin-dependent cell adhesion in vivo is characterized by the “rolling” of leukocytes along the surface of an endothelial cell

monolayer[4]. The pivotal role of fucosylation in the interaction of leukocytes with endothelium has been further illustrated by a rare genetic disease, Leukocyte Adhesion Deficiency II Syndrome (LAD II). Neutrophils in LADII lacking fucosylated ligands for E, P, L-selectins, consequently, fail to adhere to selectins and have significant defects in selectin-dependent pathophysiological events. LADII patients, thus, demonstrate a profound reduction in migration into inflamed cutaneous sites in vitro. The defect in LADII lies in the de novo pathway of GDP-fucose biosynthesis[4].

As introduced in the preceding chapters, breast cancer is the most common malignant tumor and ranks second in the mortality for malignancies in American women. Its prognosis largely depends on the extent of tumor invasion and distant metastasis [175]. Poor prognosis in breast cancer is closely correlated with enhanced invasion, metastasis and angiogenesis [118]. Elevated fucosylation has been found in mammary malignancies. The positive expression of sLex in primary breast cancer has been shown to be significantly associated with lymph node involvement and a diminished disease-free survival rate [156, 176]. As fucose-containing molecules are closely correlated with neoplastic progression, and a deficiency of fucose severely impairs the interaction of leukocytes with endothelium in patients with LAD II, a rationale has been proposed to explore the biological effects of cell adhesion events by modification of the fucose expression on tumor cells. We have, in the preceding chapter, hypothesized that decreasing the fucose expression would affect the biological behavior of breast cancer cells and provided evidence for that theory. Here we focus on extending this hypothesis to envelop the interactions between tumor cells and extracellular matrix, as well as between tumor cells and endothelial cells mediated by fucosylated glycans.

# Results

## *Alpha-L-fucosidase Decreases MDA-MB-231 Cells' Adhesion on Extracellular Matrix Components*

To investigate the possible effect of α-L-fucosidase treatment on the adhesion of MDA-MB-231 tumor cells to ECM, we performed static adhesion assays by using several extracellular matrix components including type I collagen, hyaluronic acid, fibronectin, and laminin, which are all known to contribute to breast cancer cell attachment and spreading. In these studies, we found that defucosylation by α-L-fucosidase significantly decreased the adhesion of the breast cancer cells to fibronectin, laminin, type I collagen and hyaluronic acid at essentially all time-points. The adhesion to HuBiogel®, a reconstituted human ECM mixture, also decreased after defucosylation (**Figure 15**). In contrast, the adhesion of these cells on a non-native matrix (polystyrene) did not show significant changes (**Figure 16**). These effects also displayed a curve of recovery over time.

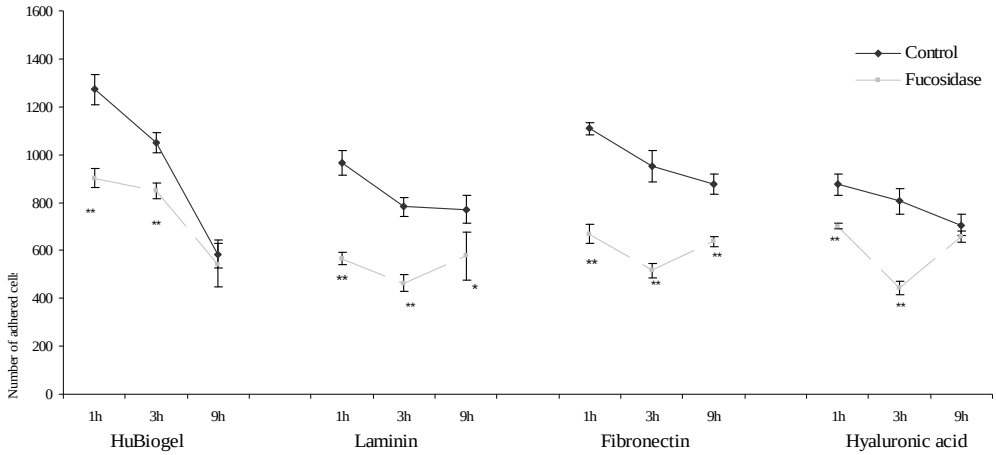



**Figure 15. Static adhesion assay of α-L-fucosidase treated MDA-MB-231 cells on extracellular matrices.** Defucosylation significantly decrease MDA-MB-231 cells adhesion to fibronectin and laminin at all three time points after treatment (1h, 3h and 9h). The adhesion to HuBiogel® and hyaluronic acid also shown significant decrease at 1h and 3h. 96-well culture plates were coated with HuBiogel®, hyaluronic acid, laminin, and fibronectin as described [62]. Cells were harvested and treated with or without fucosidase and then plated onto coated wells. Cells were left to adhere for indicated periods, washed with PBS and fixed with paraformaldehyde. Adherent cells were subsequently stained by crystal violet and counted. *, representing $P<0.05$ by the Mann-Whitney Test; **, representing $P< 0.01$ by the Mann-Whitney Test as compared to control.

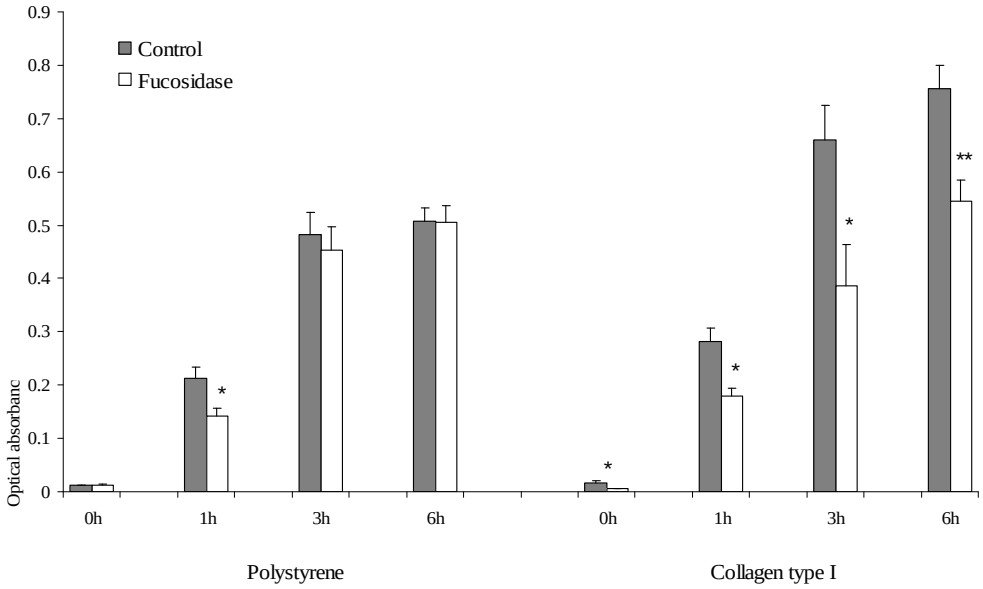



**Figure 16. Static adhesion assay of α-L-fucosidase treated MDA-MB-231 cells on polystyrene and type I collagen.** Defucosylation impaired cell adhesion on to type I collagen, while the adhesion on the plastic surface was unaffected. Tumor cells were harvested and treated with or without fucosidase, followed by plating onto 96-well culture plates with or without pre-coated type I collagen. Cells were left to adhere for the indicated times and followed by washing with PBS. Cells were fixed with paraformaldehyde and subsequently stained by crystal violet. Stained cells were dissolved in Triton X100 and the optic density was calibrated by use of an ELISA reader at 570 nm. Data were expressed as the mean ± SEM of 8 wells. *, $P<0.05$ in Mann-Whitney Test; **, $P< 0.01$ in Mann-Whitney Test as compared to controls.

We also compared the adhesion of MDA-MB-231 cells on plastic, hyaluronic acid and HuBiogel®, with or without fucosidase treatment, in the presence of antibody against CD44 and sLex. Hyaluronic acid and HuBiogel® were coated on 96-well plates and compared to untreated (Polystyrene) wells. Rabbit anti human CD44 polyclonal antibody HCAM-1 and mouse anti-human sLex monoclonal Km93 antibodies were found to block sLex-involved adhesion. HuBiogel® and hyaluronic acid were also found to promote cell adhesion in both control and fucosidase treated cells as compared to those cells growing on plastic alone. The adhesion of tumor cells

on HuBiogel® significantly decreased after treatment with fucosidase, and the sLex glycan structure appear to be involved in this adhesion process (**Figure 17**).

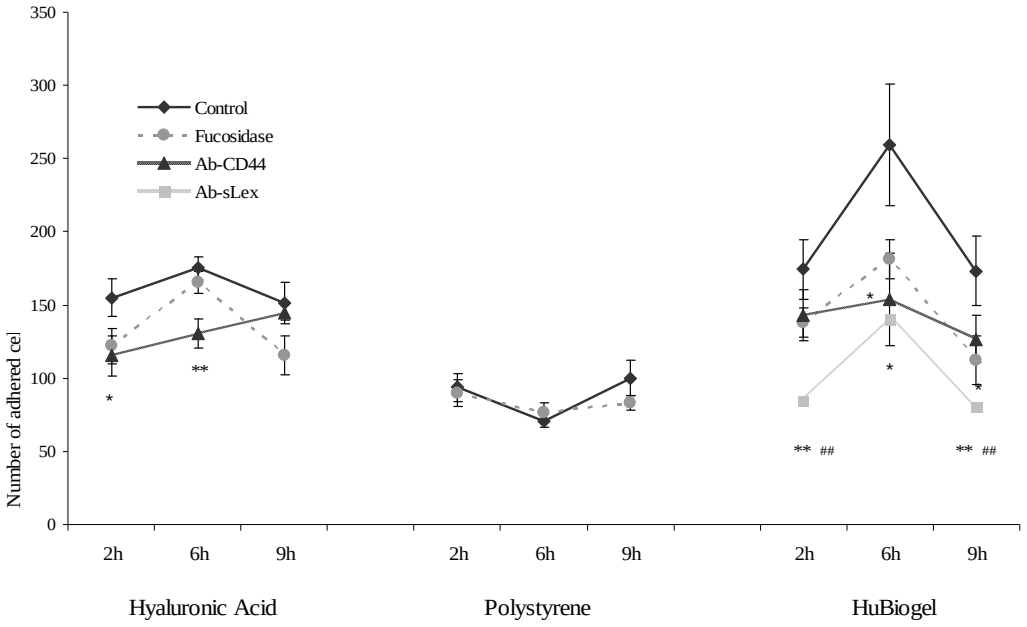
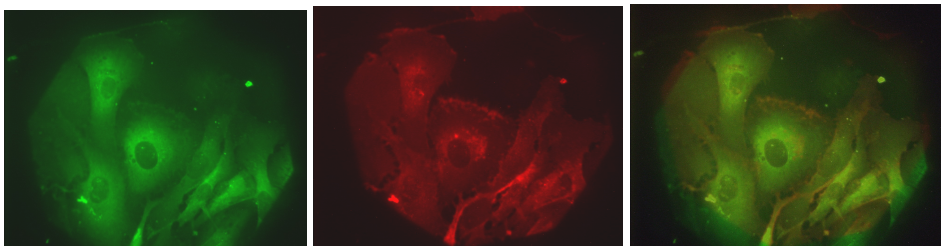



**Figure 17. Static adhesion assay of MDA-MB-231 cells on hyaluronic acid, polystyrene and HuBiogel® in the presence or absence of fucosidase and antibodies against CD44 and sLex.** The adhesion of tumor cells on HuBiogel® and hyaluronic acid, but not on polystyrene, was significantly decreased by fucosidase treatment, and the binding of mAb against sLex also interfered with cell adhesion on HuBiogel®. Culture plates were either uncoated or coated with HuBiogel® or hyaluronic acid. Cells were treated with or without α-L-fucosidase and left to adhere for different times and the number of adhered cells was stained and counted via microscopy. Data were analyzed by Mann-Whitney Test, *, $P<0.05$ comparing treated to untreated control, ** $P< 0.01$ comparing treated to untreated control. # $P< 0.05$ when groups treated with different antibodies were compared. ## $P< 0.01$ when groups treated with different antibodies were compared.

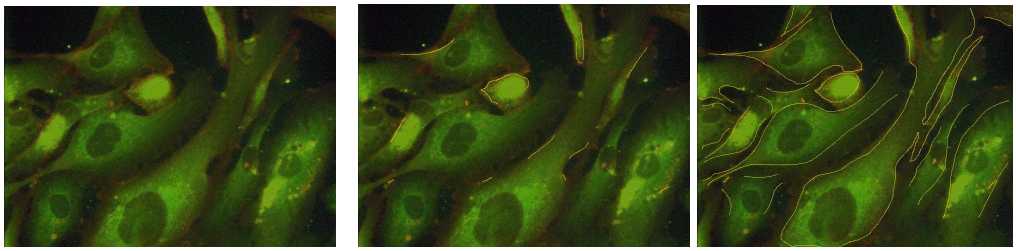
***Defucosylation Decreases the Co-localization of Fucose with β1 integrin on MDA-MB-231 cells***

In order to determine the effects of α-L-fucosidase on the glycosylation pattern of integrins on MDA-MB-231 cells' surfaces, we used indirect co-localization assays to study the co-localization of fucose with integrin subunits. The co-localization of green fluorescence conjugated secondary antibodies against integrin and red fluorescence-labeled streptavidin capturing fucose were determined as illustrated in **Figure 18**. The methodology and results of immunofluorescence co-localization of integrin and fucose are shown in **Figure 19**. Decreased co-localization of β1 integrin and fucose in the presence of fucosidase were revealed in our experiments. Meanwhile, this change was not found in parallel experiments with α*v* integrin subunits.



β1 integrin | α-L-fucose | Merged co-localization of β1 integrin and α-L-fucose

**Figure 18. Indirect immunofluorescence and co-localization of integrins with fucose.** After standard fucosidase treatment, MDA-MB-231 cells were grown on coverslips for 24 hrs; cells were then fixed in paraformaldehyde/PBS, and blocked with bovine albumin in PBS. Cells were then incubated overnight, at 4°C, with biotin-conjugated Ulex, or mAb against α*v* integrin or β1 integrin subunits. Fluorescence conjugated secondary antibodies and streptavidin were diluted respectively in the blocking solution and incubated with the cells for 1 hr, at room temperature, in the dark. Images were taken by using Olympus microscope at appropriate wavelengths.



Colocalization sites of β1 integrin  Length of Colocalization Regions  Perimeter Measure of All

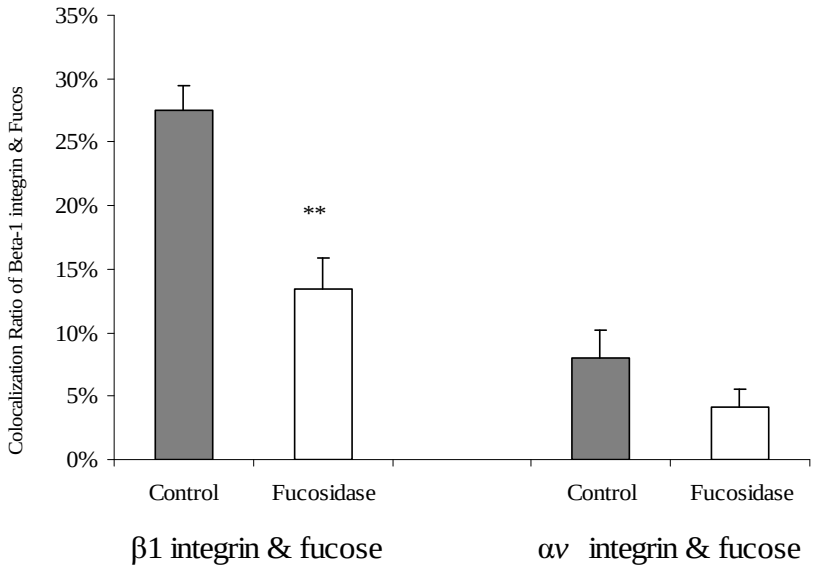



**Figure 19. Illustration of the methods and the result of immunofluorescence co-localization of integrins with fucose.** The immunofluorescence co-localization images of either β1 integrin with fucose or αv integrin with fucose were captured under microscopy. The length of the co-localized segments and the perimeter of the contour are specified and measured by Image J (NIH) software. The co-localization ratio was expressed as the ratio of the length of positive co-localization to the sum of perimeters of all recognizable cell contours. The co-localization ratios from 10 fields were calculated from each group. **, $p<0.01$ in Mann-Whitney Test.

***Alpha-L-fucosidase Treatment Down-regulates Cell Surface Expression of CD44 and CD15 on MDA-MB-231 cells***

The CD44 family of adhesion molecules mediates 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. CD44s undergo extensive post-translational modifications including glycosaminoglycans addition and glycosylation resulting in tumor cells being decorated on their surfaces by fucose. We thus, investigated the effect of α-L-fucosidase on the expression of CD44 on the surface of breast cancer cells. MDA-MB-231 cells were treated with fucosidase as described. We found fucosidase significantly decreased the detection of cell surface CD44 as evaluated by flow cytometry using phycoerythrin-labeled monoclonal antibody against the predominant form of CD44 **(Figure 20)**. This effect was lost by 24 hours, which is thought to be through cell surface reconstitution.

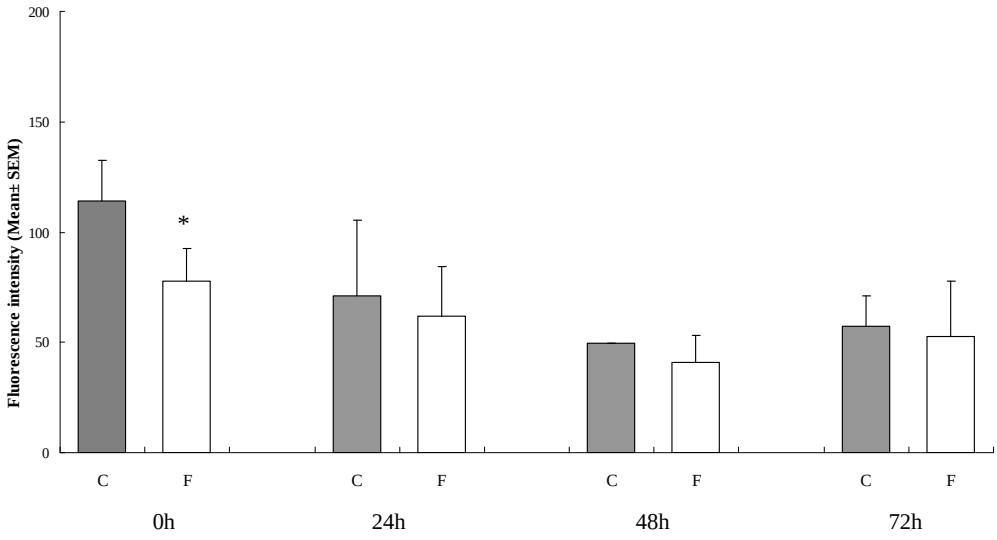



**Figure 20. CD44 changes as detected by PE labeled monoclonal antibody in a flow cytometry assay with and without fucosidase treatment.** MDA-MB-231 cells were treated with α-L-fucosidase as described in the Materials and Methods. Both groups of cells were incubated with the monoclonal PE-labeled anti-CD44 antibody, MHCD44. 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 fucosidase treated groups. At each time point the histogram represents the mean +/- SEM for 5 independent experiments. A significant difference ($p<0.05$) was appreciated only for time "0". Relatively rapid reconstitution resulted in no difference ($p>0.05$) for CD44 at all other times. ■ untreated control □ fucosidase.

Lewis X (i.e., CD15) is a fucose containing glycan participating in cell-cell and cell-matrix interactions. It was also found to be abundantly expressed on breast cancer cells as determined by flow cytometry **(Figure 21)**.

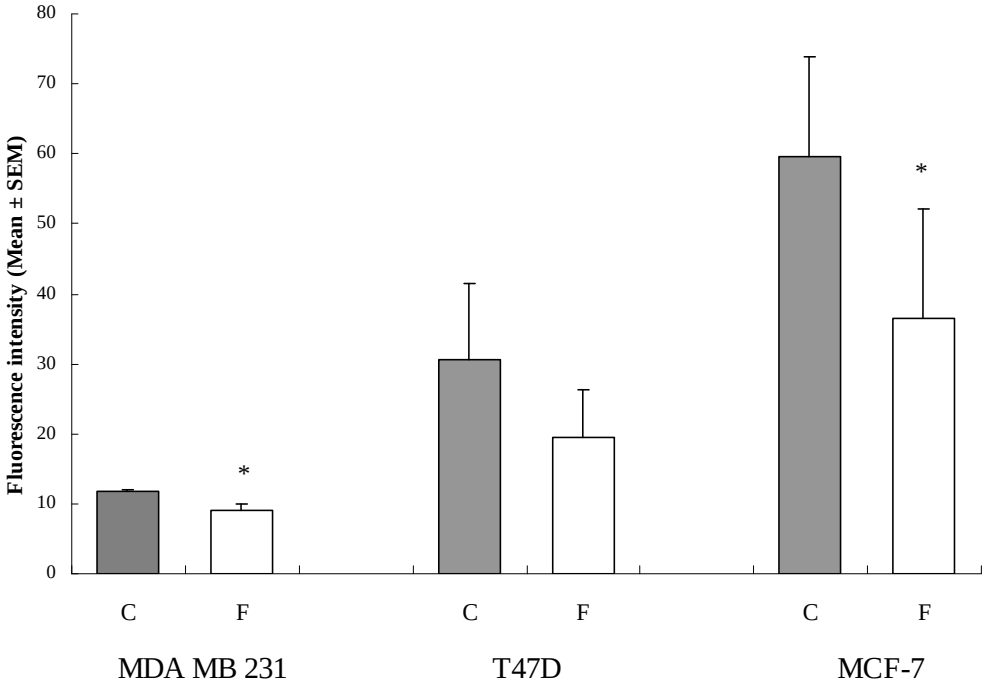



**Figure 21. Effects of fucosidase treatment on CD15 expression of human breast cancer cells as shown by flow cytometry.** CD15 expression was evaluated by flow cytometry at the time points indicated using methods as described in the Materials and Methods. The bar graphs demonstrate the mean +/- SEM of five independent experiments for the fluorescence intensity in MDA-MB-231 & T47D cells and seven experiments for MCF-7, respectively. 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 statistical significant difference. However, for T47D the difference approached significance but did not reach it. ■ untreated control □ fucosidase

### *Alpha-L-fucosidase Treatment Decreases the Sialyl Lewis X Expression on MDA-MB-231 Tumor Cells*

Previously, we have shown that α-L-fucosidase could effectively decrease the expression of α-L-fucose on MDA-MB-231 cells. As sLex is a fucose-containing tetrasaccharide glycan, it would be rational to test whether defucosylation decreases the level of sLex expression. Thus, an indirect flow cytometry method was applied to study the effect of fucosidase on sLex expression; we found α-L-fucosidase treatment

significantly decreased the expression of sLex. Defucosylation treatment significantly decreased the mean and median FL1-H FITC (representing sLex fluorescence intensity), as well as the Events above Gate (representing the approximate number of positive cells) as shown in **Figure 23**. The negative control with "cells only" and "cells plus secondary antibodies" demonstrated a very low background (data not shown).

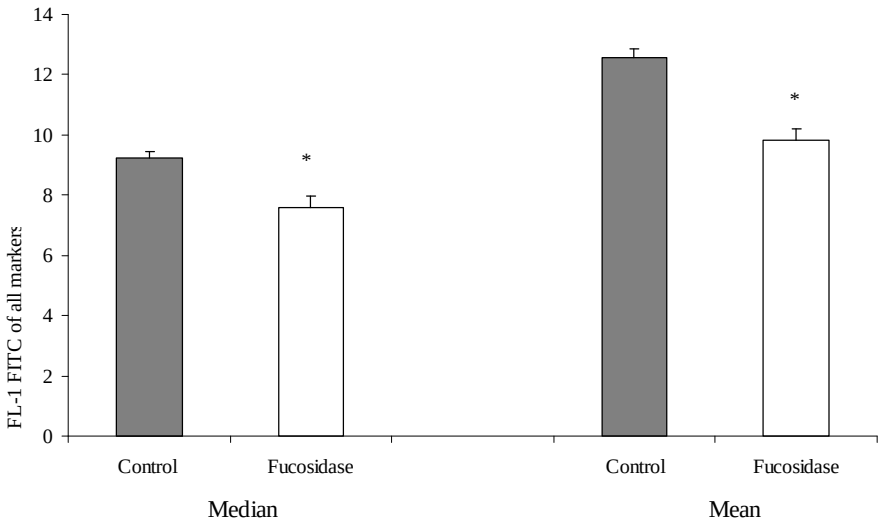



**Figure 22.**

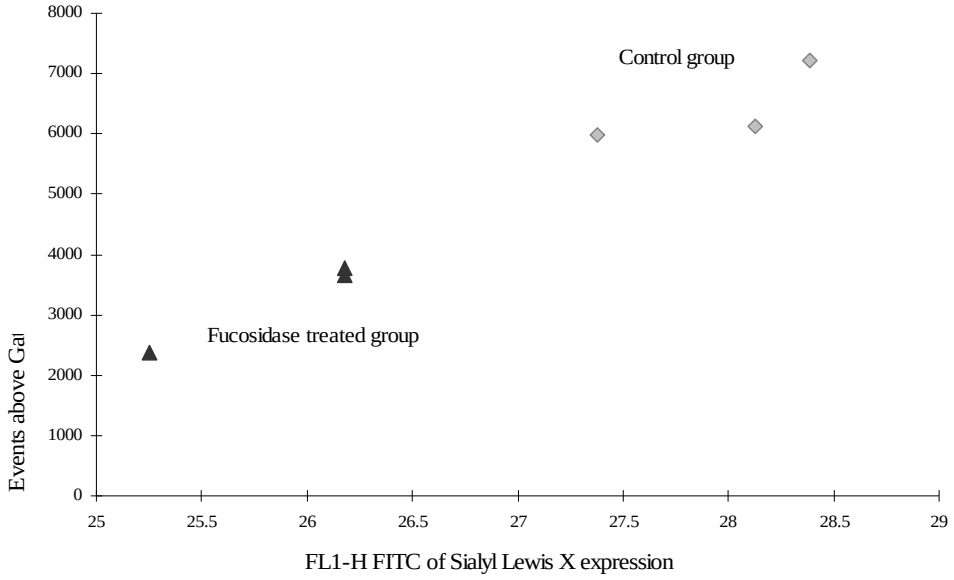



**Figure 23.**

**Figure 22-23. Flow cytometry analysis of sLex expression on MDA-MB-231 cells.** Fucosidase treatment significantly decreased the expression level of sLex. The same number of fucosidase-treated and control cells were washed and re-suspended in PBS containing BSA, then incubated with KM93 mAb against sLex. After washes, cells were incubated with of FITC-conjugated secondary antibody. Cells were again washed with PBS and fixed in paraformaldehyde. Flow Cytometry was performed as described. Each group contains 3 samples. **Figure 22** The Mean and Median of FL-1 FITC (sLex) by flow cytometry is shown (*, $p<0.05$ by the Mann-Whitney Test). **Figure 23** The distribution with regard to FL1-H FITC (Events above Gate) of three individual samples from control and fucosidase treated groups.

***Alpha-L-fucosidase Treatment Decreases the Rolling of MDA-MB-231 tumor cells on Endothelium under Flow Conditions***

Selectin-dependent cell adhesion in vivo is characterized by the “rolling” of leukocytes or tumor cells along the surface of the endothelial cell monolayer. This process is mediated by torque exerted on the cell as a consequence of a force differential between the more rapidly flowing stream near the center of the vessel (and on the luminal surface of the rolling cell) and the relatively slow flow near the endothelial cell surface (and the adjacent abluminal side of the rolling cell). These

forces, together with the rapid on and off rates of selectin-selectin ligand adhesive interactions occurring at the leukocyte (or tumor)-endothelial cell interface, causing the cell to roll along the endothelial lining of the vessel. The interaction of sLex and selectins may take place effectively even at submillimolar concentration levels.

The rolling of tumor cells on endothelium is an essential step in extravasation to form distant metastasis foci. Similar to leukocytes, the initial interaction between tumor cells and endothelium are mediated by sLex and E-selectin. Defucosylation has been found to be able to remove fucose from Lewis antigens and decreased the expression of sLex (**Figure 22-23**). It would be important to determine whether this treatment would lead to changes in the rolling behavior of defucosylated tumor cells. A flow chamber assay system were thus used to study the interaction between tumor cells and endothelium under flow conditions, providing better mimicking of the in vivo situation compared with its static counterpart.

In the first part of this experiment, we used non-labeled tumor cells and HUVEC to investigate the effects of defucosylation on the tumor cell adhesion on endothelium under flow conditions (**Figure 24**). We also use automated computer analysis including programs written by Dr. Kucik as well as algorithms developed in conjunction with commercial software companies. The combination of large amounts of image data and automated processing makes it practical to analyze a sufficient number of events to accurately calculate mean velocities, quantify acceleration events, and determine statistical significance.

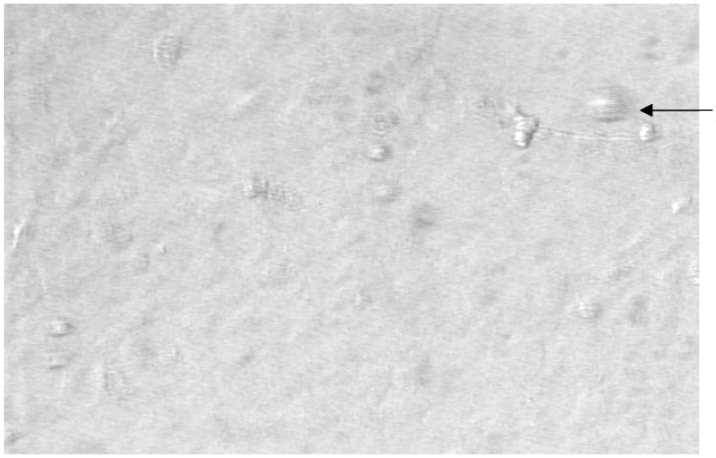



**Figure 24. A representative image captured from a flow chamber assay.** MDA-MB-231 cells were trypsinized, counted and evenly assorted into three groups; two groups were treated with fucosidase, at 37℃, for 30 and 60 minutes respectively. Control cells were incubated with only PBS for 60 minutes. Cells were then diluted in HBSS. A laminar flow chamber was constructed by using a GlycoTech® flow chamber insert and gasket inserting into the dish cultured with confluent HUVEC cells. Cells were injected into the flow chamber, in HBSS, at controlled physiological shear stress using a programmable syringe pump. Cells were viewed on an Axiovert100 microscope. The rolling cells can be distinguished by its characteristic motion pattern.

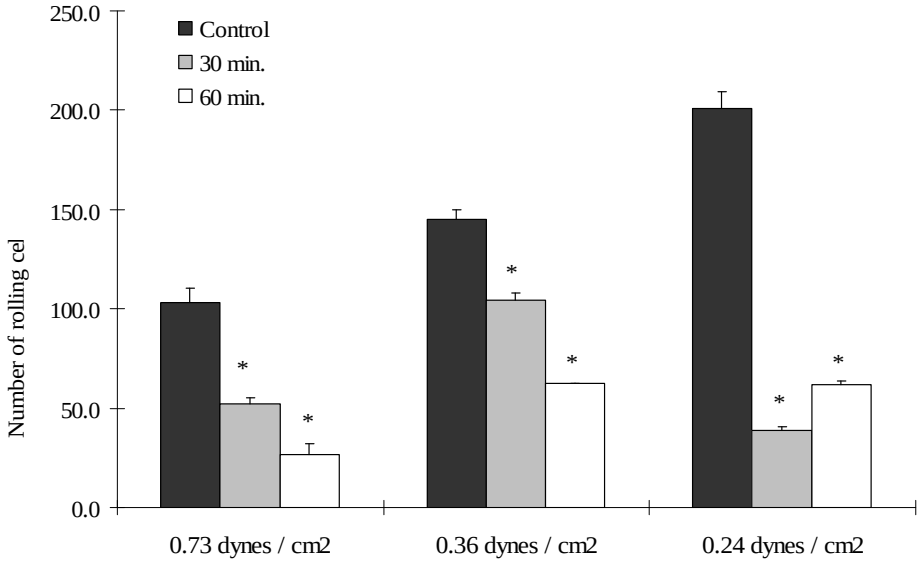



**Figure 25. Flow chamber assay demonstrating a significant decrease of MDA-MB-231 cells rolling on HUVEC after α-L-fucosidase treatment.** The number of tumor cells rolling on HUVEC was found to be decreased under three different sheer stress levels, at **30 min**, and at **60 min** of fucosidase, on HUVEC (*, $p<0.05$ by Mann-Whitney Test). There is also a significant difference between **30 min** and **60 min** of treatment ($p<0.05$ by the Mann-Whitney Test).

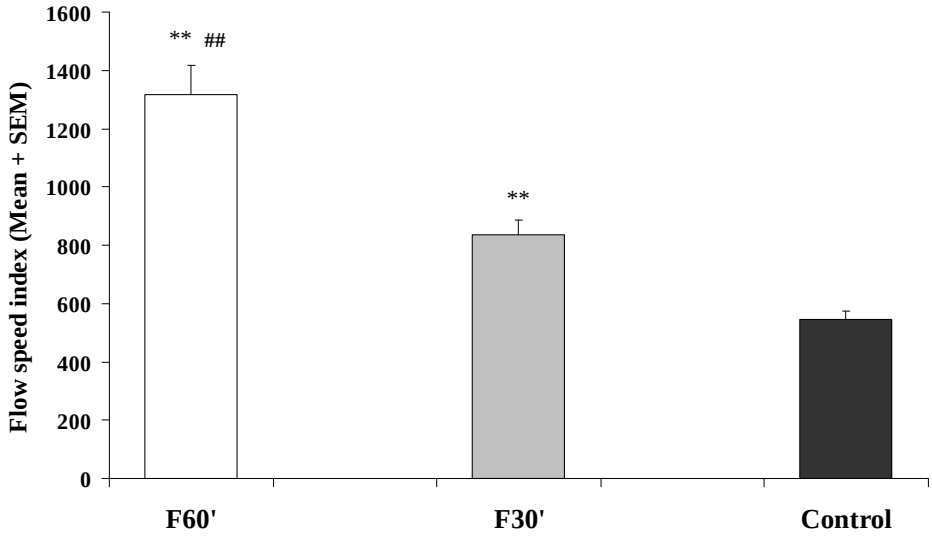



**Figure 26. The effects of α-L-fucosidase on tumor cells' flow speed on HUVEC.** The flow speeds of individual cells were recorded and calculated as described before. Defucosylated cells had a weaker interaction with HUVEC and thus flowed faster. Compared with the control, the flow speed of MDA-MB-231 cells significantly increased after standard 30 minutes of treatment (**, Mann-Whitney test, $p<0.01$). 60 minutes of treatment with fucosidase produced an even more prominent increase (**, Mann-Whitney test, $p<0.01$). There is also a significant difference between 30 and 60 minutes of treatment (##, Mann-Whitney test $p<0.01$).

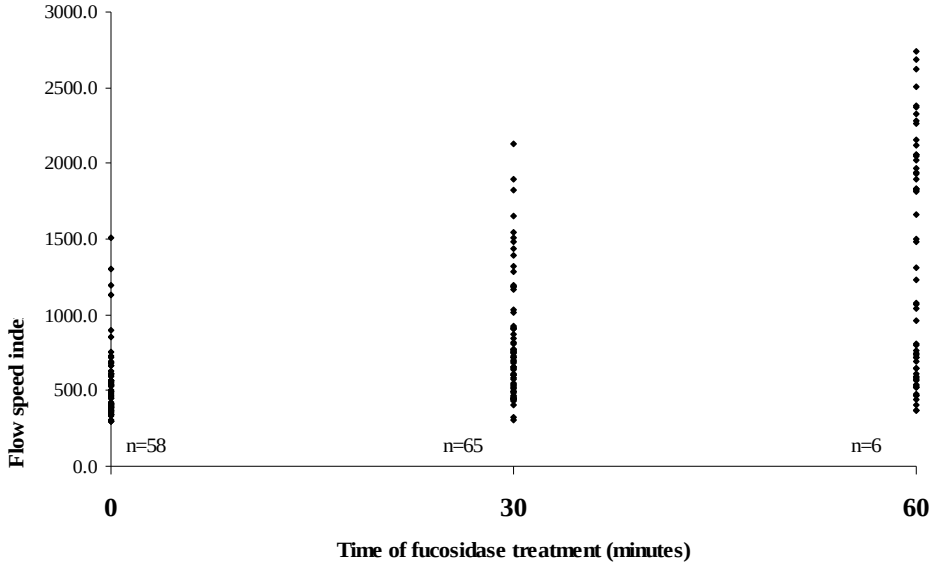



**Figure 27. The scatter distribution of the flow speed of MDA-MB-231 cells after fucosidase treatment.** More cells tend to flow faster after defucosylation, which was correlated with the time of treatment with fucosidase. See Methods for details.

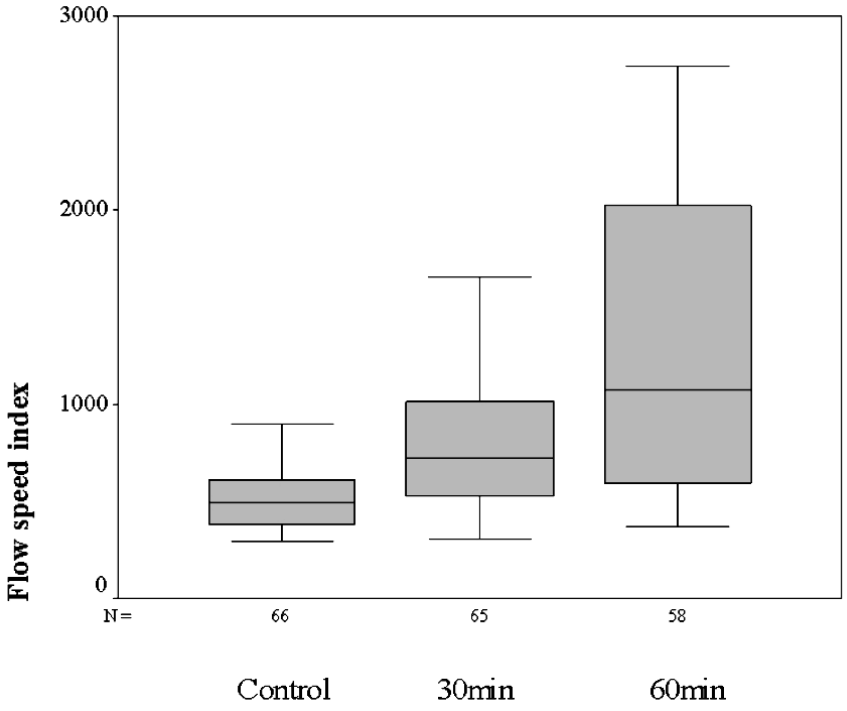



**Figure 28. Box-plot of the flow speed of MDA-MB-231 cells after fucosidase treatment** (Control, 30 minutes and 60 minutes of fucosidase treatment). In the box plot, the box stretches from the lower hinge (defined as the 25$^{th}$ percentile) to the upper hinge (the 75$^{th}$ percentile); median, minimum and maximum are also diaplayed.

***Fucosidase Treatment Decreases the Static Adhesion of MDA-MB-231 cells on HUVECs***

Not only was the rolling on endothelium affected by defucosylation, we have also identified that the static adhesion of MDA-MB-231 cells to endothelium in vitro was also inhibited by α-L-fucosidase treatment. The adhered MDA-MB-231 cells on HUVECs could be distinguished by their different shapes (**Figure 29**), which make it possible to quantitatively measure the adhesion of tumor cells on endothelium in vitro.

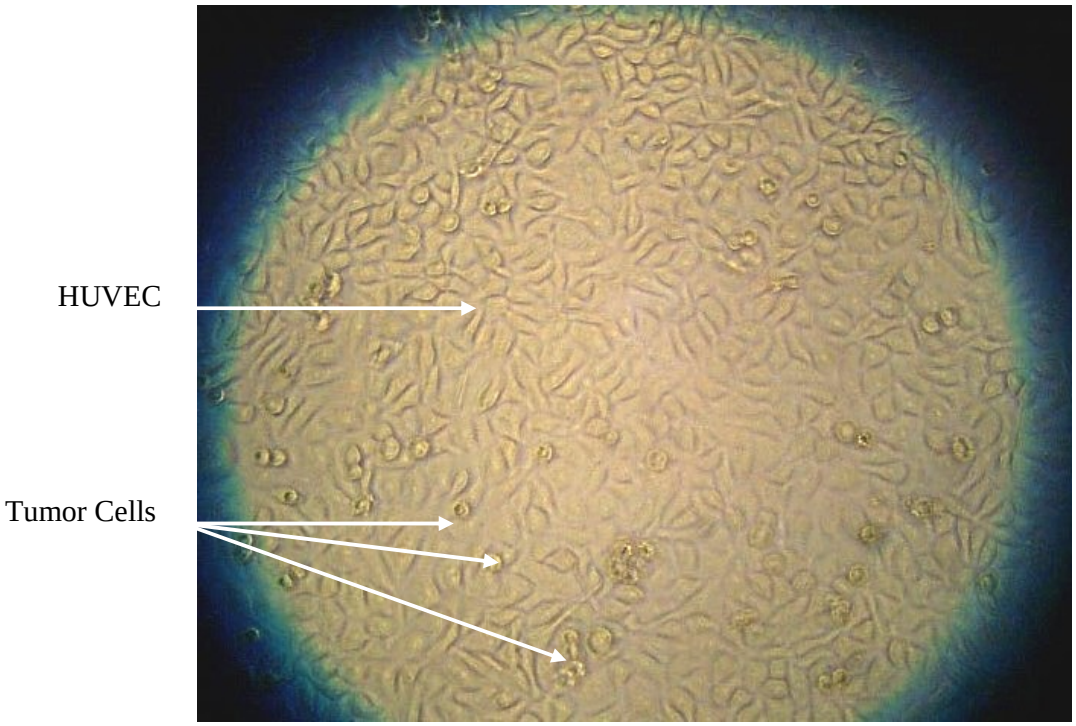



**Figure 29. The static adhesion assay of MDA-MB-231 cells on HUVEC.** Fucosidase treated and control cells (5000/ml) were seeded onto the confluent HUVEC monolayer cultured in 96 wells-plates. After the indicated time of co-culture, the wells were washed by PBS. The adhered tumor cells were counted under microscopy with the aid of a grid eyepiece. The adhered MDA-MB-231 cells displayed different morphology from HUVEC as indicated by the arrows and could be easily identified and counted by their different shapes.

The importance of sialyl Lewis x was also illustrated by adding sLex specific antibody into the adhesion assay system (**Figure 30**).

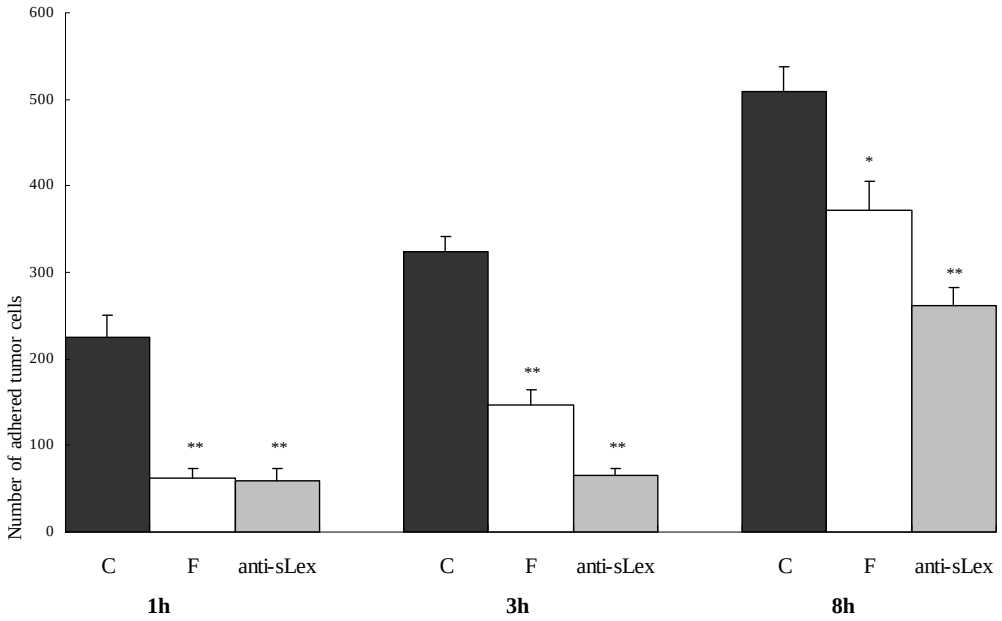



**Figure 30. Defucosylation significantly decreased the MDA-MB-231 cells adhesion on HUVEC under static conditions.** Km93 anti-sLex antibody also significantly inhibited the adhesion on endothelial cells. Data were analyzed by Mann-Whitney Test, *, $P<0.05$ represents the treated group as compared to the control, ** $P< 0.01$ represents the treated group as compared to the control.

***Fucosidase Treatment Decreases the Rolling of MDA-MB-231 on E-selectin, P-selectin and ICAM-I***

To further study the effects of α-L-fucosidase on tumor-matrix interactions, we sought to study the effect of defucosylation on tumor cells rolling under more standard and simplified conditions. Thus, a flow chamber assay with fluorescence labeling was applied. E-selectin, P-selectin and ICAM-I were used to coat flow chamber plates, and labeled cells could be easily differentiated by their different colors under fluorescence microscopy **(Figure 31)**. The variance in the number of

rolling cells revealed different interactions with individual matrix components. Using this method, we have identified that fucosidase treatment impaired the rolling of MDA-MB-231 cells on all 3 adhesion molecules, i.e. on E-selectin (**Figure 32)** and ICAM-I **(Figure 33)**, and on P-selectin but only under low shear stress conditions **(Figure 33)**.

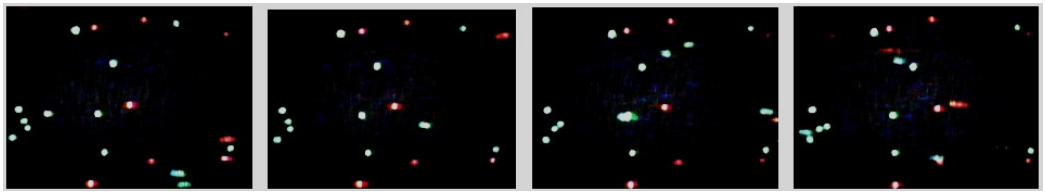


**Figure 31. Consecutive image frames captured from a representative flow chamber assay with fluorescence labeling.** Dots represent individual MDA-MB-231 cells. Tissue culture dishes were coated with purified substrates as described. Fucosidase-treated tumor cells were labeled with CellTrace$^{TM}$ Calcein Red Orange, while tumor cells in the control group were labeled with Calcein green. After incubation with fluorescence dyes and washing thoroughly, 5ml of each group cells were thoroughly mixed. The mixture of cells with different colors of fluorescence were applied to the flow chamber following the same procedure as described above, except video recording was performed under fluorescence microscopy. The number of rolling cells labeled **red** (fucosidase treated) and labeled **green** (control) at the various time intervals were counted, respectively.

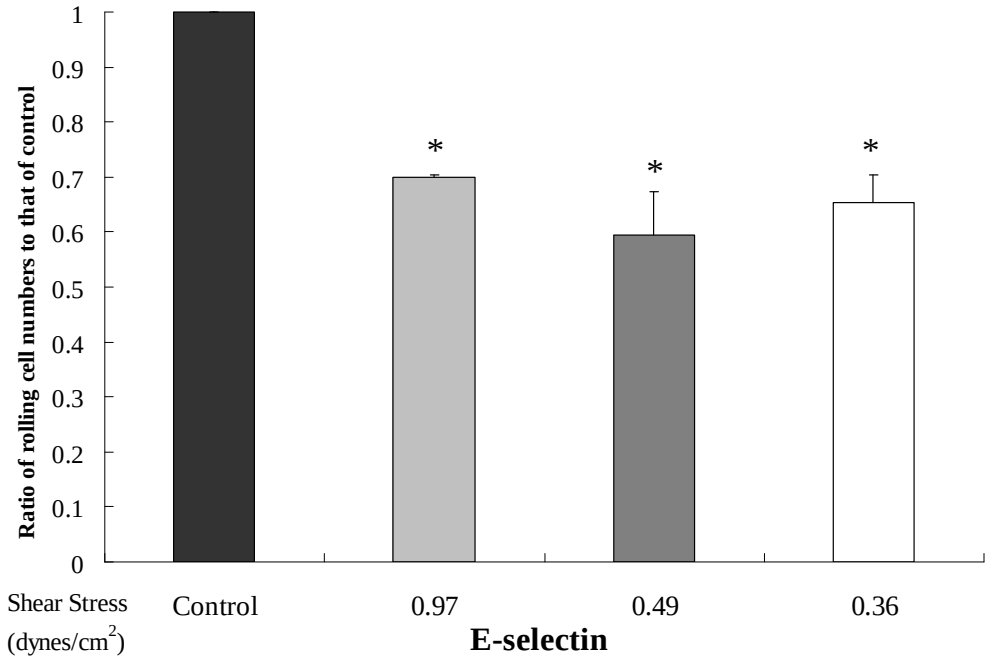



**Figure 32**

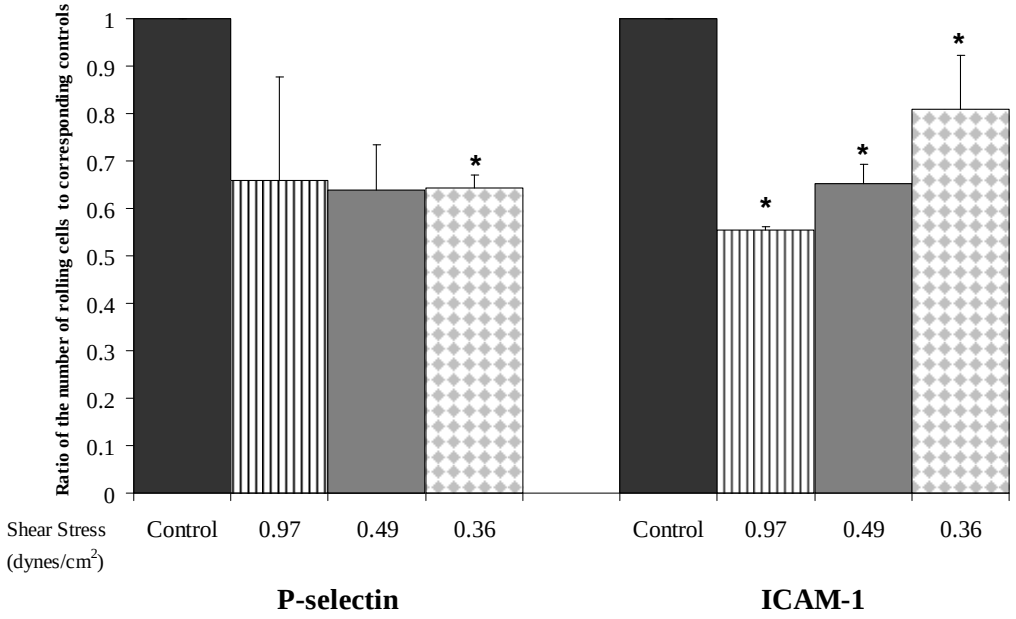



**Figure 33**

**Figure 32-33. Fluorescence-labeled flow chamber assay on purified adhesion molecules** (**Figure 32** E-selectin, **Figure 33** P-selectin and ICAM-I). The fluorescence-labeled flow chamber assay was conducted as described. Alpha-L-fucosidase treatment decreased the rolling of MDA-MB-231 cells on all purified adhesion molecules under all three levels of sheer stress for E-selectin and ICAM-1, as well as at the "0.36 dynes/$cm^2$" group for P-selectin. Data were analyzed by Mann-Whitney Test, *, $p<0.05$ represents the treated group compared to the control, ** $p< 0.01$ represents treated group compared to the control group.

In conclusion, we provide evidence to support the hypothesis that alteration of the expression level of a single monosaccharide, i.e. α-L-fucose, alters the interactions between tumor cells with matrix and with endothelium in vitro. Our data revealed that defucosylation decreased tumor cells adhesion on fibronectin, hyaluronic acid, laminin and HuBiogel®. We also found defucosylation reduced the detection of specific glycans by mAbs directed against CD15 or CD44. The co-localization of fucose on β1 integrin also decreased. We also investigated the interaction of tumor cells with endothelial cells under both static and flow conditions. Again, α-L-fucosidase treatment decreased the expression of sLex and inhibited the rolling of tumor cells on endothelium and on adhesion molecules, which in turn, increased the flow speed of human breast cancer cells.

## Discussion

Patients with localized breast cancer have a 5-year survival rate of 98%, while the survival rate drops to 26% in those patients already having distant metastases

upon diagnosis [177]. Thus, early detection and interference with tumor progression, i.e., invasion and subsequent metastasis, is critical to improve prognosis. Tumor progression is generally accompanied by concurrent alterations in virtually every aspect of glycosylation [178, 179]. Voluminous evidence has proven that breast cancer is not an exception. Abnormal glycosylation patterns further, are known to signal the transformation of breast cancer to a metastatic phenotype [53].

In the previous chapter, we identified high levels of α-L-fucose on human breast cancer cells. As we reviewed, fucose-containing glycans are known to participate in many physiological processes including constituting the major blood group antigens, mediating leukocyte-endothelial adhesion, fertilization, as well as ontogenesis and differentiation [180]. The importance of fucosylation has been further demonstrated in a rare genetic disease, Leukocyte Adhesion Deficiency (LAD) type II, in which the terminal fucose moieties are severely impaired, leading to a profound defect in leukocyte rolling on endothelium [181]. Under multiple pathological settings, fucosylation has also been found to be elevated in various kinds of tumor and could often be correlated with tumor progression, e.g., up-regulated fucosyltransferase VII genes were correlated with lymph node metastasis and the poor prognosis of patients with breast cancer [182]. Thus, we hypothesized that reducing the “fucose-burden” by exogenous fucosidase would interfere with the very interactions modulated by fucosylated glycans and glycoproteins. We also showed that defucosylation was not cytotoxic under the conditions used, but mainly impaired crucial cell-ECM and cell-cell interactions via altered cell signaling, which in turn, disturbed important tumor functions.

Fucosylations, we now suggest, indirectly modify adhesion molecules, including integrins, selectins and CD44. Instead of targeting a specific protein, alterations in glycosylation patterns decorating various adhesion molecules accumulate and generate considerable effects on cellular adhesion to ECM components. We have identified significant changes in the adhesion properties of these defucosylated cells. Under static conditions, the adhesion of MDA-MB-231 cells on simple matrices such as fibronectin, laminin, type I collagen, hyaluronic acid and complex ones such as HuBiogel®, were significantly decreased by α-L-fucosidase treatment **(Figure 15)**.

There have been many reports suggesting that glycosylation modulates the functions of integrins, which mediate the interactions of tumor cells with fibronectin, laminin and the collagens. In metastatic human melanoma cells, β-1,6- branched glycans on $\alpha_3$, $\beta_1$ integrin subunits have been shown to affect the ligand binding of $\alpha_3\beta_1$ integrin and to modulate tumor cell adhesion [183]. Glycosylation also directly affects β1 integrin maturation, subunit assembly and binding activity [184]. β-1,6-GlcNAc branching protects $\beta_1$ integrin from degradation and enhances delivery to the plasma membrane that, in turn, subsequently promotes cell migration and Matrigel invasion [185]. Further, changes in N-linked β-1,6 branching on the glycosylated $\beta_1$ integrin subunit has been demonstrated to alter the cell-matrix adhesion and migration by modulating integrin clustering and subsequent signal transduction [186]. In contrast, down-regulation of the expression of β-1,4-galactosyltransferase V has been found to promote the maturing and stability of cell surface integrin β1 and enhance the adhesion ability of neoplastic cells to fibronectin while modulating the level of focal adhesion kinase phosphorylation [187] .

In this chapter, we provide evidence for found decreased co-localization of fucose with the β1 integrin subunit after fucosidase treatment, but not with the $\alpha v$ integrin subunit (**Figure 19**). This decreased fucosylation associated with $\beta_1$ integrin may have contributed to the altered human breast cancer cells' adhesion on fibronectin and other matrix components. It's also possible that the fucosidase treatment modified other cell surface adhesion molecules, as similar to the role that fucose plays on integrins, the hypersialylation of β1 integrins in colon adenocarcinoma up-regulates attachment to collagen I and laminin [188]. Different glycan structures on other integrins also show certain discrepancies in terms of adhesion. In contrast to the role of fucose, desialylated $\alpha_3\beta_1$ integrin from human bladder T24 carcinoma cells exhibits significantly higher fibronectin-binding capability [127] and removal of sialic acid by neuraminidase treatment enhances the cell adhesion of Burkitt's lymphoma cell to galectin-1 [189].

Similar to our findings, manipulating the fucose-generating enzyme, FX, affects global cellular fucosylation and alters the interaction of colorectal cancer cells with extracellular matrix components such as fibronectin [190]. The FX enzyme is also known to modulate the adhesive interactions between colorectal cancer cells and endothelial cells by controlling the production of fucosylated Lewis antigens [191] . Generally, glycosidase or glycosylation inhibitors have also been found to affect the adhesive functions of integrins. Tunicamycin has been reported to inhibit glycosylation on $\alpha_v\beta_5$ and $\alpha_v\beta_6$ integrins and to impair the adhesion of HT29-D4 colon adenocarcinoma cells to vitronectin and fibronectin [192]. Similarly, deglycosylation has been shown to induce dissociation or alter association of $\alpha_5\beta_1$ subunits resulting in concomitant loss of fibronectin binding activity [193]. Inhibition of glycosylation is also known to result in aberrant glycosylated $\beta_1$, $\alpha_1$, $\alpha_3$, $\alpha_6$, and $\alpha v$ integrin subunits

while significantly decreasing the adhesion of HT-29 human colon adenocarcinoma cells to laminin, type IV collagen and fibronectin [194]. Increasingly, evidence highlights the importance of glycosylation on tumor adhesion mediated by integrins and this work supports this contention.

Another adhesion-associated family of molecules, CD44 is widely distributed among different cells and tissues and plays important roles in many biological and pathological processes. Specifically, CD44 is known to up-regulate migration, invasion and metastatic activities [139]. N-/O-glycosylation and glycosaminoglycan (GAG) carried by CD44 are important for the communication between ECM and intracellular protein components. Changes in N- and O-glycosylation of CD44s modulate its cleavage [195] and adhesion on hyaluronic acid [196]. Immediately after treatment, the observed decreased detection of CD44 (**Figure 20**) may have arisen via two possible pathways. Firstly, glycosylation have considerable effects on the masking, exposure or modification of antigenic epitopes. Alteration of glycosylation may impair its recognition by antibodies. Removal of N-glycosylation has already been shown to impair the immunoreactivity of mAb against pan-CD44 [136]. The second possible argument is that exogenous events which alter cell-cell interactions lead to perturbations of one or more signaling pathways, which, in turn, cause the down-regulation of CD44 expression. Further experiments examining CD44 mRNA and protein levels will be helpful in illuminating the mechanisms during the observed changes.

As we have shown in **Figures 15** and **17**, CD44 mediated adhesion on hyaluronic acid significantly decreased after fucosidase treatment. A number of investigators have also found glycosylation affected the binding of CD44 with its

ligands, principally, Hyaluronic Acid (HA). Examples include the increased expression of N-acetylglucosamine on CD44 enhancing mouse melanoma cells' adhesion to HA to promoted tumor growth and metastasis [197] and inhibition of N-glycosylation decreasing CD44 mediated-adhesion to HA by ovarian cancer cells [196] and by neuroblastoma cells [198]. Similar to the role of sialic acid in the modulation of adhesion mediated by integrins, removal of sialic acid by neuraminidase increased the adhesion to HA by ovarian cancer cells [196]. Furthermore, a combination of neuraminidase and O-glycosidase treatment rendered CD44 reactive to antibodies which previously failed to recognize its expression on tumor cells [139]. Perturbation of HA-CD44 binding is known to suppress the PI3-kinase/Akt pathway [199]. These reports also suggest a possibility that, the detected changes of the expression of CD44 by antibodies, may indeed arise from alterations in the glycosylation pattern of CD44, which justify more research using other approaches.

Cell adhesion molecules, including intercellular cell adhesion molecule-1 (ICAM-1), E-selectin and P-selectin, has been studied extensively in the field of inflammation. Basic functions include their ability to recruit leukocytes onto the vascular endothelium. It's now clear that some circulating cancer cells may also utilize similar machinery to disseminate and extravasate, building towards the metastatic phenotype. The selectins are a family of adhesion molecules expressed on endothelial cell and leukocytes, mediating leukocyte-leukocyte interactions and leukocyte-endothelium interactions. The interaction of L-selectin expressed on leukocytes with its environment is one of low affinity with rapid "on-and-off" switching. Thus, this drives the adhesion of leukocytes to the vascular endothelium and modulates leukocyte-leukocyte rolling. P-selectin is found both within endothelial cells and platelets, and is involved in leukocyte recruitment, trafficking,

wound healing and blood clotting. Ligands for L- and P-selectin include fucosylated structures, mainly the sLex moiety [4]. Through L- and P-selectin, tumor cells often form complexes within thrombi and with platelets and leukocytes in the bloodstream, facilitating metastases and evasion of the immune system [4].

As we have discussed previously in the text, the expression of selectins and their Lewis antigen ligands are known to be correlated with tumor progression. It has been found that MDA-MB-231 human breast cancer cells, themselves, can induce the expression of E-selectin on endothelium [200]. In normal breast epithelial cells, sLex and sLea are rarely expressed, while they are seen to be increased in primary breast carcinoma and metastatic foci. Further, it has been shown that the expression of E- and P-selectins have also been found to be elevated in tumor tissues [201]. Serum level of sLex and soluble E-selectin have similarly been shown to be elevated in patients with advanced and recurrent breast cancer, especially in those with distant metastases [202]. The expression of sLex in primary breast cancer tissues has been correlated with the poor prognosis of patients and with lymph node metastases [203]. Compared with primary cancer, metastatic breast cancer cells isolated from bone marrow have also been reported to express higher amounts of Lex , sLex and Ley [204]. This combined evidence strongly suggested a prominent role for sLex in neoplastic progression of breast cancer, which prompted us to investigate the effects of fucosidase on the fucose-containing glycan, sLex.

Using flow cytometric analysis, we were also able to identify a decreased expression of sLex on the cell surface of the MDA-MB-231 human breast cancer cells after treatment with fucosidase **(Figure 22-23)**. This same general decrease was also found in the expression of Lex **(Figure 21)**. The effectiveness of fucosidase in

modifying Lewis antigen expression has previously been reported. In fact, fucosidase treatment has been shown to be able to completely abolish the recognition by Lex-specific antibodies [205]. Presumably, the epitope of sLex recognized by the mAb Km93 was disrupted by the hydrolysis of the terminal α-1, 3-fucose after fucosidase treatment. Recently, it was reported that sLex appears to directly participate in epithelial migration and repair [206]. We also found that the KM 93 sLex antibody interfered with the adhesion of breast tumor cells to the HuBiogel complex ECM (**Figure 17**), and further, this was linked to the cumulative effects on the incorporated sLex carried by various adhesion molecules. Clearly further studies are needed to better define the functional roles of sLex structure in mediating the adhesion process beyond that associated with rolling on endothelium.

Lewis antigen determinants are rigid structures, which generally maintain the same conformation in the free and bound states [207]. Under external forces, the intermolecular dissociation between sLex and selectin domains are mainly determined by the separation of fucose from calcium which drive conformational changes and adhesive functionality of selectin-ligand interactions [208]. The CRD domain of E-selectin is known to coordinate a single calcium ion interacting with two hydroxyl groups on fucose (**Figure 34)**, one of the four monosaccharides constituting the tetrasaccharide glycan, sLex [209]. Intermolecular dissociation between sLex and E-selectin was mainly determined by separation of fucose from $Ca^{2+}$ in selectin-ligand systems [208]. Replacement of these hydroxyl groups by hydrogen abrogated E-selectin binding [210] and the presence of fucose at the α-1,3-position was essential for the ligand function of selectins [211]. Thus, removal of fucose from selectin ligands would presumably alter the conformation and impair the stability of selectin-ligand interactions under external forces.

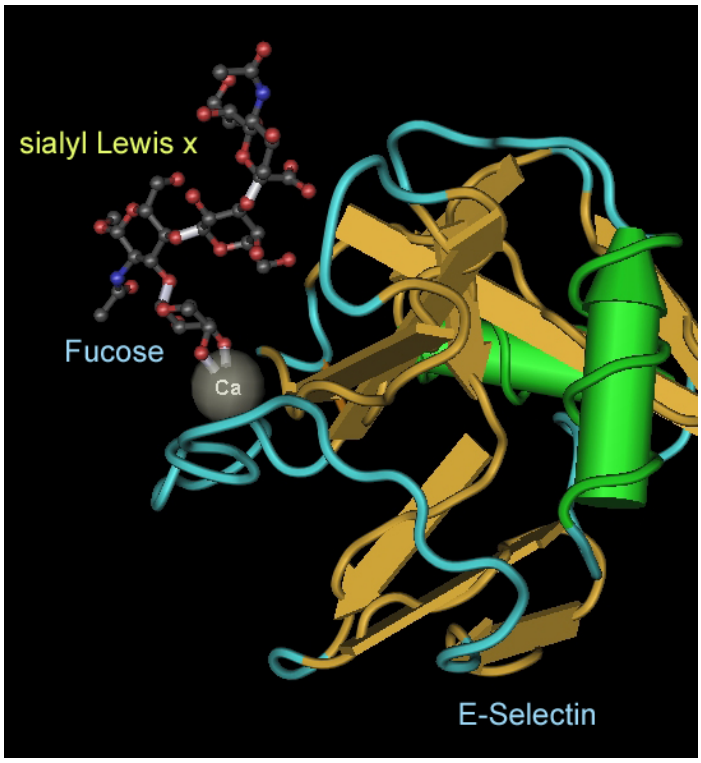



**Figure 34. Illustration of the interaction between human E-selectin domains binding with sLex,** showing the arrangement of sLex, juxtaposed to the calcium through hydroxyl groups (figure created based on the work of Somers et. al [212], see Appendix for the License Agreement)

Others have previously shown that α-1,3-Fucosyltransferase-VII-deficient cells display significant reduction in their interaction with P- and E-selectins under flow conditions [213]. Further, inhibition of glycosylation in Lewis lung carcinoma cells has similarly been seen to reduce the expression of sLex, thereby, impairing P-selectin-dependent cell adhesion in vitro and decreasing formation of metastases in vivo [214]. Benzyl-α-GalNAc, an O- glycosylation inhibitor, has also been found to suppress the expression of sLex and sLea on tumor cells and thereby reduce their adhesion to endothelial cells or platelets [215], while decreasing their binding to E-selectin [216]. Down-regulation of the fucosylation generating enzyme, FX enzyme, as we have alluded to previously, affects global cellular fucosylation, decreases adhesion to activated endothelial cells and to recombinant E-selectin, and alters the interaction

of cancer cells with extracellular matrix components [191]. These observations parallel the findings that we uncovered in our study. These observations thus provided the rationale for us to set up an experimental model which more closely mimicked the physiological state.

We, thus, utilized a non-passive model of breast cancer cells movement within the vascular tree during the late stage of neoplastic progression. A high temporal- and spatial-resolution flow system was utilized which is capable of capturing images at high speed and resolution making it possible to capture accurate positional measurements of individual cells' velocity and acceleration. Tumor cells with different fluorescence labeled tags provided a more comparable approach to study the flow condition of breast cancer cells on identical matrices **(Figure 31)**. By using this flow chamber assay system, we have identified a decrease rolling of breast cancer MDA-MB-231 cells on endothelial cells, in vitro, under flow condition. This study further suggests the possibility that defucosylation resulted in a low level of fucose on sLex, which in turn, impaired the interaction of these tumor cells with endothelium, as well as with purified E-, P-selectins (at low shear stress) and ICAM-1 under flow conditions **(Figure 32-33)**. In addition to reducing the expression of sLex, fucosidase may also affect many other fucose-containing glycans including other members of the Lewis antigen family (Lex, sLea, etc. see **Figure 14**) and numerous fucose-incorporated glycoproteins and glycolipids, which have the capability to act as ligands for ICAMs and selectins. For example, down-regulation of sLea has previously been shown to decrease the adhesion of highly metastatic colorectal cancer cells to endothelial cells and to recombinant E-selectin [217]. Sialyl fucosyl poly-N-acetylgalactosamine gangliosides also mediates E-selectin-dependent rolling and tethering under physiologic shear stress conditions [218].

Similar to our findings, down-regulation of sLex decreased human hepatocarcinoma 7721 cell adhesion to HUVEC, and sLex specific KM93 antibody significantly abolished the cell migration and invasion of these cells [65]. Compared with a sialyl residue, fucosyl residues appear more critical for human hepatocarcinoma H7721 cells' adhesion to fibronectin and laminin, but less to their adhesion to HUVEC. Noticeably, cell adhesion to HUVEC, migration, and invasion can all be inhibited by the mAb against sLex [219], which is consistent with our findings **(Figure 29)**. On the other hand, treatment of the mixed population of cells found within human cord blood with GDP-fucose and exogenous α-1,3 fucosyltransferase VI, was seen to increase sLex expression, augment binding to P- and E-selectin, and improve cell rolling on P- and E-selectin under flow conditions [220]. Binding of cancer cells to E-selectin has also been shown to initiate signal transduction pathways affecting metastatic abilities. Related to this phenomena, interaction of T84 colon cancer cells to E-selectin in vitro is known to significantly elevate intracellular $Ca^{2+}$ concentrations, increase tyrosine phosphorylation, stimulate tumor cell actin reorganization, increase collagenase secretion and induce cell migration [221]. Decreased interaction with selectin by fucosidase treatment, also is presumed to affect these functions downstream of sLex-selectin interactions.

Other fucose-containing Lewis family glycans have also been implicated in the progression of breast cancer. Lex (i.e. CD15) is recognized as a tumor antigen and is known to participate in many processes involved in oncogenesis, development, differentiation and cellular adhesion. The expression of Lex in breast cancer is commonly found to be localized at the leading edge of an invading tumor or at the outer edge of carcinoma in situ, suggesting a potential role in invasiveness and metastasis [222] and is further found to be correlated with the grade, clinical stage,

recurrence and prognosis [223]. Glycoproteins serving as Lex-carriers include CEA-related cell adhesion molecule 1 [85] and cadherin [65]. Lex also mediates adhesion of human breast carcinoma cells to endothelium [224].

As fucose is one of the three monosaccharides comprising Lex **(Fig 14)**, it was not unexpected to find the detection of Lex decreased after defucosylation **(Figure 21**), similar to the findings with sLex. A similar role was identified for sLea, as transfection of cells with antisense cDNA for α-1,3/4-fucosyltransferase eliminated the expression of sLea and completely abolished their adhesion to E-selectin [168]**.** Interestingly, the glycosylation carried by the CD44 splice variant(s) on LS174T colon carcinoma cells, was found to possess E-selectin ligand activity, providing a novel functional role for CD44 in cancer metastasis [225, 226], while also building a bridge to our finding concerning the expression of CD44 on MDA-MB-231 cells after fucosidase treatment **(Fig 20)**.

In contrast to the exogenous defucosylation by enzymatical hydrolysis, considerable efforts have been sought to diminish cell surface fucose levels by modulating fucosyltransferases expression. The antisense transfection of several fucosyltransferase genes has in fact, result in a substantial reduction of sialyl Lewis antigens' expression on cell surfaces, and consequently to inhibit their adhesion to E-selectin, decreasing metastases in human BxPC-3 pancreatic tumor cells [227] and HT-29 LMM colon carcinoma cells [228]. Direct interference with the interaction between sLex and E-selectin is also an intriguing target and other investigators have shown that a high level of 'shredded sLex', in the serum of patients with pancreatic cancer could be seen to reduce SW1990 cell binding to E-selectin [216].

In this chapter, we have thus presented our findings which would enrich our understanding as to the functional role that fucosylation plays in the cross-talk

between cells and their matrices. Further, we have identified the effectiveness of defucosylation on tumor cell functions in a physiological-mimicking situation as related to breast cancer cell neoplastic progression.

# CHAPTER 5

## CONCLUSIONS

The application of glycosylated proteins as tumor markers for the diagnosis and treatment of tumors is not novel, but the functional significance of glycans in tumor development and neoplastic progression has only recently been unveiled. A more precise localization and depiction of the structure and function of cancer associated glycans has become available thanks in part, to the advancement in glycobiology molecular and cellular techniques. A rationale has been accepted by a growing number of investigators that by interfering with the expression of glycosylation, it becomes feasible to inhibit tumor develop and progress. Altered glycosylation in cancers has in fact, become a target for both experimental therapy and clinical trials. Farthest down this pathway are the family of specific pharmaceuticals developed to block selectin-mediated adhesive events. The trend in cancer glycotherapy remains the development of even more specific glycosylation inhibitors and the delivery of an efficient system of therapeutic genes that modulate the glycosylation sites expressed on tumor cell surfaces. In addition, an even popular approach is to analyze glycan profiles in selected cancers so as to reveal new glycan biomarkers with improved accuracy.

A glycosynapase model of carbohydrate-bearing membrane microdomains has been established as the controlling functional unit in carbohydrate-dependent cell adhesion and it has been shown that the sLex and sLea antigens confer upon cells a key “prometastatic glycosylation phenotype” [229]. Targeting the machinery for

processing tumor cell surface oligosaccharides, new drugs have been developed to inhibit metastasis [230]. Further, synthetic selectin-ligand mimetics have been shown to act as competitive inhibitors of glycan mediating functions, and cell-permeable, small-molecule oligosaccharides have been identified which can alter metabolic pathways and disturb the biosynthesis of target glycans. Systemic administration of AcGnG-NM, a disaccharide-based inhibitor of glycosylation has been shown to be able to inhibit the biosynthesis of sLex associated with spontaneous lung dissemination of tumor cells without interfering with the normal physiological expression and functions of selectin-sLex dependent responses [214]. Mimics of sLex have also been designed and synthesized to inhibit tumor cells' interaction with E- and P-selectin [231], which further outcompete sLex/a antigens expressed on tumor cell surfaces to prevent initial vascular adhesion [232]. In addition, competing disaccharides have been generated which can block synthesis of selectin ligands and subsequent adhesion of cells to selectins through decreased expression of α-2,3-sialylated and fucosylation oligosaccharides [233]. Lastly, the initial arrest of tumor cell in the lung vasculature has been reported to be remarkably inhibited by exogenous liposomal sLex [234].

Certain obstacles in the application of glycotherapy of cancer patients do exist. In contrast to the highly-specific and fully potent function of protein/enzyme targets, the consequences of altering glycosylation may result in essentially undetectable and non-specific changes. Even within a particular class of proteins, the effects of altering glycosylated residues are highly variable and unpredictable. In addition, identical glycosylation changes have been found to have markedly different effects on different proteins or lipids. This is only heightened by the knowledge that the examination of

these entities by either in vivo or in vitro means, in and of itself, may yield different result from the natural, in-situ state.

In the setting of a glycosynapase microdomains, our findings provide a direct approach to disrupt the interaction and modify this “prometastatic glycosylation phenotype” mimicking the in vivo physiological milieu, thus providing a basis for more clinically-oriented approaches in the near future. A clinical scenario could be visualized, based on our study, where by circulating sLex-diminished carcinoma cells would bear a diminished capacity to build an interaction with the vascular endothelium and predictably, generate less metastases, especially if one were to deliver the defucosylating drug during or immediately post-operationally. Exogenous application of glycosidases may also be potentially suitable for the prevention of tumor dissemination within certain luminal organs, for example, the urinary bladder or common bile duct.

## Summary

Modification of a certain monosaccharide on membrane glycoproteins may provide a profound “canister-like” effect on various aspects of tumor function. Innovative research in tumor glycobiology should provide us with new weapons to modulate tumor invasion and metastasis and thereby help to lower the mortality in patients with cancer. A further understanding of the expression of malignancy-associated glycosylation events and the detailed molecular mechanisms whereby they mediate cellular events will, in turn, help us to identify additional targets of such glycan-mediating interactions, all of which should boost the development of more

glycan-based therapies and increase the possible choices for physicians to improve the prognosis of patients with malignancies in general and with breast cancer specifically.

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