

Review Article

The emerging importance of α -L-fucose in human breast cancer: a review

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Abstract: Breast cancer cells incorporate the simple sugar alpha-L-fucose (fucose) into glycoproteins and glycolipids which, in turn, are expressed as part of the malignant phenotype. We have noted that fucose is not simply a bystander molecule, but, in fact, contributes to many of the fundamental oncologic properties of breast cancer cells. Here, we summarize the evidence from us and others that fucose is necessary for key functions of neoplastic progression including hematogenous metastasis, tumor invasion through extracellular matrices including basement membranes and up-regulation of the Notch signaling system, with implications for epithelial-to-mesenchymal transition and activation of breast cancer stem cells. Additionally, certain breast cancer biomarkers are fucose-rich while a well-known marker of breast cancer progression, soluble E-selectin, is a known counter-receptor of fucosylated selectin ligands. We provide illustrative examples and supportive evidence drawn from work with human breast cancer cell lines *in vitro* as well as clinical studies with human pathologic material. And finally, we discuss evidence that fucose (or its absence) is central to the mechanisms of action of several experimental targeted therapies which may prove useful in breast cancer treatment. We propose that alpha-L-fucose is essential in order to construct first, the malignant and then the metastatic phenotype of many human breast cancers. This knowledge may inform the search for novel treatment approaches in breast cancer.

Keywords: Fucose, selectin, biomarker, Notch, trastuzumab, breast cancer

Introduction

The complete sequencing of the human genome has brought forth major advances in our understanding of the biology of disease. Nevertheless, subsequent studies in this “post-genomic era” have revealed new complexities, new challenges and new pitfalls in biological research. For example, the genetic code has shown limited utility in predicting post-translational modifications such as the synthesis of glycoproteins from their protein precursors. Interest in biology beyond the genome has fostered new studies that seek to understand these complex biological processes which are seemingly not directly encoded by the genetic code.

Post-translational modifications involving carbohydrates are the province of the interdisciplinary field of glycobiology, “the study of the structure,

biosynthesis, biology, and evolution of saccharides (sugar chains or glycans) that are widely distributed in nature, and the proteins that recognize them [1].” Contributions to glycobiology have been made by investigators from more traditionally-defined fields such as biochemistry, genetics and immunohematology (to name but three). The evolution of glycobiological concepts, technology and knowledge has progressed substantially over the past 3 decades, including much early pioneering work carried out in Japan. During that time period, an immense fund of knowledge was developed which supports the role of carbohydrate structures in disease states, including many human cancers. There is now clear assurance that carbohydrates are essential in manifesting the malignant and metastatic properties as well as functional behaviors of human cancer cells.

We have previously reviewed the evidence that

one particular simple sugar, alpha-L-fucose, is a critically important molecule in neoplasia [2]. More recent research on human breast cancer suggests that alpha-L-fucose is not simply a bystander molecule, but is a functionally important pathophysiological effector. Accordingly, we propose that alpha-L-fucose is an essential component of the malignant and metastatic phenotypes of many human breast cancers, and that this knowledge can inform the search for improved therapeutic agents. We developed this thesis based on experimental results obtained from multiple human breast cancer cell lines *in vitro* and from pathologic material derived from human patients. In asserting the significance of alpha-L-fucose, we recognize that there are other sugars of importance in breast cancer, other pathological metabolic pathways, and other therapeutic approaches to which alpha-L-fucose is irrelevant. Our aim is to persuade the reader of the special (and ultimately practical) importance of alpha-L-fucose in this disease process.

The field of glycobiology has developed its own history, conceptual framework and terminology. It is not our aim to give a comprehensive recounting of these facts. Here, we present only those key terms and concepts which are needed to understand the relationships between alpha-L-fucose and breast cancer. The definitions are quoted or paraphrased from the NCBI Bookshelf online text, *Essentials of Glycobiology* (2nd edition), except as otherwise noted [1]. Free online access to this text is available at <http://www.ncbi.nlm.nih.gov/books/NBK1908/>.

Terms and concepts

Glycan

The currently accepted generic term for “any sugar or assembly of sugars, in free form or attached to another molecule, used interchangeably with saccharide or carbohydrate.”

Alpha-L-Fucose

A six-carbon deoxy-sugar in which a hydroxyl group at the carbon 6-position is replaced by a hydrogen atom (**Figure 1**). Fucose is utilized exclusively in the L-configuration in mammals. This sugar is enzymatically synthesized in mammalian cells and is also recovered by cells from extracellular sources by a specific transmem-

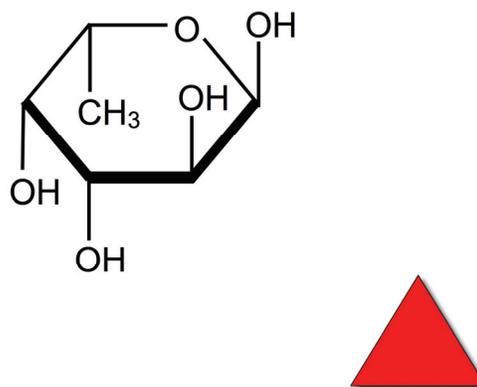


Figure 1. Alpha-L-fucose is the only L-sugar utilized in mammals and is a 6-deoxyhexose. By convention, fucose is represented by a red triangle in diagrams of glycan structures.

brane carrier and intracellular salvage pathway. Since alpha-L-fucose is the only form which is relevant in humans, we will hereafter refer to the sugar simply as fucose.

Fucose trafficking in cells

When fucose is attached by a fucosyltransferase enzyme via a glycosidic bond to a biomolecule, that molecule is said to be fucosylated. When fucose is cleaved by a fucosidase enzyme from a biomolecule by hydrolysis of the glycosidic bond, the molecule has been defucosylated. Fucose can be added to existing glycans to yield more complex glycans. This is seen, for example, in the synthesis of small carbohydrates such as the Lewis antigens (see below). Fucosylation is carried out on complex and highly-branched glycans as well. A special form of fucosylation, i.e., direct glycosidic addition of fucose to a protein, is carried out by the protein-O-fucosyltransferase (abbreviated Pofut) enzyme family. Protein-bound fucose can be elongated by glycosidic attachment of another sugar to fucose, with subsequent sequential additions of sugars to form a glycan chain. Core-fucosylation is a specialized modification of glycans which is of particular importance in antibody-dependent cellular cytotoxicity, abbreviated ADCC. Fucose-containing glycans are often expressed in many copies on a single glycoprotein molecule; the glycoprotein is then said to be decorated by the glycans. A single glycoprotein can be decorated by more than one type of fucosylated glycan.

Tumor-associated carbohydrate antigens

Cell surface glycan antigens which are associated with transformation to a malignant phenotype. Abbreviated TACA, these antigens may be attached to lipids or proteins which are thus designated as glycolipids or glycoproteins.

Lewis antigens

A class of small glycans, originally described as minor blood group antigens in a population of human patients (the Lewis family) with hematologic disorders. Lewis antigens have since been recharacterized as “histo-blood group antigens” in view of their expression on normal and malignant epithelial cells of various origins [3]. The most common Lewis antigens are composed of a small number of constituent simple sugars (3 or 4), of which 1 or 2 moieties are fucose. Some of the Lewis antigens are further modified by addition of 1 or 2 sulfate groups. Certain Lewis antigens are overexpressed in well-characterized human breast cancer cell lines and also in certain pathological material from patients. **Table 1** summarizes the structures of those Lewis antigens associated with breast cancer as well as several additional fucosylated antigens of known importance in breast cancer.

Lectins

A class of proteins which (a) bind specifically to particular glycan structures, with the further requirements that the proteins (b) are not immunoglobulins (except for certain members of the immune superglobulin family) and (c) are not enzymes that act on the glycan as a substrate, nor transport system receptors for the glycan. Lectins are part of the general class of “agglutinins” discovered over 100 years ago in plants; lectins are now known to be widely expressed in animals including humans. The term “lectin” is derived from the Latin *lectus* (“meaning to pick, choose or select”). A special class of lectins, the selectins, has been de-

<u>Antigen</u>	<u>Structure</u>
Lewis X	
Sialyl Lewis X	
Lewis Y	
Lewis A	
Sialyl Lewis A	
Lewis B	
H type 2	
LNFP III	
Globo H	

Fucose
 Galactose
 N-acetylglucosamine
 Glucose

N-acetylglucosamine
 Sialic acid
 Mannose

Table 1. Structures of the fucosylated cell surface antigens reviewed in the text. The symbols for common monosaccharides follow the convention as described in *Essentials of Glycobiology*, 2nd edition [1] and reproduced in part below. The symbol “R” denotes the glycoprotein or glycolipid carrier to which the antigen is bound. For simplicity, only the glycosidic bond involving fucose (α2, α3 or α4) is indicated for each antigen. LNFP III is the abbreviation for Lacto-N-Fucopentaose III. Note that LNFP III is terminated by the Lewis X antigen. Lewis X, sialyl Lewis X and Lewis Y are synthesized from the “Type II” precursor (N-acetylglucosamine linked β(1,4) to galactose). Lewis A, sialyl Lewis A and Lewis B are synthesized from the “Type I” precursor (N-acetylglucosamine linked β(1,3) to galactose).

scribed and is known to be fundamentally important in inflammation and cancer metastasis. Fucose is an essential component of most of the “natural” ligands for the selectins, and fucose is also incorporated into aberrant selectin ligands expressed by multiple human transformed carcinoma cell lines.

Nonreducing end of a glycan

In the this context, it indicates the chemical polarity of a glycan structure which is linked to a protein or lipid. The nonreducing end is the “outermost end [or outer terminal end] of an oligosaccharide or polysaccharide chain, which is opposite to that of the reducing end.” The reducing end refers to that end of the glycan chain which is covalently attached to the protein or lipid carrier. The importance of this distinction is that fucose is most commonly incorporated into a glycan at the nonreducing end. Thus localized to the outer terminus of the glycan chain, fucose is exposed to the microenvironment and is therefore expected to account for much of the glycan’s identifying properties as would be detected by lectins or antibodies.

The “Sugar Code” or “Glyocode”

Just like other biomolecules such as nucleic acids, glycans carry information in the form of a code which is inscribed in the glycan’s 3-dimensional structure and cellular distribution. The information in this code can be interpreted (“decoded”) by lectins which then trigger signal transduction cascades for intercellular signaling. The concepts are most closely associated with the pioneering work of Sharon and Gabius [4,5]. There are naturally-occurring plant and animal lectins which recognize fucose. Although the normal physiological roles of these fucose-specific lectins are incompletely understood, several fucose-binding lectins have proved useful as tools in research on human cancers [6,7]. Such lectins have been shown to recognize aberrantly fucosylated ligands expressed on cancer cell surfaces.

The Notch signaling system

An evolutionarily conserved, complex coordinated system of intercellular and intracellular signaling molecules in which signal transduction is mediated by Notch ligands and Notch receptors [8]. Initially discovered in *Drosophila*

melanogaster, the Notch signaling system has proved to be of great importance in developmental biology in many organisms including humans, as well as in regulation of cell fates, mature tissue homeostasis and maintenance of stem cells. Notch receptors and Notch ligands are glycoproteins. The “canonical” Notch signaling pathway is mediated by regulated proteolysis [9]. Intercellular activation of the cell surface Notch receptor leads to proteolytic cleavage and release of the Notch intracellular domain, which is translocated to the nucleus, ultimately resulting in transcriptional activation of specific Notch target genes. Dysregulation of Notch signaling (including overexpression of ligands or receptors or both) has been documented extensively in human breast cancer cell lines and in human patient material. Current evidence supports a juxtacrine or autocrine mechanism of Notch ligand/Notch receptor activation in human breast cancers. Mammalian Notch receptors require fucosylation in order to be fully activated by Notch ligands.

The cancer stem cell hypothesis

Stem cells are defined as “cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation [10].” The existence of such cells was proven in the hematopoietic system, and it was subsequently shown that stem cells in human AML appeared to have arisen from normal stem cells which had undergone malignant transformation. The current cancer stem cell hypothesis holds that cancer is primarily a disease of stem cells which have accumulated mutations over a long period of time, finally undergoing malignant transformation, while retaining important stem cell properties including self-renewal, proliferation and capacity for multilineage differentiation. Similar to normal stem cells, which are rare in the mature organs and tissues, cancer stem cells are believed to constitute only a small fraction of the cells in a malignant tumor. Since normal stem cells tend to be resistant to chemotherapy and radiation when compared with mature cell types from the same tissues, it is thought that cancer stem cells share these properties and can therefore survive treatment and give rise to recurrent disease. It is now suspected that cancer therapies, to be effective, must kill cancer stem cells even though they may constitute only a small minority of the tu-

mor cell burden in the human patient. In referring to a therapy for CML resulting in an early but non-durable response, Jones and others wrote: "This pattern of clinical activity would be analogous to cutting a dandelion (or other weed) off at ground level—it may appear to produce the desired effect, but only elimination of the root will actually prevent the weed from re-growing [11]." This "dandelion hypothesis" of cancer stem cell treatment neatly sums up the argument for new treatments which specifically target cancer stem cells. Therapies directed at cancer stem cells will depend on identification of the stem cell phenotypes by means of appropriate cancer stem cell markers, which might also serve as surrogate treatment markers. Identification of cancer stem cell markers has therefore been a high priority. Several potentially fucosylated cell surface glycoproteins, particularly CD44, are regarded as putative stem cell markers in human breast cancer.

Models and tests of tumor cell invasion

The process of cancer cell invasion into its microenvironment has been extensively studied, and discrete events in the process are recognized. In 1986 Liotta hypothesized the main steps in tumor cell invasion into the extracellular matrix (ECM) environment. These included (1) adhesion or attachment to ECM components, (2) dissolution or proteolysis of the ECM, and (3) locomotion of the tumor cell into the region of matrix which has been modified by proteolysis [12,13]. Adhesion is known to involve specific molecular components of the ECM. Tests of static adhesion of tumor cells can be carried out utilizing purified ECM components, especially laminin and type IV collagen, the 2 major proteins of the basement membrane, and hyaluronic acid and fibronectin, key constituents of the ground substance. Dissolution or proteolysis involves local degradation of the attachment proteins and structural collagenous proteins within the ECM by tumor cell-associated proteases such as the type IV collagenases, matrix metalloproteinases (MMPs) and serine proteases. Activity of these enzymes can be assayed by gel zymography assays. "Motility" includes locomotion (often in the presence of a molecular gradient) along with remodeling of the ECM and cytoskeleton of the cancer cell, invadopodia protrusion, and attachment to and detachment from ECM components.

Tests of dynamic invasion processes can be carried out *in vitro* using 3-dimensional complex extracellular matrices which are intended to mimic the tissue microenvironment confronted by cancer cells (reviewed in [14]). In a typical experiment, cancer cells are placed in the upper well of a Boyden-like chamber and allowed to invade through an artificial basement membrane (an ECM layer of predefined thickness) in the presence or absence of chemoattractants. Counts of the invading cells along the underside of the membrane or in the bottom of the chamber are made by light microscopy upon completion of the desired time interval for invasion. Examples of complex extracellular matrices include Matrigel[®], a protein mixture which is derived from EHM mouse sarcoma cells and contains laminin, collagen and various growth factors and matrix-degrading proteases; and HuBiogel[®], an all-human matrix which contains laminin, fibronectin and collagens I, III and IV, but is devoid of the major known growth factors and proteases.

Fucose's role in defining the general cancer phenotype, invasion and metastasis: pertinent background material

Converging lines of evidence support a role for fucose in the general cancer phenotype including invasion and metastasis. This evidence comes from (1) developments in the general field of glycobiology, (2) validation of the "inflammatory cascade" model of leukocyte-endothelial cell interactions, (3) development of the analogous "metastatic cascade model, and (4) the role of fucose in cancer generally.

Glycobiology with relevance to cancer in general

Cell surface glycans on diseased cells have been examined in work dating back to the 20th century. Studies first appearing in the 1960s included assays examining the binding of cancer cells to plant-derived lectins which were specific for particular sugars or small groups of sugars [15, 16]. Later investigations utilized monoclonal antibodies raised against human tumor cell lines or primary material. These studies revealed drastic differences in the glycan expression of neoplastic cells as compared to the glycans within or surfacing the corresponding normal cells. These glycan changes were observed in cell lines derived from many adenocarcinomas, certain melanomas and some leu-

kemias and lymphomas. The broad scope of this phenomenon was noted by Hakomori in 1984, in a comprehensive review which introduced the concept of the “tumor-associated carbohydrate antigen,” or TACA [17]. He summarized the then-known changes, both quantitative and qualitative, in cell surface glycolipids and glycoproteins of transformed cells in comparison to their non-transformed counterparts. As results of further studies accumulated over the next decade, it became apparent that the TACAs were not merely adornments of oncogenic transformation, but were in fact active participants in the cellular recognition, adhesion and signaling activities of transformed cells. In a 1996 seminal paper, “Tumor Malignancy Defined by Aberrant Glycosylation and Sphingo (glyco)lipid Metabolism,” Hakomori proposed that “aberrant glycosylation, expressed in glycosphingolipids and glycoproteins in tumor cells, is implicated as an essential mechanism in defining stage, direction, and fate of tumor progression [18].” In 2002, Hakomori further proposed the concept of the glycosynapse to encompass the adhesion/signaling duality of many cell-cell interactions in which glycans are essential [19]. The information-carrying capacity of glycans came to be appreciated in this timeframe as was the corresponding role of lectins in deciphering this information [20]. By 2010, glycans had been shown to be intrinsically important in the pathobiology of most of the common human malignancies [21].

The central role of selectins in the inflammatory cascade

The selectins are a specialized class of cell surface lectins which function as vascular cell adhesion/signaling molecules in normal human physiology. These lectins are found in humans on endothelial cells, leukocytes and platelets [22]. Selectins bind to “natural ligands” expressed on cell surface glycoproteins, and most of these ligands contain fucose incorporated in the form of Lewis antigens. The selectins and their ligands are known components of the “inflammatory cascade” model which was initially proposed to account for leukocyte-endothelial and leukocyte-platelet interactions in inflammation and is generally accepted as accurate [23].

At sites of inflammation, infection or vascular injury, local proinflammatory or pathogen-

derived stimuli render the luminal vascular endothelial surface attractive for leukocytes. This innate immune response consists of a well defined and regulated *multistep cascade* involving consecutive steps of adhesive interactions between the leukocytes and the endothelium. According to this model [23], selectins and their ligands are critical in the initial weak “tethering” and “rolling” adhesion of leukocytes to the endothelium. This step enables the subsequent firm adhesion of leukocytes to endothelial cells and, thereafter, leukocyte transmigration from blood vessels into tissues. Platelets are also involved in the immune response via selectins.

Studies of the three best characterized selectins had progressed independently prior to 1989. These cell surface molecules had early on been known to be lectins, but were designated by multiple and often confusing names. In 1989, structures of these 3 lectins were published nearly simultaneously [24]. This led to the realization that all 3 were genetically related and possessed similar structural features, including a specific “carbohydrate recognition domain.” In the interest of clarity the “selectin” nomenclature was introduced in 1991 [25], designating the 3 types of lectins according to the cells in which they were discovered, i.e., E-selectin on endothelial cells in blood vessels, L-selectin on leukocytes, and P-selectin on platelets (P-selectin is now known to be expressed on endothelial cells as well). Meanwhile, 5 different groups independently showed that sialyl Lewis X was the critical determinant of E-selectin ligand activity [26]. Interestingly, sialyl Lewis X had first been described as a tumor-associated carbohydrate antigen nearly a decade earlier [27]. One of the first reviews of naturally-occurring selectin ligands reported that many of these ligands contained fucose at the non-reducing ends of glycans on glycoproteins or on glycolipids [28]. Sialyl Lewis X was recognized by all three selectins, as were certain other lactosamine variants of sialyl Lewis X and sialyl Lewis A. There were initial reservations as to which of the many molecules with selectin-binding activity were the “real” biologically-relevant selectin ligands *in vivo* [29]. Subsequent studies with dynamic flow models *in vitro* and with animal models *in vivo* confirmed the identities of the biological selectin ligands. In 2008, an immunoregulatory role for alpha-fucosidase was proposed based on *in vitro* and *in vivo* models of leukocyte migration in inflammation [30]. It is

now established that selectins and their ligands are essential, rate-limiting components in the recruitment of circulating blood cells to sites of inflammation.

Fucose in cancer and the role of the metastatic cascade

The first description of Lewis blood group antigens was published in 1947 [31]. In the early 1950's the Lewis A "substance" was isolated and was shown to include fucose as one of its constituent sugars [32]. Other Lewis antigens were later characterized, and fucose was shown to be the immunodominant sugar in each of the fucosylated Lewis antigens and in the H antigen [33, 34]. In 1964, investigators detected fucose embedded with a lipid derived from human gastric and bronchogenic adenocarcinomas [35]. In 1971, a glycolipid from a "carcinoma" was shown to contain lacto-N-fucopentaose III, which is now known to contain the Lewis X antigen [36]. From this the term "fucolipid" was derived to describe this new class of tumor-associated molecules. Ironically, plant lectins specific for fucose including *Lotus tetragonolobus* and *Ulex europaeus* variants were used to demonstrate fucose on malignant cells.

The sialylated Lewis antigens, sialyl Lewis A and sialyl Lewis X, were first identified as tumor-associated carbohydrate antigens [37, 38]. The suggestion was made that such carbohydrate structures on glycolipids and glycoproteins were "oncodevelopmental antigens." [39]. Hakomori in 1992 argued that Lewis X and related structures were adhesion molecules, raising the possibility that cancer cells could adhere to selectins [40]. The following year, Kannagi's group showed that sialyl Lewis X and sialyl Lewis A supported adhesion of multiple types of transformed cells to vascular endothelium, including carcinomas of colon, lung, pancreas, liver and stomach as well as a teratocarcinoma and 3 leukemias [41]. The authors proposed that sialyl Lewis A and sialyl Lewis X were critical in adhesion of tumor cells to endothelial cells. The concentration of Lewis antigens at the invading front was shown in clinical material from human colon cancers to also be critical [42].

The 1990's showed remarkable progress in carbohydrate biology relevant to cancer. Abnormal expression of Lewis antigens and other fucosylated cell surface molecules was documented in

human carcinomas of multiple primary sites and in Hodgkin lymphoma, melanomas and certain leukemias. In 1998, based on our review of these studies, we proposed that fucose was a potentially critical molecule in neoplasia, including those of the breast [2].

The 1990's also brought refinement of the "metastatic cascade" concept, i.e., the presumed sequence of events by which epithelial derived tumor cells establish metastases via a hematogenous pathway. A condensed description can be summarized as follows: Neoplastic cells undergo sequential genetic and epigenetic events which allow them to survive growth, invasion through basement membranes, intravasation into the blood stream, transport to a distant site, arrest in the vascular wall, extravasation through the vessel into surrounding parenchyma, and growth at a distant site [43]. Multiple workers took note of the similarity of the metastatic cascade to the inflammatory cascade, and hypothesized that circulating tumor cells might make use of the selectins as part of the arrest, extravasation and invasion processes. The demonstration of possible selectin ligands in many cancers suggested the feasibility of this conjecture [44].

In addition to their cell adhesion properties, selectins have been shown to possess signal transduction capabilities [45]. In 1998, Lorenzon and colleagues showed that leukocyte adherence to endothelial cells resulted in endothelial cell activation, and that this effect could be blocked by monoclonal antibodies against selectins or sialyl Lewis X [46]. Ligation of selectins by fucosylated selectin ligands was soon shown to have several effects: initiation of intracellular signaling within endothelial cells, regulation of cell-cell interactions, and even activation of signaling pathways within leukocytes which presented the ligands to the selectins [47]. One of the "glycosynapses" defined by Hakomori holds that binding of certain selectin ligands to selectins (e.g. an E-selectin ligand binding to E-selectin) triggers the activation of signaling pathways, thereby leading to phenotypic changes in both the signaling cell and the signal-receiving cell *in vivo* [19].

In 2001, a human hematopoietic cell CD44 glycosylation variant was shown to be a physiological ligand for E-selectin [48]. Also in 2001, we proposed that ablation of fucose on cancer cells

was a potential therapeutic strategy for human malignant neoplasms [49]. In 2004 the fucose-generating FX enzyme was shown to control the adhesive properties of colon cancer cells [50]. In 2005 CD44 was demonstrated to be an E-selectin ligand expressed on human neutrophils [51]. In the same year it was shown experimentally that sialyl Lewis A and sialyl Lewis X function in cancer cell adhesion and probably also in cell signaling to promote angiogenesis [52]. In 2007, another group showed that CD44 modified with sialyl Lewis X on colon cancer cell lines could ligate all 3 key selectins [53]. Also in 2007, a paper from Eastern Europe summarized the role of fucosylation of glycoconjugates in health and disease [54].

In 2008, several review articles provided forceful generalization about selectins and their ligands in cancer. Nakahara and Raz reviewed the role of lectins and their ligands in tumor progression and metastasis [55]. Witz reviewed the evidence that selectins and their ligands were central components in carcinoma metastasis, and he labeled the selectin - selectin ligand interaction an "axis of evil" in cancer [56]. Other authors independently concluded that E-selectin regulates tumor cell migration under flow conditions, and that extravasation of leukocytes in comparison to tumor cells shared several of the same major steps [57]. A 2008 review reported on "The Biological Function of Fucosylation in Cancer Biology" with specific attention to core fucosylation [58]. In 2009 the paper "Alpha 1,3 fucosyltransferases are master regulators of prostate cancer cell trafficking" was published, based on *in vivo* models of bone marrow endothelium binding and inflamed post-capillary venule binding. CD44 was one of the proven scaffold proteins decorated with sialyl Lewis X [59]. Finally, the generalization that "selectins promote tumor metastasis" was advanced in a 2010 review article with that title [60]. Thus, the role of particular Lewis antigens in metastasis has gained broad acceptance among researchers in cancer metastasis.

The role of fucose in the breast cancer phenotype, invasion and metastasis: evidence

In 1988, an NMR spectroscopy group found that a rat mammary carcinoma's metastatic potential *in vivo* was nearly abolished by enzymatic removal of fucose from the cells before tail vein injection in a rat model of metastasis.

This was an early indication of the direct importance of fucose in breast cancer [61]. In 1991, the fucose-containing CD15 antigen (Lewis X) was found to be expressed on 3 human breast cancer cell lines (MCF-7, SK-BR-3 and BT-20). The antigen was detected by the PM-81 monoclonal antibody, which was then under consideration as a bone-marrow purging agent [62]. This was followed in 1994 with evidence that the Lewis Y antigen was expressed on the invasive 3396 human breast cancer cell line, as shown by the BR96 anti-Lewis Y monoclonal antibody. The Lewis Y was highly enriched on cell surface structures ("microspikes" and "ruffled membranes") which were involved in cell locomotion [63]. The BR96 antibody was a potent inhibitor of cancer cell migration *in vitro*, suggesting that Lewis Y had a role in breast cancer cell motility.

In 1996, Kannagi's group showed that human breast cancer cells adhered to E-selectin on vascular endothelial cells, and that sialyl Lewis X expression on the cancer cells was required to mediate the adhesion. Six human cancer cell lines were used in these studies (MCF-7, T-47D, ZR-75-1, MDA-MB-231, BT-20, SK-BR-3). The authors proposed that sialyl Lewis X was a cell adhesion molecule involved in the metastatic spread of human breast cancers [64]. The following year, a study from Finland examined surgical specimens from 12 patients with invasive breast cancers and metastatic lesions in axillary lymph nodes. The primary breast cancers showed high expression of sialyl Lewis X and sialyl Lewis A in tumor epithelium and endothelium, and higher levels of one of both antigens in lymph node metastases in 9 of the 12 cases. Interestingly, E-selectin expression was found to be elevated in primary breast tumor endothelium. The authors interpreted these findings as evidence that breast cancer cells expressing sialyl Lewis X and sialyl Lewis A had an increased ability to form new metastases [65]. In 1998, a monoclonal antibody directed against human breast cancers, FC-2.15, was shown to recognize Lewis X [66]. Also, the human breast cancer line MCF-7 was found to express the gene for the fucosyltransferase enzyme that completes the synthesis of sialyl Lewis X [67].

Clinical-pathological studies continued to suggest a relationship between invasive breast cancer prognosis and overexpression of fucosylated Lewis antigens. A 2000 study of 43 patients

showed an association of sialyl Lewis X and Lewis B expression with metastatic disease, and an association of Lewis Y expression with stage T4 disease [68]. A 2002 study of 87 radical mastectomy patients showed that expression of sialyl Lewis X was associated with lymph node metastasis and shorter survival times [69]. A 2005 study of 660 patients with operable invasive breast cancer was carried out with a monoclonal antibody that recognized either of the fucosylated antigens Lewis Y or Lewis B [70]. High expression of Lewis Y/B was found more often in tumors of higher grade and poor prognosis, and overexpression of Lewis Y/B in node-negative cancers was associated with significantly decreased patient survival.

The last 6 years have brought further evidence for the role of fucosylated glycans in cultured human breast cancer cell lines *in vitro*. Most of these studies include the triple-negative, basal type, highly metastatic MDA-MB-231 human breast cancer cell line. A 2006 study of the glycan profile of MDA-MB-231 cells showed that the enzymes for synthesis of sialyl Lewis X including fucosyltransferases were expressed [71]. In 2007, Lewis X was shown to mediate adhesion of MCF-7 breast cancer cells to activated endothelial cells [72]. The authors proposed that a newly described scavenger-type C-type lectin (SRCL), expressed on endothelial cells, might be partly responsible for binding to Lewis X on MCF-7 cells. The same group later showed that CD98hc is the major carrier of Lewis X and Lewis Y on MCF-7 cells [73]. ErbB2 receptors have been shown to carry Lewis Y antigens in the HER2 positive SK-BR-3 human breast cancer line; treatment with an anti-Lewis Y antibody results in growth inhibition and down-regulation of ErbB2 expression on SK-BR-3 cells [74].

Between 2008 and 2011, 5 papers showed additional direct evidence of the role of fucosylated ligands in human breast cancer, particularly as expressed on CD44 variants. One group examined the ability of metastatic breast cancer cells to carry out transendothelial migration *in vitro*. This group found that the metastatic MDA-MB-231, MDA-MB-435 and MDA-MB-468 breast cancer cells transmigrated across endothelial cell monolayers in a manner that depended on E-selectin. The non-metastatic breast cancer cells MCF-7 and T47D showed only minimal invasion. The metastatic cells expressed CD44

while the non-metastatic cells expressed little CD44 [75]. The authors showed that the invasive ability of the metastatic cells required the expression of an E-selectin binding glycan, sialyl Lewis X, as a decoration of the cell surface CD44 splice variant 4, i.e., CD44v4. This study also showed that the selectin ligands on MDA-MB-231 cells were localized to the migrating front of the cell in "spiking" cellular protrusions. This finding was reminiscent of the 1994 study which showed that Lewis Y antigens were concentrated on microspike and ruffle structures of invading 3396 breast cancer cells [63].

A paper from our group showed alterations in human breast cancer adhesion-motility in response to changes in cell surface glycoproteins displaying alpha-L-fucose moieties [76]. This study used the highly metastatic MDA-MB-231 cell line as well as MCF-7 and T47D breast cancer lines. Studies were carried out before and after treatment of cells with alpha-L-fucosidase (EC 3.2.1.51), which cleaves fucose bonded to glycolipids and glycoproteins. Tests included flow cytometry to assess antigen expression as well as assays of proliferation and of extracellular matrix invasion. Fucosidase treatment reduced the expression of Lewis X, Lewis Y and CD44; the last was suspected to be a carrier of Lewis antigens. Fucosidase treatment did not impair cancer cell viability or proliferation under the conditions employed in these experiments. Fucosidase treatment markedly reduced the invasion by MDA-MB-231 cells into a complex all-human extracellular matrix material (HuBiogel). Invasion was also reduced by treatment with the *Ulex europaeus* lectin (which recognizes fucose in the Lewis Y antigen or H blood group). When treatment included a fucosidase inhibitor along with fucosidase, the invasion of the ECM by breast cancer cells was largely restored. Inhibitors of glycosylation also reduced the ability of MDA-MB-231 cells to invade the ECM. Interestingly, fucosidase treatment led to a reduction in activity of matrix metalloproteinase 9 (MMP-9) which is secreted by breast cancer cells and functions to degrade extracellular matrix material.

A second paper from our group further studied the effects of defucosylation of breast cancer cells [77]. Tests included assays of static adhesion to purified ECM components (fibronectin, laminin, hyaluronic acid, Type I collagen) and human umbilical vein endothelial cells (HUVECs)

as well as to Hubiogel human ECM material. Static adhesion by MDA-MB-231 breast cancer cells was significantly reduced in all of these assays by fucosidase treatment. Antibodies to sialyl Lewis X and CD44 both reduced the adhesion of tumor cells to HuBiogel and the ECM component hyaluronic acid. Reduction in cell surface sialyl Lewis X was also significantly decreased as documented by flow cytometry.

A test of dynamic tethering and rolling of breast cancer cells on HUVEC endothelium was then carried out at 3 different physiological rates of shear stress. For all 3 shear stress conditions, fucosidase treatment caused reductions of the number of rolling MDA-MB-231 cells. These effects were greater when the duration of MDA-MB-231 cell incubation with fucosidase was increased from 30 min to 60 min. Flow chamber assays showed that fucosidase treatment led to decreased binding of cells to purified E-selectin, P-selectin and ICAM-1. Additionally, immunohistochemistry of MDA-MB-231 cells showed colocalization of fucose with β 1 integrin, which was markedly reduced by fucosidase treatment. Taken together, results from our laboratory showed that fucosidase caused extensive changes in the glycan phenotype of MDA-MB-231 cells, with significant reductions in adhesive and invasive properties.

A 2010 study showed that CD44 is in fact a carrier of the fucosylated H2 antigen (CD173) and the Lewis Y antigen (CD174) on 3 breast adenocarcinoma cell lines: MDA-MB-231, MDA-MB-435, and MCF-7 [78]. Surgical specimens from 15 patients also showed frequent expression of CD173 and CD174. Of those cells that expressed these 2 fucosylated antigens, more than 95% co-expressed CD44. The authors concluded that “fucosylated histo-blood group antigens are markers of breast cancer-initiating cells.”

A recent (2011) paper identified membrane glycolipids (specific gangliosides) as E-selectin ligands on 2 human breast cancer cell lines: BT-20 and MDA-MB-468 [79]. The cells' E-selectin binding abilities were proven by the measuring of the tethering and rolling of breast cancer cells under physiological flow conditions, using a dynamic shear stress model with activated HUVEC endothelium. Extracted membrane glycolipids from the 2 breast cancer cells were immobilized on Petri dishes, and HUVECs then

were perfused over the lipid spots, confirming that the glycolipids did in fact bind E-selectin on endothelial cells. In the case of BT-20 cells, the glycolipid ligands expressed sialyl Lewis X and sialyl Lewis A as verified by specific antibody testing. In the case of MDA-MB-468, the E-selectin glycolipid ligands were sialylated but did not carry sialyl Lewis X as expressed on the known glycoprotein E-selectin ligands they tested (PSGL-1, CD43, CD66 and PCLP). The authors postulated that novel, as-yet-unidentified sialylated gangliosides account for the E-selectin binding activity in their assay. Of note, the authors did not test for CD44v4 but report that such studies were in progress in their laboratory.

Thus as of early 2011, there is clear evidence for the involvement of fucose, as incorporated into Lewis antigens, in the phenotypic, invasive and metastatic properties of multiple human breast cancer cells *in vitro*. Multiple well-characterized breast cancer cell lines express these antigens, and sialyl Lewis X is particularly associated with the more invasive and metastatic cell types. CD44, a putative marker of breast cancer stem cells, has been shown to function as a scaffold for sialyl Lewis X in several of the cell lines. Clinical studies with patient specimens have provided evidence that overexpression of these fucosylated Lewis antigens is correlated with metastasis and poorer patient prognosis.

The role of fucose in Notch signaling in breast cancer

Notch signaling controls many cell fate decisions during embryonic life, including laterality and segmentation, and is also critical for maintaining homeostasis in mature cells. The Notch signaling cascade is highly conserved throughout metazoan biology and appears to have originated at the time of the “Cambrian Explosion” in evolution of body morphology and complexity [80-82]. Insects and vertebrates share many of the same Notch cascade components, so that knowledge gained from insect models has improved the understanding of Notch signaling in mammals including humans. The “Notch mutation” was first detected in *Drosophila melanogaster* nearly a century ago, but the complex Notch signaling system of receptors, ligands, successive proteolysis and crosstalk

has only been understood in the past 2 decades [83]. In parallel with these modern discoveries, abnormal Notch signaling has been detected in multiple human cancers. The most studied example is T-cell acute lymphoblastic leukemia (T-cell ALL) [84]. More recently, aberrant Notch signaling has been implicated in cancers of the colon and rectum [85], stomach [86], liver, [87], lung [88], prostate, bladder and kidney [89] and skin [90], as well as in gliomas [91] and several pediatric malignancies [92]. In view of these findings, it is not surprising that pathological Notch signaling has been documented in human breast cancers [93, 94].

Abnormal Notch signaling in breast cancer is mediated by overexpression of either Notch ligands and/or their receptors, or alternatively, underexpression of the native Notch inhibitor designated as “Numb” [95]. Importantly, abnormal Notch signaling in breast cancer is *not* due to a constitutively activated Notch receptor (as has been demonstrated in T-cell ALL) [96]. Assays conducted on surgical breast cancer specimens and human breast cancer cell lines have shown elevation of Notch ligands and receptors, as well as molecular evidence of Notch signaling, in aggressive breast cancer variants [97, 98]. The pertinent molecules include the “canonical” Notch ligands, especially Jagged1 (JAG1); Notch receptors, predominantly Notch1 and Notch4; and “downstream” target molecules including Hes, Hey, Slug, Cyclin D and cleaved Notch intracellular domain [99, 100].

Fucosylation of the Notch receptor’s extracellular domain is a critical step in constructing the functional Notch receptor [101, 102]. Each Notch extracellular domain, or ECD, contains many (29 to 36) Epidermal Growth Factor-like (EGF-like) repeats with consensus motifs suitable for fucosylation or glycosylation [103]. The enzyme protein-O-fucosyltransferase 1 (Pofut1) carries out the direct addition of fucose to EGF-like repeats on the Notch ECD. The attached fucose then serves as the substrate for synthesis of short but functionally important oligosaccharides. This process, referred to as elongation, is initiated by the Fringe enzyme, which adds N-acetylglucosamine to fucose that is bonded to the EGF-like repeats [104]. The mature glycoforms of the Notch ECD are then able to respond to Notch ligands of the Jagged and Delta classes. Mammalian cells which lack Pofut1 will stably express cell surface Notch receptors but, in almost all cases, the ECDs do not

bind Notch ligands, or they may transduce Notch signals weakly or not at all. Mammalian Notch receptors require fucose for optimal sensitivity to Notch ligands. Although the presence of fucose in breast cancer Notch receptors has not yet been evaluated directly, an absence of fucose would be fundamentally unprecedented in this highly conserved signaling system. It, thus, seems reasonable to expect that fucose is required for optimal Notch signaling in breast cancer.

Multiple competing groups have studied Notch signaling in breast cancers with aggressive phenotypes or poor prognosis. These workers have shown direct causal relationships or strong associations between abnormal Notch signaling and specific malignant characteristics in these breast cancers. Pathological Notch signaling in breast cancer, which we now presume to be regulated by fucose, has been linked with:

1. Emergence of the basal-like breast cancer subtype, increased risk of recurrence in node-negative patients, and poor overall survival [105, 106];
2. Governance of breast cancer stem cell activity including self-renewal, maintenance of stem cell characteristics, and enrichment of breast cancer cell populations with stem cells [107-111], and conversely, reduction of the stem-cell-like population when Notch signaling is inhibited [112];
3. Association with the “triple-negative” phenotype and induction of the epithelial-to-mesenchymal transition [113-115];
4. Transcription of genes leading to uPA elevation [116];
5. Regulation of tumor angiogenesis, and in particular, multiple components of the VEGFR system [117, 118];
6. Transcription of the Myc oncogene, co-expression of Notch1 with Myc in a significant fraction of human breast cancers studied, and co-expression of Notch3 with Myc in a human cell model of inflammatory breast carcinoma [119-121];
7. Initiation and maintenance of osteolytic bone metastases in breast cancer [122-124];

8. Promotion of chemoresistance and prevention of apoptosis [125, 126]

9. Oncogenic crosstalk with other developmental signaling cascades including Wnt and Hedgehog, and with oncogenic kinases and transcription factors including Ras, PI-3k/Akt, mTOR and NF- κ B [127-129];

10. Transcription of genes leading to HER-2 elevation [130, 131]. Korkaya and Wicha have, in fact, proposed that Notch and HER-2 in breast cancer stem cells constitute an “axis of evil,” suggesting that abnormal Notch signaling, overexpression of HER-2 and maintenance of breast cancer stem cell characteristics may be different but related components of the same underlying process [132].

Pathological Notch signaling has been implicated in multiple other human diseases including spondylocostal dysostosis, Alagille Syndrome, CADASIL syndrome, multiple sclerosis, and, as noted above, T-cell ALL and numerous cancers of solid organ origin [133-135]. Several glycobiochemists, having ascertained the importance of fucose control over Notch signaling in these diseases, have recommended the pharmacological therapeutic targeting of Pofut1 and Fringe – the enzymes, as noted above, which respectively fucosylate Notch and elongate fucose after its attachment to Notch. Given the detrimental effects of Notch signaling in breast cancer, and the expected role of fucose in modulating these effects, we suggest that fucose-directed interventions might offer new therapeutic options for human breast cancer, especially for treatment-resistant variants.

Fucose as a biomarker for human breast cancer

With the advent of personalized medicine and individualized breast cancer therapy, the era of “one size fits all” treatment is drawing to a close. It is envisioned that molecular markers from an individual breast cancer patient will enable a more refined assessment of that patient’s disease. The ideal cancer biomarkers would have clinical utility for assessing factors such as individual prognosis, predicted response to therapy, pretherapeutic assessment of treatment toxicity and quality of life, all at a cost which is sustainable in the long term. Despite intense research efforts, documented in thousands of publications, the ideal breast can-

cer biomarker has yet to be identified. In a 2007 Special Article, the American Society of Clinical Oncology (ASCO) determined that only 8 molecules (and certain gene expression assays) met their criteria for useful tumor markers in breast cancer [136]. Clearly, there is a need for reliable new tests to aid in formulating treatment plans and to inform patient counseling in these decisions.

Proposed biomarker strategies for breast cancer include multigene parameter screens, circulating tumor cell identifiers, circulating cell-free DNA and microRNA analyses, and systems biological approaches, to name but a few [137]. With rare exceptions, the possible role of glycan biomarkers in breast cancer has been overlooked [138]. As we have, here, documented the extensive role of fucose in human breast cancer, it is reasonable to ask whether fucose may be directly or indirectly attached to molecules with biomarker significance. There are, in fact, specific molecules with the potential to fulfill the ASCO criteria for tumor markers in human breast cancer. In some cases the fucosylated molecules themselves are candidate biomarkers. In other cases the candidate biomarkers are “accessory molecules” to fucose, having functions in signaling, adhesion, metabolism or antibody detection of fucose, but lacking fucose themselves. Properties of fucose-related biomarkers include: association with a basal cell phenotype, local recurrence, distant metastasis, cancer-initiating cells, progression-free survival, and sensitivity to trastuzumab therapy as well as response to other forms of treatment.

We have categorized these candidate biomarkers into 5 groups, in ascending order of the technical complexity required for their detection.

1. Small oligosaccharide glycans which contain fucose. These glycans were discussed previously and their structures are listed in **Table 1**. To recapitulate, these include the Type II structures Lewis X, sialyl Lewis X and Lewis Y, as well as the Type I structures Lewis A, sialyl Lewis A and Lewis B. Other fucosylated oligosaccharide glycans include the H blood group structures, Lacto-N-Fucopentaose III and the Globo H antigen.

2. Cell surface glycoproteins which are known or suspected to serve as scaffolds for decoration by fucose or Lewis antigens. These glycopro-

teins include CD44, CD98hc, Notch receptors, CD147 and podocalyxin.

(a) CD44 is considered to be a marker of breast cancer and, most recently, a marker of breast cancer stem cells (CSCs) [139]. There is prior evidence from other systems in which CD44 is decorated by sialyl Lewis X antigens; these decorated glycoforms are important selectin ligands in normal leukocyte physiology [51] and have been identified on the human prostate carcinoma cell line PC3 [59] and the human colon carcinoma cell line LS174T [140]. The fucosylation status of CD44 in breast cancer is therefore of particular interest.

Several studies have in fact demonstrated fucosylation of CD44 in breast cancer cell lines. Fucosylation of standard CD44 (CD44s) and CD44v4 has been documented in the aggressive, triple-negative metastatic breast cancer cell lines MDA-MB-231, MDA-MB-435 and MDA-MB-468 [75-77]. These *in vitro* studies suggest that fucosylation of CD44 is required for invasion and metastasis. Furthermore, CD44 has recently been shown to be a carrier of 2 additional fucosylated antigens – the H2 antigen (CD173) and Lewis Y (CD174) – in the human breast cancer cell lines, MDA-MB-231, MDA-MB-435 and MCF-7, and in human tumor samples from multiple patients [78]. The authors of this last study concluded that H2 and Lewis Y, carried on CD44, are markers of cancer initiating cells. Based on these findings, it is tempting to speculate that CD44 glycoforms in breast cancer stem cells might, in general, be decorated with fucose-bearing glycans such as sialyl Lewis X.

(b) The CD98 heavy chain, CD98hc, is a type II transmembrane glycoprotein with a molecular weight of about 82 kDa. CD98hc is also known as SLC3A2 and 4F2 (or 4F2hc) antigen. CD98hc has two known functions. First, CD98hc can associate with certain integrins that regulate their own functions [141], and second, CD98hc can form a complex with the catalytic CD98 light chain (LAT1) to generate the functional “system L,” which carries out sodium-independent transport of neutral amino acids across cell membranes. Inhibition of system L inhibits growth *in vitro* of the human breast cancer cell lines MCF-7, ZR-75-1 and MDA-MB-231 [142].

CD98hc has been proposed as a biomarker of

human breast cancer [143] based on tissue microarray studies with material from 245 patients with invasive breast cancer. In 2009, CD98hc was shown to be a major carrier of Lewis X antigens in the human breast cancer cell lines MCF7 and SK-BR-3. There was relative paucity of Lewis X antigens on CD98hc from T47D and ZR-75-1 cells and almost no Lewis X expression on CD98hc from MDA-MB-231 cells [73]. The authors postulated that Lewis X-decorated CD98hc might serve as a ligand for the recently described endothelial lectins of the scavenger receptor class, thus supporting metastasis by breast cancer cells. Parenthetically, CD98hc has also been shown to be a major scaffold for Lewis X (CD15) on Reed-Sternberg cells in all human Hodgkin's lymphoma cell lines tested to date [144].

(c) Notch1 and Notch4 receptors have been proposed as human breast cancer biomarkers for diagnosis and prognosis [97, 145, 146]. Notch extracellular domains have been shown to be polyfucosylated in mammalian cells. As noted earlier, while Notch fucosylation has not yet been tested in breast cancer cell lines or patient material, it is overwhelmingly likely that fucose, in fact, is present. We also suspect CD147 and podocalyxin to be potentially fucosylated biomarkers in breast cancer for the reasons given below, in part because each one is a member of a molecular family that has been shown to carry fucosylated Lewis antigens.

(d) CD147 is a widely expressed, multifunctional, highly glycosylated transmembrane protein with Ig superfamily properties. This molecule, first termed “basigin,” was cloned as a carrier of Lewis X from teratocarcinoma tissue [147]. In 1993, basigin was assigned the CD number 147 [148]. A search of GenBank has shown identical coding sequences for CD147, basigin, HAB18G, and EMMPRIN [149]. Thus, the different designations of these *proteins* are regarded as synonymous, although different *glycoforms* are possible. For simplicity we will refer to this molecule as CD147.

CD147 is believed to promote malignant progression in multiple human cancers [150, 151]. As far back as 2008, it was asserted that CD147 was “a novel universal cancer biomarker for diagnosis and prognosis [152].” The role of CD147 in breast cancer has been specifically considered. A 2004 study of 1,816 human

breast cancer tumor samples showed that CD147 expression was significantly correlated with poor histological grade, mitotic index, tumor size and tumor-specific survival in human breast cancer. These findings thus suggested a crucial role of CD147 in breast cancer progression [153]. In a study of 1,117 human breast cancers, CD147 expression was positive in over 67% of tumor tissue samples, compared with 5.2 % in normal epithelial tissues [14]. More recently, CD147 was studied by immunohistochemistry in 1,637 breast tissue samples, and selected cases were studied by *in situ* hybridization [154]. Overexpression of CD147 was associated with local recurrence, distant metastases, reduced disease-progression free survival and shorter overall survival.

In view of the biomarker potential of CD147, its glycosylation status is relevant. Different CD147 glycation isoforms have been shown in breast cancer cell lines in which CD147 is a carrier of Lewis X glycan structures (MCF-7) or is not a carrier (MDA-MB-231) [155]. CD147 has been shown to be an E-selectin ligand in neutrophils [156]. It seems reasonable to suspect that CD147 may be a carrier of Lewis X in breast cancer. Further studies with different breast cancer cell lines and human tissue specimens are needed to confirm this suspicion.

(e) Podocalyxin was first detected in the podocyte cell glycocalyx within normal renal calyces [157] but has since been shown to be present in other tissues, including luminal breast epithelial cells [158]. Podocalyxin, a 140 kDa glycoprotein, belongs to a family of sialomucin transmembrane glycoproteins which includes podocalyxin, the closely-related Podocalyxin-Like Protein (PCLP) [159], endoglycan and CD34. Endoglycan was demonstrated to be a carrier of sialyl Lewis X and a functional ligand for vascular selectins *in vitro* [160, 161]. CD34 is a well-characterized L-selectin ligand [162]. Podocalyxin and PCLP have been shown to have selectin-binding activity in models other than breast cancer [163, 164].

Podocalyxin and PCLP have been recognized as potential biomarkers in breast cancer [165] and other human cancers [166]. Podocalyxin-like protein is a major scaffold for sialyl Lewis X in human prostate cancer PC3 cells and is considered a candidate E-selectin ligand [59]. Results with LS174T colon carcinoma cells show that

cell surface expressed PCLP is a carrier of sialyl Lewis X and is a functional E-selectin ligand [167]. These precedents suggest that podocalyxin in breast cancer may be decorated with sialyl Lewis X. Human breast cancer podocalyxin has not yet been tested for expression of Lewis antigens or fucose.

Our laboratory has employed a broad-spectrum fucosidase treatment in a nontoxic and reversible process for removing fucose from the surface of viable human breast cancer cells [76, 77]. The method was adapted from an earlier paper by Holmes *et al.* [61]. Fucosidase treatment has been used to test the importance of cell surface fucose using *in vitro* and *in vivo* models. Decisive effects on small glycan antigens and several cell surface carrier glycoproteins were documented. This treatment could be employed in a variety of breast cancer models systems in order to provide a prompt indication of fucose's functional role in those systems. In our experience, testable hypotheses and predictions were readily assessed and expeditious. For example, in our studies with MDA-MB-231 cells, fucosidase treatment was seen to impair cell adhesion, invasion, metastasis and binding of anti-CD44 antibodies, without affecting viability, proliferation or ability to synthesize and replenish fucosylated cell surface antigens. Results are to date unknown in regard to resistance to treatment, and could not be predicted readily, since fucosyltransferase activity has been linked to chemotherapy resistance in other models systems [168-170].

3. Accessory molecules which are related indirectly to fucose-expressing molecules in breast cancer. These include JAG1, sE-selectin, serum alpha-L-fucosidase and circulating anti-Globo H antibodies.

(a) JAG1: The Jagged-1 (JAG1) Notch ligand binds to the fucosylated Notch receptor extracellular domain, thus activating the Notch signaling cascade with resulting synthesis of downstream targets. Overexpression of JAG1 in human breast cancer is an independent prognostic indicator associated with basal phenotype, recurrence in lymph node-negative breast cancer and poor median survival and 10-year survival [97, 105].

(b) sE-selectin: E-selectin is the endothelial receptor for natural selectin ligands; native E-

selectin ligands contain the fucosylated sialyl Lewis X oligosaccharide [171]. Breast cancer cells are known to trigger neosynthesis of E-selectin via release of stimulatory growth factors. Under such stimulation, endothelial cells also synthesize a soluble form of the E-selectin molecule, sE-selectin, which is shed into the circulation [172]. Levels of circulating sE-selectin have been shown to rise during progression of invasive breast cancer [173, 174]. Conversely, sE-selectin has been shown to decrease in parallel with effective chemotherapy [175]. Thus, sE-selectin has been utilized in clinical trials as a surrogate treatment marker in patients with breast cancer [176].

(c) Serum alpha-L-fucosidase: In 2009, a study was carried out to identify predictive biomarkers for response to trastuzumab. Twenty-four patients with HER2-positive breast cancers were selected to undergo trastuzumab monotherapy. Plasma samples were analyzed with mass-spectrometric techniques [177]. This study found that a higher plasma fucosidase (FUCA) activity level was correlated with a favorable progression-free survival. The authors concluded that elevated FUCA activity may be a predictive biomarker of sensitivity to trastuzumab therapy.

(d) Circulating plasma antibodies to Globo H: Glycan microarrays have been developed which detect attomolar levels of anti-glycan antibodies in human plasma [178]. Investigators employed the microarrays to study plasma samples of 58 breast cancer patients and 47 healthy blood donors, aiming to detect antibodies that bound to Globo H or its truncated fragments [179]. A very significant difference in anti-Globo H levels between patient and normal plasma samples was shown. The authors propose that the Globo H microarray may serve as a new platform for early detection of breast cancer and to monitor the immune response to carbohydrate epitopes after vaccine therapy or during the course of cancer progression.

4. Serum glycan profiles. Advanced glycobiology techniques have shown differences between serum glycan profiles of breast cancer patients and those of normal control subjects. A relatively restricted number of circulating proteins show increased fucosylation in breast cancer patients, with apparent relationship to the clinical stage.

(a) A 2008 study by Kyselova and others used mass spectrometry methods to generate “glycomic maps” in order to identify sets of glycans associated with malignant transformation in breast cancer [180]. Six human cell lines (2 invasive, 4 noninvasive) were employed initially. Specific glycoproteins of interest were identified. Thereafter, serum samples were collected from 82 breast cancer patients (stages I-IV) and 27 disease-free volunteers and their serum glycomic maps were constructed and compared. The differences in glycomic maps revealed substantial increases of fucosylation in relation to malignant transformation.

(b) Another 2008 study described “a strategy to reveal potential glycan markers from serum glycoproteins associated with breast cancer progression [181].” The technique coupled high-performance liquid chromatography (HPLC) with mass spectrometry to evaluate N-glycans of total serum glycoproteins from human subjects. Serum samples from 18 patients with stage IV breast cancer and 18 cancer-free controls were analyzed, with longitudinal followup of 10 of the patients. Sialyl Lewis X and other fucosylated glycans were identified on several glycoproteins. The authors concluded that increased fucosylation was the most significant change observed in breast cancer patient sera, in agreement with the study of Kyselova *et al.*

(c) A 2010 mass spectrometric study by Cho *et al.* analyzed glycoproteins in plasma from a small number of breast cancer patients in comparison with pooled plasma from 100 control subjects [182]. This study identified a panel of 19 glycoproteins which carried Lewis X, sialyl Lewis X or both, at levels 3-fold or greater in cancer patients compared with normal controls. The sialyl Lewis X-overexpressing proteins included plasminogen, vitronectin, clusterin, histidine-rich glycoprotein and apolipoprotein A-I.

(d) A fourth study (in 2010) used HPLC to assess whether levels of specific serum glycans could identify women with aggressive breast cancer at an early stage. The serum glycoproteins from 52 breast cancer patients and 134 women with benign breast disease were studied. The combined levels of 3 specific glycans (2 of which carried sialyl Lewis X) were significantly higher in the sera of patients with lymph node metastases when compared with sera from patients without node involvement. [183].

5. Glycan gene expression signatures. Lastly, in a study of tumor material from 90 breast cancer patients, glycan gene expression was tested using RNA expression and microarray technology, and results were analyzed by hierarchical clustering [184]. Expression analysis revealed that mRNA levels for many of the glycan-related genes differed significantly between normal and malignant breast tissue. The authors tested 2 cohorts of patients across all stages of breast cancer. Upregulation of pertinent enzymes in one or both cohorts was reported for core fucosylation (FUT8) and for steps involved in synthesis of sialylated Lewis antigens (B4GALT1, B4GALT2, B4GALT3). Borderline significant higher expression of the gene for a fucosyltransferase (FUT6) in tumors was detected in one cohort.

Fucose-related biomarkers in breast cancer are thus known or suspected based on *in vitro* studies as well as results obtained with patient-derived material. Fucosylation of cell surface molecules is unlikely to be a random or fortuitous bystander modification, since functional roles in cell-cell and cell-extracellular matrix interactions have already been documented. The significance of circulating fucosylated glycoproteins remains to be fully elucidated.

The role of fucose in defining the cellular cytotoxicity of trastuzumab

HER2 (Human Epidermal growth factor Receptor 2) is a membrane glycoprotein which is overexpressed in at least 20% of human breast cancers. The 185 kilodalton glycoprotein is a Type I membrane-spanning tyrosine kinase. HER2 signaling becomes activated upon dimerization of the HER2 glycoprotein with either another HER2 or another member of the HER family (reviewed in [185]). Thus activated, HER2 triggers a cascade of intracellular signaling events which promote an aggressive phenotype including cell proliferation, differentiation, migration, invasion, angiogenesis and decreased apoptosis. Overexpression of HER2 carries independent *prognostic* significance for risks of disease progression, relapse and mortality. HER2 overexpression can be assessed by a number of different methods including immunohistochemistry, fluorescence *in-situ* hybridization (FISH) and other emerging molecular methods such as chromogenic *in-situ* hybridization (CISH) [186]. The endpoint is most often reported as either positive or negative

without further quantification. Equivocal findings may be resolved by testing one molecular method against another with FISH serving typically as the “gold standard”.

Trastuzumab, also known as Herceptin®, is a humanized monoclonal IgG1 antibody directed against the extracellular domain of HER2. The clinical introduction of trastuzumab was a major advance in breast cancer therapy which has achieved longer disease-free survival and longer overall survival in many patients who test positive for HER2 (reviewed in [187]). It has now been explored in many other tumor types, adenocarcinoma of the stomach perhaps being the best known. Thus, HER2 status has *predictive* significance in identifying candidates for trastuzumab treatment.

Trastuzumab's 2 discrete antitumor effects are exerted through the 2 main structural components of the antibody, i.e., the “variable” FAB portion and the “constant” FC portion. First, trastuzumab's FAB portion binds to the HER2 molecule's extracellular domain. This prevents HER2 from dimerizing with another HER molecule, thereby interrupting HER2 signaling in that cell. Once the trastuzumab has become bound to HER2, a second antitumor mechanism comes into play. The FC portion of trastuzumab engages the *activating FC receptor* (FC γ R1IIa) on an effector cell, usually a natural killer (NK) cell. The doubly-bound trastuzumab molecule thus physically tethers the NK cell to the cancer cell and instigates a lytic attack on it. Trastuzumab has also been reported to mediate complement-directed cytotoxicity *in vivo*, but the predominant cytotoxic effect of trastuzumab is ascribed to ADCC.

Trastuzumab and other therapeutic antibodies share a structural feature that is critical to their effectiveness: a specific oligosaccharide which is covalently linked to the FC region at asparagine 297 (Asn297) in each of the 2 heavy chains [188]. This oligosaccharide, a mannosylchitobiose core, is “core-fucosylated” with a single fucose sugar attached in an α (1,6) linkage as shown in **Figure 2** [189]. Fucosyltransferase VIII catalyzes the “core fucosylation” to the core oligosaccharides located in the Fc region; further elongation (of the mannose moieties of the core structure) is carried out by other glycosyltransferases. In comparison with trastuzumab's molecular weight of roughly 185 kilo-

α -L-fucose in human breast cancer

Core fucosylation of the Fc portion of trastuzumab

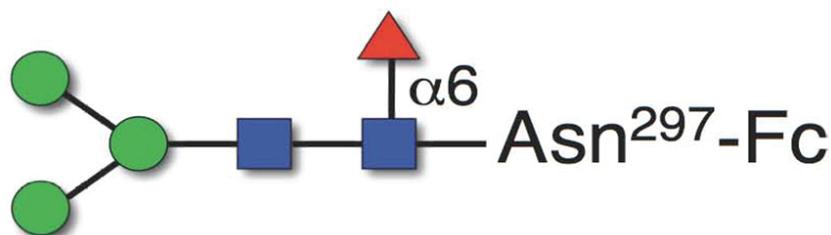


Figure 2. The mannosyl-chitobiose “core” is illustrated, with fucose attached in an $\alpha(1,6)$ glycosidic linkage to N-acetylglucosamine, the first sugar in the core structure. The core is bonded to asparagine 297 in both heavy chains of the Fc portion of trastuzumab. Fucosyltransferase VIII is the enzyme which catalyzes the addition of fucose to the core structure. The fucosylated core is usually elongated by enzymatic addition of sugars to the branched mannose moieties. This leads to stepwise construction of oligosaccharide chains which are attached to the core via mannose.

daltons, the molecular weight of 2 fucose moieties is trivial at about 328 daltons (slightly more than 0.2% of the mass of the trastuzumab molecule). As we will show, the functional significance of fucose is far out of proportion to its small size.

The value of trastuzumab in breast cancer therapy is well established. Nevertheless, there are limitations to the drug's effectiveness [190]. First, patients whose tumors show low, heterogeneous or equivocal expression of HER2 are not candidates for trastuzumab therapy since the effectiveness in this setting is unsatisfactory. Second, more than 50% of HER2-positive patients show *de novo* (primary) resistance to trastuzumab therapy. Third, in patients who have been successfully brought into remission, secondary resistance to trastuzumab emerges frequently, often after just one year of therapy. Fourth, plasma IgG proteins compete with antibodies for binding to the activating FC receptors on NK cells, thus diminishing the cytotoxic effects of the antibodies [191]. Fifth, the large required doses of trastuzumab are very costly, raising serious concerns for the economic sustainability of this form of therapy [192]. However, recent studies of post-translational glycosylation of antibodies have provided important insights into the cytotoxic effects of antibodies. Thanks to this research there are solutions at hand which may overcome the limitations of trastuzumab in a decisive fashion. Fucose is the central subject of this research.

In 2000 Clynes *et al.* studied the antitumor effects of several therapeutic antibodies, including trastuzumab and rituximab, in the presence of immune effector cells. The antibodies' FC portions' interactions with FC γ receptors could either increase or inhibit cellular cytotoxicity against the target cells. FC interactions with *activating* FC γ receptors led to increased cellular cytotoxicity, while FC interactions with *inhibitory* FC γ receptors led to the reverse. The authors noted that activated ADCC contributed substantially to the antitumor effects of the antibodies [193]. Soon thereafter, Shields *et al.* applied these principles in a study of NK cells' FC receptor interactions with a novel form of trastuzumab which had been depleted of core-fucose [194]. This fucose-depleted trastuzumab had been synthesized in a Chinese hamster ovary (CHO) cell line deficient in FUT8, the gene for the fucosyltransferase VIII enzyme. The fucose-depleted trastuzumab was then tested for its cytotoxic potency against the HER2 over-expressing breast cancer cell line, SK-BR-3. Depletion of fucose from trastuzumab led to (1) improved binding of the modified trastuzumab to the *activating* Fc receptors on NK cells, and (2) a 43-fold increase in potency of ADCC against the breast cancer cells *in vitro*.

Nearly simultaneously with the above-mentioned work, similar investigations of fucose-depleted rituximab were underway in Japan and pertinent results were accrued. In 2003 Shinkawa *et al.*, working with rituximab, selec-

tively removed or added specific sugar moieties to the FC portion of a modified anti-CD20. Only removal of fucose produced significant enhancement of cytotoxic potency against the CD20-overexpressing human B lymphoma cell line Raji [195]. This improved ADCC was also shown to be independent of functional polymorphisms of the FC receptors (i.e., FC γ R1IIa) on human peripheral blood monocytes [196]. Physical chemistry studies of fucose-depleted rituximab proved its markedly greater affinity for the FC receptor compared with standard rituximab [197]. The affinity enhancement was ascribed to a more favorable enthalpy of binding ($\Delta H \sim 5\text{-}6$ kcal/mole lower than for the standard rituximab), with an increase in the binding association constant by a factor of at least 20. In a recent review, Raju offered this generalization: "The high affinity between nonfucosylated antibodies and FC γ R1II receptors appears to result from productive interactions between receptor carbohydrate attached at Asn162 [of the FC receptor] and regions of the FC that are only accessible in the absence of a core Fuc [fucose] residue. Since the core Fuc residue does not make any hydrophobic or hydrophilic interactions and is highly flexible, *its presence appears to sterically hinder Fc receptor binding*" (emphasis added) [198].

Workers in Japan confirmed that fucose-negative trastuzumab showed superior cytotoxic potency *in vitro* utilizing blood samples both from healthy volunteers and from breast cancer patients [199]. The promising future of fucose-negative trastuzumab was noted [200]. By 2006-2007 it was clear that fucose-free monoclonal antibodies showed considerable promise for immunotherapy in human patients. One review entitled "Non-Fucosylated Therapeutic Antibodies: the Next Generation of Therapeutic Antibodies" summarized the excellent results and the biological rationale for this major shift in focus [201]. The authors noted that "fucosylated therapeutic antibodies spoil the nonfucosylated antibody-induced ADCC," emphasizing that the standard monoclonal antibodies trastuzumab and rituximab were *potent competitive inhibitors of ADCC* when these standard antibodies were used in mixtures with non-fucosylated antibodies. The authors proposed that future therapeutic IgG antibodies should consist only of non-fucosylated forms, that is, excluding the standard fucosylated forms of trastuzumab and rituximab which are currently

in clinical use. In this same time frame, pharmaceutical companies recognized that "antibody engineering," including fucose depletion, could produce therapeutic antibodies with enhanced effector function [202-205].

It was previously shown (in an animal model) that plasma IgG probably inhibited the cytotoxic effect of rituximab [191]. It was later reported that human blood IgG inhibited the cytotoxic effect of therapeutic IgG antibodies, specifically including trastuzumab, and that, therefore, high serum trough levels of trastuzumab were required to maintain therapeutic effectiveness [206]. Subsequent studies of non-fucosylated forms of rituximab and other antibodies showed that they do, in fact, evade the inhibitory effects of plasma IgG on Fc receptor binding [207-209].

In 2010 an industry group reported "Superior In Vivo Efficacy of Afucosylated Trastuzumab in the Treatment of HER2-Amplified Breast Cancer [210]." This antibody was synthesized by CHO cells deficient in FUT8. A second group from the same company reported a new method of producing FUT8 gene deletion in CHO cells using zinc-finger nucleases (ZFN treatment), yielding completely nonfucosylated antibodies [211]. The authors predicted industrial bioproduction of fucose-free antibodies with their method: "We conclude that ZFN treatment can be broadly compatible with bioprocess cell line development and look forward to the large-scale manufacture of nonfucosylated monoclonal antibodies."

The potential importance of fucose-negative trastuzumab may be better appreciated from a hypothetical future perspective. It seems plausible that the enhanced ADCC effects shown experimentally should be similarly attainable in humans. If we extrapolate into the future and consider fucose-depleted trastuzumab as the "new normal" trastuzumab, then by comparison, the conventional "old" trastuzumab can achieve only a modest (i.e., relatively suboptimal) maximum cytotoxic effect. In order to achieve this suboptimal response, a markedly higher dose of "old trastuzumab" is required. This phenomenon is best understood by realizing that *conventional trastuzumab is an antagonist of natural killer cells*. Strictly speaking, the fucosylated FC portion of conventional trastuzumab is a strong partial antagonist of binding to the FC receptor on NK cells, albeit with modest

partial agonist activity. A beneficial, though suboptimal, clinical effect of conventional trastuzumab may require up to a 100-fold greater dose to the patient in contrast to the “new trastuzumab.”

In view of the predictable clinical advantages of fucose-depleted trastuzumab, we might reconsider the limitations of trastuzumab for human use:

Candidates for trastuzumab therapy

Fucose-free trastuzumab shows enhanced ADCC experimentally, such that satisfactory cytotoxic potency can be attained with lower doses of the agent. Other fucose-free antibodies have been shown to trigger ADCC at *lower antigen density thresholds on the target cells*, in contrast to the standard fucosylated antibodies [212]. These results suggest that patients with equivocal, heterogeneous or low HER2 expression may become candidates for therapy with fucose-free trastuzumab.

Trastuzumab resistance

Escalating doses of fucose-free trastuzumab might overcome resistance (primary or secondary) in those tumors which express detectable HER2, based on the superior cytotoxic potency which might be achieved with this agent. Indeed, studies *in vitro* and *in vivo* have shown that trastuzumab-resistant breast cancer cells retain their sensitivity to ADCC killing [213]. This “brute force” approach – enhanced cytotoxic potency leading to increased cancer cell killing – may prove beneficial in cases of trastuzumab resistance related to compensatory signaling pathways, altered downstream signaling, MUC4 steric hindrance or competition by soluble HER2 [214]. Pertinent to this discussion, a recent clinical study showed a correlation between NK function and response to trastuzumab in metastatic breast cancer patients [215].

Native IgG interference

Fucose-free trastuzumab has greater affinity for Fc receptors on NK cells and thus can be expected to evade the inhibitory effects of plasma proteins. This evasion of native IgG interference is predicted to achieve high cytotoxic potency in the clinical setting, most likely with relatively lower doses of the antibody.

Cost of treatment

Effective doses of fucose-free trastuzumab will presumably require a much lower mass of the trastuzumab glycoprotein, at lower cost. This should provoke a reconsideration of cost-effectiveness calculations for trastuzumab therapy [192, 201].

While work on low-fucose trastuzumab was underway, contemporaneous research was conducted on other fucose-depleted therapeutic antibodies. It is now clear that the enhanced cytotoxic potency of these antibodies is a general phenomenon which is not restricted to trastuzumab alone. Results with other fucose-depleted antibodies are similar to those obtained with trastuzumab, typically including 100-fold increases in ADCC potency *in vitro*. These fucose-depleted immunotherapeutic antibodies include anti-RhD [216, 217], anti-CD19 [218, 219], anti-CD20 (rituximab) [220, 221], anti-CD30 [222], anti-CD317 [223] and anti-CCR4 [224]. The compelling advantages of nonfucosylated antibodies, and the importance of FC region modifications, are now amply documented [198, 225-228]. Pharmaceutical companies, having recognized the strategic importance of fucose-depletion technology, have either developed or licensed technology for producing such antibodies [229]. Phase I clinical trials are underway for 4 candidate nonfucosylated IgG monoclonal antibodies: anti-CCR4, anti-CD20, anti-CD30 and anti-RhD [224, 226, 230].

Discussion

Concise summary

Among the post-translational changes which accompany malignant transformation, disruption of the *glycan integrity* of the transformed cell is well documented, and thus the relevance of glycobiology in cancer is assured. In the case of breast cancer in particular, the simple monosaccharide fucose is overexpressed in the form of tumor-associated carbohydrate antigens, thus contributing to the glycan phenotype associated with malignant transformation and progression. In addition, fucosylated selectin ligands are overexpressed on aggressive and metastatic cell lines, indicating their ability to exploit selectin-binding processes in the metastatic cascade. Clinical studies with human breast cancer tissues show correlations be-

tween Lewis antigen overexpression and higher-grade disease, metastasis and poor overall prognosis.

Aberrant Notch signaling is frequent in human breast cancers. As described above, it is virtually certain that fucose is required for canonical Notch signaling in human breast cancer. It follows that fucose control of Notch is critical in mediating malignant behaviors such as basal cell type, breast cancer stem cell activity, osteolytic metastases, chemoresistance, prevention of apoptosis, HER2 elevation and poorer prognosis.

Fucose is present or very likely to be present in various biomarkers of breast cancer. One of the “accessory molecule” biomarkers, sE-selectin, has been shown to be an effective surrogate treatment marker in clinical trials. Emerging methods of biomarker discovery, especially serum glycan profiling, have already shown elevated fucosylation of specific serum proteins in breast cancer patients. We hypothesize that emerging fucosylated biomarkers will provide reliable, clinically-useful tests which will aid in tailoring treatment for the individual patient.

Fucose-depleted variants of trastuzumab show markedly improved ADCC potency against HER2-positive breast cancer cells in comparison with conventional trastuzumab. The emergence of these agents is likely to broaden the clinical indications for trastuzumab therapy in breast cancer, hopefully with the advantage of lower cost as well.

Future possibilities

New biomarker discovery strategies aim to achieve robust results by combining existing “omics” approaches, e.g., glycomics with proteomics [231], glycomics with lipidomics [232] and glycomics with genomics [233]. In a recent Leading Edge Essay in *Cell* (“Glycomics Hits the Big Time”) [234], Hart and Copeland summarize recent advances in glycobiology which demonstrate the scope and scale of glycan functional roles in health and disease. The authors suggest that convergent application of glycomics with other ‘omics technology, assisted by bioinformatics, will lead to discovery of new biomarkers and treatment options. For example, the fucosylated fraction of α -fetoprotein (AFP) has been approved by the FDA as a clinical bio-

marker of hepatocellular carcinoma [235, 236]. Similarly, the fucosylated fraction of prostate-specific antigen (PSA) appears to be increased in patients with prostate cancer compared with benign prostatic hyperplasia [237]. The concept that sugars such as fucose can serve as “molecular switches,” regulating glycoprotein shape and lectin affinity, has been advanced by Gabius and colleagues [238, 239]. The molecular switch hypothesis may help to explain why fucose-depleted trastuzumab is so much more efficacious than standard trastuzumab.

The role of selectins in the metastatic cascade has raised the possibility that novel therapeutic interventions – “anti-adhesive therapies” – might abrogate metastases [240]. In the 2007 paper, “Targeting Selectins and Selectin Ligands in Inflammation and Cancer,” Barthel *et al.* consider several possible strategies including neutralizing monoclonal antibodies, competitive ligand inhibitors or metabolic carbohydrate mimetics [241]. Early reports of anti-adhesive therapy *in vivo* are promising. A recent study in an animal model of sickle cell crisis was carried out with a synthetic pan-selectin inhibitor (a mimic of sialyl Lewis X) [242]. This small molecule, GMI-1070, which contains a fucose-mimicking portion, effectively competes for the carbohydrate docking domains of E-, L- and P-selectins, with the aim of inhibiting proinflammatory and procoagulant responses. Treatment with GMI-1070 reversed acute vascular occlusions in sickle cell mice. An accompanying Commentary (“Mightier than the Sickle Cell”) considers the potential importance of this therapeutic approach, which is scheduled to enter Phase 2 clinical trials [243]. Presumably, pan-selectin blockers could be similarly effective in preventing the establishment of metastatic lesions by competing with tumor cells for selectin binding sites [244]. This conjecture has yet to be tested in human subjects. Heparins, including low molecular weight heparins lacking anticoagulant activity, have been shown to inhibit metastases by blocking L-selectin and P-selectin binding *in vivo* [245, 246]. Decreased formation of tumor/leukocyte/platelet thrombi is believed to account for the antimetastatic effects [247].

Conclusion

Human breast cancer diagnosis and treatment have progressed markedly in recent decades. Knowledge of breast cancer pathophysiology

has grown to encompass new diagnostic molecular methods, and better treatment options are entering clinical practice. Nevertheless, the understanding of breast cancers' pathophysiology is far from complete. We have presented evidence that alpha-L-fucose is essential for construction of the malignant and metastatic phenotypes in many human breast cancers. Fucose is integral to two "Axes of Evil" relevant to breast cancer: the selectin-selectin ligand axis proposed by Witz, and the HER2-Notch-breast cancer stem cell axis proposed by Korkaya and Wycha. In addition, we have drawn attention to the future clinical utility of fucose-depleted trastuzumab – again underscoring the importance of fucose in biology. We hope that readers will better appreciate the importance of fucose in human breast cancer, as well as in its treatment. We have cited review literature frequently so that the interested reader can locate more complete explanations of the topics that we have touched upon. Finally, we regret that we were unable to cite many worthy publications due to space limitations.

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