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Harnessing cancer cell metabolism for theranostic applications using metabolic glycoengineering of sialic acid in breast cancer as a pioneering example

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Abstract

Abnormal cell surface display of sialic acids – a family of unusual 9-carbon sugars - is widely recognized as distinguishing feature of many types of cancer. Sialoglycans, however, typically cannot be identified with sufficiently high reproducibility and sensitivity to serve as clinically accepted biomarkers and similarly, almost all efforts to exploit cancer-specific differences in sialylation signatures for therapy remain in early stage development. In this report we provide an overview of important facets of glycosylation that contribute to cancer in general with a focus on breast cancer as an example of malignant disease characterized by aberrant sialylation. We then describe how cancer cells experience nutrient deprivation during oncogenesis and discuss how the resulting metabolic reprogramming, which endows breast cancer cells with the ability to obtain nutrients during scarcity, constitutes an “Achilles’ heel” that we believe can be exploited by metabolic glycoengineering (MGE) strategies to develop new diagnostic methods and therapeutic approaches. In particular, we hypothesize that adaptations made by breast cancer cells that allow them to efficiently scavenge sialic acid during times of nutrient deprivation renders them vulnerable to MGE, which refers to the use of exogenously-supplied, non-natural monosaccharide analogues to modulate targeted aspects of glycosylation in living cells and animals. In specific, once non-natural sialosides are incorporated into the cancer “sialome” they can be exploited as

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Conflict of interest

There are no other competing financial interests.

epitopes for immunotherapy or as chemical tags for targeted delivery of imaging or therapeutic agents selectively to tumors.

Keywords

Biomarkers discovery; Nutrient utilization; Nutrient deprivation; Cancer metabolism; Glycan sialylation; Sialic acid glycoengineering; metabolic glycoengineering

1. Introduction

Cancer is a leading cause of mortality in developed nations (in several regions of the USA it has overtaken cardiovascular disease as the leading cause of mortality) and is threatening to become the planet's number one killer in the next generation [1, 2]. Despite extensive efforts over the past several decades dating from the declaration of President Nixon's five year war on cancer declared, survival rates for this disease have barely improved. Halting progress in the war against cancer has heightened urgency for new approaches for identifying biomarkers and developing new treatments [3–5] that will allow (1) better prediction of cancer risk; (2) earlier diagnosis; (3) classification of the degree of malignancy to facilitate individualized, patient-appropriate therapy; and (4) methods to monitor therapeutic effectiveness. Ideal biomarkers will be readily translated into the clinic, be easily combined with *in vivo* cancer imaging technologies [6], and will be effective at discerning the metastatic spread of cancer, which results in the formation of aggressive secondary tumors in distant organs that ultimately results in the death of most cancer patients [7–9].

This report describes prospects for how cell-surface glycosylation, which has long been recognized as distinguishing feature of cancer in general, can be exploited for the discovery and exploitation of new and improved biomarkers and therapeutic options with a focus on breast cancer for two reasons. First, breast cancer remains a leading cause of death for women worldwide and especially in developed nations [10]. For example, in 2012, there were 1.7 million new breast cancer cases representing about 12% of all new cancer cases and 25% of all cancers in women worldwide with the highest incidence rate in North America with ~92 cases per 100,000 people in the United States and 80 in Canada. The second reason is that breast cancer has been used to pioneer the study an underappreciated aspect of glycan biosynthesis, which are connections between glycosylation and abnormal glucose metabolism now broadly associated with many (or most) types of cancer. These provocative findings suggest that nutrient deprivation illuminates and exacerbates glycosylation changes in cancer and can provide a foundation for new diagnostic and therapeutic approaches.

Connections between nutrient availability, metabolism, and carbohydrate structures that serve as putative cancer biomarkers are a major focus of this article, we first (in **Section 2**) provide a general overview of glycosylation in cancer and then (in **Section 3**) spotlights sialylation and *O*-GlcNAc, which are aspects of glycosylation that are particularly sensitive to the nutrient status of cancer cells. Subsequently, the role of nutrient deprivation and metabolic flux of nutrients in the *in vivo* cancer microenvironment will be described accompanied by prospects to exploit metabolic glycoengineering techniques to discover,

identify, and image biomarkers and develop new diagnostic and therapeutic approaches for breast cancer (in **Section 4**).

2. Glycosylation in breast cancer

2.1. Abnormal glycosylation is a ubiquitous feature of cancer

Abnormal glycosylation is long-standing feature of cancer. Aberrations in glycan display in cancer were described more than four decades ago when the Hakomori laboratory linked altered glycosphingolipid profiles with metastatic cancer [11–15]. Extensive documentation of carbohydrate structures associated with cancer – often referred to as “tumor-associated carbohydrate antigens” or TACAs for short – was available by the early 1990s [16–18]. Since then ongoing efforts to catalog TACAs across cancers of various tissue origins and stages have shown general trends as well as, in many cases, nuances that hold promise for highly specific diagnosis and therapy. In this report we discuss general features of abnormal glycosylation found in cancer and use breast cancer as a specific example where TACAs play a major role in malignancy and oncogenic progression [19].

Tumor-associated carbohydrate antigens occur at varying degrees of abundance and distribution across normal, benign, and malignant tissues [19–22]; successful biomarker discovery depends on the capture and identification of these TACAs in an efficient and meaningful manner followed by correlation of these levels with the stage of cancer progression. The complexities of glycosylation make the discovery and validation of TACAs a challenging task based on several factors, a few of which are mentioned here. First, the target glycans are not primary, template-based gene products but rather depend on the under-, over-, or neo-expression of glycosyltransferases, glycosidases, and other relevant glycan-processing enzymes; as a result TACA production cannot be predicted in a rigorous manner but in some cases can be estimated from mathematical modeling approaches [23, 24]. Second, multiple glycans can decorate a single site on a protein backbone (e.g., a “glycosite” as explained in detail elsewhere [25]) and distinct glycans can be embedded within glycan chain backbone (e.g., “LacNAc” or Lewis X). Finally, although glycans are typically regarded as post-translational modifications (PTMs) of proteins, the sugar structures themselves can be post-synthetically modified (e.g., via acetylation, phosphorylation, or sulfation), thereby increasing chemical diversity and complicating analysis.

The identification of TACA biomarkers has accelerated in recent years by improvements in “glycoscience” technologies – especially analytic methods to identify and characterize glycans from biological samples – that have helped to shed light on how glycans are differentially expressed in cancerous cells, tissues, and body fluids compared to their healthy counterparts [26–34]. Accordingly, it is now recognized that a distinguishing feature of breast cancer is an altered glycan profile that includes: 1) synthesis of truncated glycans (Figure 1a), 2) exposure of Lewis antigens (Figure 1b), 3) increased branching of N-linked glycans and 4) changes in sialylation (Figure 1c). This information has made it clear that in addition to serving as correlative biomarkers of breast cancer, TACAs play active roles in driving breast cancer progression [23]. Examples of the roles of glycosylation in cancer, which are described more detail throughout this report, include regulation of cell surface

signaling (e.g., EGFR [35]), facilitation of metastasis [20], and systemic effects resulting from nutrient deficiency (e.g., calchexia [36] or secreted glyco-genes (e.g., sialidases [37])). The inherent complexity of glycosylation pose many daunting challenges when attempting to understand the wealth of nuances introduced in TACAs during oncogenic transformation but at the same time offer unparalleled opportunities for the discovery of unique biomarkers.

2.2 Sialylation plays a dominant role in diversifying breast cancer TACAs

Cell surface glycans in human cells are generally assembled from nine different monosaccharide “building blocks” (as described in detail elsewhere [38]). The addition of one of these sugar moieties, sialic acid – an unusual nine carbon monosaccharide – to the terminal monosaccharide of a glycan is referred to as “sialylation.” This widespread and highly heterogeneous modification has been particularly strongly tied to many aspects of breast cancer progression; indeed common TACAs associated with breast cancer are almost always sialylated (Figure 1). Overall, the several dozen biochemical reactions that determine sialylation in human cells (Figure 2) provide many opportunities for “something to go wrong” in disease; indeed, dynamic changes in the sialylation of glycans have been observed in many disease states beyond cancer ranging from obesity to neurological disorders to muscle disease [39–42].

Human cells diversify presentation of sialic acid on glycoconjugates in multiple ways that tune biological activity. Heterogeneity arises from several factors beginning with the “site occupancy” of terminal galactose or GalNAc residues that serve as sites for sialylation; as few as 5% (or less) and as many as 90% of such sites – even when produced by a single cell type – can be decorated with this sugar [43, 44]. Additional structural diversity is provided by the glycosidic linkage to the underlying Gal β 1,3(4)GlcNAc/Glc penultimate glycan termini. For glycoproteins, a single sialic acid typically is attached to the underlying glycan structure through either an α 2,3- and α 2,6- bonds (Figure 2); in glycolipids an α 2,8-linked sialic acid can be attached to an underlying α 2,3-linked sialoside as exemplified by the conversion of ganglioside GM3 to the breast cancer marker GD3 [45]. In specialized cases that are highly restricted in healthy adult tissue, an α 2,3-linked sialic acid can be extended with multiple copies of this sugar sequentially attached to each other through α 2,8-bonds, forming polysialic acid. Polysialic acid (PSA), which is associated with several types of cancer [46–51] including highly malignant breast tumors [49, 52].

In addition to the presence or absence of a sialic acid moiety and the linkage by which the sugar is attached to an underlying glycan, additional diversity arises from more than 50 different chemical modifications to the sialic acid template found in nature (Figure 3) [53]. Human cells are dominated by the 5-acetamido-2-keto-3,5-dideoxy-D-glycero-D-galactononic acid form of sialic acid (which goes by the shortened name *N*-acetylneuraminic acid or “Neu5Ac”). The substitution of the acetyl amino group on ring carbon 5 with the glycolyl moiety yields what has been called the “non-human” form of sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), which interestingly, occurs in human cancers at higher levels than found in healthy tissue [54]. Another minor form of sialic acid – with an O-acetyled C8 hydroxyl group (see Figure 1) – converts the pro-apoptotic ganglioside GD3 into a molecule that protects cancer cells from apoptosis and can contribute to drug

resistance [55–57]; another way that GD3 can contribute to cancer progression is through conversion to ganglioside GD2 (also shown in Figure 1) that triggers the epithelial-mesenchymal transition (EMT) and metastasis in breast cancer [58].

Key facets of glycan sialylation are now understood in considerable depth (e.g., as outlined in Figure 2), for instance the “sialome” (i.e., the collective complement of all sialic acids [59]) displayed on a cancer cell represents as many as 10^{10} residues per cell [60, 61] but although important aspects of the complexity and diversity of the sialome are now understood at a rudimentary level [62], many questions remain. For example, to date there are no unifying sialylation signatures across different types of breast cancer cells for diagnostic use; in particular “glycosite” profiles (an example of this method is provided for pancreatic cancer cells [43, 44]) that could be used to distinguish different stages of breast cancer remain lacking. Another unresolved challenge is how the regulation of complex patterns of sialylated glycans act collectively and individually to determine specific biofunctions of a glycoprotein or glycolipid receptor [63–66]. For example, sialylation decreases the attachment of breast cancer cells to the extracellular matrix, thereby increasing the migration, dissemination, and ultimately the metastatic capacity of these cells [67, 68]. On a global cellular level, non-specific effects of sialic acid have been attributed to the negative charge carried by this sugar that – due to their juxtaposition on the cell surfaces – lead to electric repulsion and prevention of cell-cell adhesions [69]. On a more detailed level, this effect is exemplified for PSA-bearing TACAs that contribute to metastasis due to the large size, hydration, and multiple negative charges of this homopolymeric form of sialic acid [47] (note PSA is depicted in Figure 2). Finally, the metastasis has been linked specifically to α 2,6-linked sialic acids on integrins [70, 71], while similarly charged α 2,3-linked glycoproteins do not show such activity, suggesting that highly discriminating receptor ligand interactions also play a role. Taken together these changes to TACA sialylation across multiple size scales and biochemical mechanisms in breast cancer cells that combine to increase metastatic potential have become better understood and appreciated in recent years. This information supports the intriguing possibility – although it has not yet reached full fruition – that sialylated glycans could serve as particularly attractive biomarkers for the clinical diagnosis of breast (and other types of) cancer. In our opinion, however, a missing element that hinders efforts to translate glycan markers into clinically-useful biomarkers for breast cancer lies in the relative neglect of “metabolism” because not only is the expression of glycosyltransferases (e.g., sialyltransferases as outlined in Figure 2) important in determining TACA composition (discussed next in **Section 2.3**), but emerging evidence suggests that metabolic flux also plays critical roles.

2.3 Biosynthesis of sialylated TACAs – the comparative roles of glycogene expression and metabolic flux

Until recently, it has been generally assumed that TACAs that constitute potential cancer biomarkers result from genetic changes to the enzymes that biosynthesize or otherwise process glycans (it is estimated that 1 to 2% of the human genome, or ~250 to 500 “glycogenes,” are involved in this process [72]); the subset of these genes include the several dozen biochemical reactions that determine sialylation in human cells (Figure 2). The complexity of glycan biosynthesis, combined with a lack of a template or proof-reading

mechanisms found in nucleic acid and protein synthesis, provide many opportunities for “something to go wrong” in disease. In particular, altered sialoglycan profiles found in disease are thought to primarily result from the skewed expression of sialyltransferases resulting in a distinctive profile of sialylated TACAs in breast cancer (typically increased sialylation accompanies cancer but sometimes decreased sialylation has been reported) [42, 73, 74]. To elaborate briefly, if none of the sialyltransferases that produce α 2,8-linked sialylglycans (as shown in Figure 2) are transcribed in a cell, neither ganglioside GD3 (Figure 1) or PSA can be produced; this is the situation in many types of healthy cells that do not produce these TACAs. In cancer, the neo-expression of the requisite sialyltransferase enables the production of these (or other) TACAs and is considered to be a key event in oncogenic progression. An example of this phenomenon is provided by our recent characterization of breast cancer cells where rapid increases in sialyltransferase expression was observed during recovery from nutrient deprivation [75, 76].

It is becoming increasingly clear, however, that transcriptional control of various subsets of the family of 20 sialyltransferase genes found in different types of human cells (Figure 2) does not fully explain changes to TACAs observed in cancer. For example, we recently showed that increased flux through the sialic acid biosynthetic pathway under conditions that had minimal impact on glycogene expression increased the abundance of several dozen TACAs [43, 44]. This result was surprising based on the long-held assumption that under most conditions (e.g., as used to create early mathematical models of sialylation [77]), metabolic flux is not a limiting factor because cellular levels of CMP-Neu5Ac have been assumed to exceed the requirements needed for sialic acid biosynthesis. Furthermore, flux into the sialic acid biosynthetic pathway – rather than being controlled by availability of the usually abundant UDP-GlcNAc feedstock – is instead regulated by feedback inhibition of UDP-GlcNAc 2-epimerase/ManNAc 6-kinase (GNE) by CMP-sialic acid [78]. This feedback system ensures that when levels of CMP-sialic acid drop below a certain threshold, inhibition of GNE is alleviated thereby rapidly restoring necessary levels of the “building block” for sialylation. It is interesting to note that during dysfunction of feedback as occurs the disease sialuria [79, 80], or bypass of inhibition by delivery of “high flux” ManNAc analogs into cells [81–83], levels of the monosaccharide form of this sugar increase by 100-fold or more (e.g., from low micromolar levels to as high as millimolar concentrations) [81, 84, 85]. Levels of CMP-sialic acid, by contrast, only increase modestly (e.g., by 2-fold), indicating that a substantial metabolic bottleneck exists in the sialic acid biosynthetic pathway that prevents correspondingly high overproduction of CMP-Neu5Ac [86, 87].

A second factor that suggested that CMP-sialic acid levels do not regulate cell surface sialylation is that this compound is synthesized in the nucleus, translocated to the cytosol, and subsequently imported into the Golgi apparatus through an antiport transporter that requires that one molecule of CMP be transported out of the lumen of the Golgi for each CMP-sialic acid that is imported [88]. This mechanism – if strictly enforced – constitutes a second bottleneck that suggests that even when increased levels of CMP-sialic acid are produced by a cell, the extra substrate would not be able to enter the lumen of the Golgi where sialyltransferases are localized. As a counterpoint, there now are indications that the Golgi CMP-sialic acid transporter is “leaky,” allowing import of CMP-sialic acid without the concurrent export of CMP albeit at a slower rate [89]. The “leaky” nature of this transporter

is borne out by sialic acid supplementation strategies (as discussed in **Section 4.3** below) where increased production of CMP-sialic acid does have an impact on sialylation, increasing cell surface levels of this sugar [43, 90, 91].

Based on the strengthening evidence that enhanced flux into the sialic acid biosynthetic pathway and the subsequently increased CMP-sialic acid levels can modulate the biosynthesis of sialylated glycans, we briefly discuss another source of sialic acid pathway metabolites in addition to *de novo* synthesis. In particular, the salvage of exogenous sialoglycoconjugates and the cycling of sialoglycans between the cell surface and internal cellular compartments [92] provides another source of CMP-Neu5Ac precursors through the removal of sialic acid moieties from internalized entities by sialidases (e.g., NEU1-4 or Klotho [93], Figure 2) after which the liberated sialic acid can re-enter the biosynthetic process. Some reports indicate that sialic acid lyases (enzymes that can be over-expressed in cancer) convert recycled sialic acids to the corresponding form of ManNAc [94, 95], which re-enter the sialylation process at an earlier stage than Neu5Ac. Either way, sialic acid released from extracellular glycoconjugates during lysosomal degradation can re-enter the cytosol and be used as a substrate for another round of cell surface sialylation [71, 96, 97]. In addition to lysosomal sialidase activity, sialidases also act on cell surface sialoglycans, which – by modifying cell surface receptor-ligand interactions – can have a profound fate on cell behavior and fate [98]. Similarly, sialidases exist that can liberate sialic acids from adjacent cell-matrix glycoconjugates and thereby regulate glycan shedding, plasticity, and/or degradation [37, 99, 100]. Finally, sialidases emanating from cancer cells can be shed into systemic circulation, where they can cleave sialic acids from serum glycoproteins, which results in the faster clearance of these proteins from circulation. Overall the remodeling of sialylation by the combined action of sialyltransferases and sialidases plays numerous important roles in maintaining health or succumbing to various diseases [101].

In addition to enzymes that act directly on sialic acid (e.g., the sialyltransferases and sialidases shown in Figure 2 and discussed above), sialylation can be indirectly affected through the availability acceptor substrates such as the Tn antigen [102]. Interestingly, a yin-yang situation exists where “premature” sialylation in breast cancer results in truncated mucin-type O-glycans associated with breast cancer (e.g., sTn production, Figure 1, results in truncated mucin-type O-glycans). This example illustrates how the complexity of sialylation goes beyond the enzymes as depicted in Figure 2 that biosynthesize or otherwise process this sugar to potentially involve the overall complement of several hundred genes involved in glycosylation. Compounding the challenge of unraveling and understanding sialylation in specific and glycosylation overall, it is becoming increasingly evident that “energetics” – exemplified by the Warburg effect where cancer cells greatly-increased amounts of glucose – also modulates glycan biosynthesis. In our opinion, the impact of metabolic flux on cell surface glycosylation including the biosynthesis of TACAs has not been adequately explored, especially in the context of nutrient deprivation [103]. Because of the relative dearth of information describing how aberrant energy metabolism alters cell surface glycosylation (including sialylated TACAs), this article will cover this topic next by providing a brief overview of metabolic challenges faced by a nascent tumor (in **Section 3.1**) and then give a description of how the Warburg effect is linked to glycosylation via the

hexosamine biosynthetic pathway and downstream “*O*-GlcNAc” modification of intracellular proteins (in **Section 3.2**).

3. Contributions of energy metabolism – Interplay between the Warburg effect, cancer development, and glycosylation

3.1 Abnormal energy metabolism (aka the Warburg effect) is a hallmark of cancer

The development of cancer is a complex and varied process that will be described briefly using breast cancer as an illustrative example (additional information can readily be found elsewhere including the widely cited “hallmarks of cancer” review that outlines ten key characteristics of cancer [104], which includes our current focus on altered cellular energetics). Almost all (~95%) incidences of breast cancer originate from epithelial cells of the surrounding large ducts and terminal duct lobular units (TDLUs) whereas non-epithelial malignancies of the breast, which arise in connective tissue, are rare [105]. The mammary glands and breast tissues are exposed to complex hormone influences that contribute to the structural and functional maintenance of the breast but also, upon experiencing genetic mutation, can alter epithelial cell homeostasis in a way that initiates a series of transformation events that ultimately results in cancer. The oncogenic progression of an early stage breast cancer cell is facilitated by the accumulation of additional genetic changes along with successive rounds of mutation, selection, and clonal expansion resulting in the formation of a primary breast tumor [106]. A common feature of nascent cancer cells – especially in very early stages before vascularization – is an ability to acquire necessary nutrients under conditions of nutrient scarcity and utilize these nutrients to maintain growth and to build new biomass [104, 107–110].

Not surprisingly, early stage cancer cells – which exist in an oxygen deficient milieu before vascularization – out of necessity take advantage of the innate ability of human cells to ferment glucose anaerobically to obtain energy [111, 112]. Normal cells typically metabolize glucose to pyruvate via glycolysis and – in the presence of sufficient oxygen – utilize the resulting pyruvate for proficient ATP production (obtaining ~32–34 ATP equivalents from a single molecule of glucose) through oxidative phosphorylation in the mitochondria. By comparison, during oxygen deficiency (hypoxia) when oxidative phosphorylation is not possible, normal cells convert excess pyruvate to lactate obtaining only two ATP equivalents from glucose resulting in (1) increased glucose consumption, (2) decreased oxidative phosphorylation, and (3) accompanying lactate production. When a closely related phenomenon occurs in tumor cells, it is known as the ‘Warburg effect’ after the German physiologist Otto Warburg who first described “aerobic glycolysis” in cancer more than 90 years ago [113–116].

Although cancer cells share a similar collection of metabolic “machinery” with their normal counterparts that enables both cell types to ferment glucose for energy production, these components function in vastly different ways depending on the input of a small, but critical, number of regulators. For example, cancer cells generally preserve and utilize a limited capacity for oxidative phosphorylation (hence the term “aerobic glycolysis”) that augments ATP production (i.e., cancer cells often obtain four ATP equivalents instead of two from a

molecule of glucose during fermentation of this sugar). A second nuance is that although lactate production occurs during both healthy and disease-related glucose fermentation [117], in healthy individuals this potentially byproduct is quickly “detoxified” by the Cori cycle but in cancer can exacerbate cachexia [36]. Another difference between cancer and normal cells is that the Warburg effect in cancer is initiated not only by hypoxia but also by critical oncogenes such as phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) as well as hypoxia inducible factor-1 (HIF-1) [118]; moreover, cancer cells retain the Warburg effect even when oxygen is restored in part through cross-talk between the well-known oncogene c-MYC and down-stream glucose metabolism [119]. These features reflect the fact that the increased influx of glucose into cancer cells is not only driven by energy requirements of the cell but, on the contrary, is finely tuned by pathways that respond to extracellular stimuli such as growth factor-mediated signaling [120–124].

3.2 The HBP links glycosylation with later stage cancer progression

The retention of the Warburg effect upon tumor vascularization and the restoration of oxygen benefits cancer cells in several possible ways. For example, from 5 to 15% of incoming glucose is diverted into the pentose phosphate pathway, providing a cancer cell with various small molecule metabolites required for cell division and proliferation. Another potential benefit is that if a cancer cell again faces oxygen deprivation (e.g., in a non-vascularized portion of a rapidly growing tumor or upon metastasis to a distant site in the body), it will still be equipped to deal with hypoxia. Increasingly compelling evidence indicates that the deviant metabolism that characterizes cancer cells [125–129] can facilitate oncogenic progression in considerably more complex and non-obvious ways as well. For example, if a nascently-transformed breast epithelial cell remains within the basement membranes of the TDLUs, it is classified as non-invasive carcinoma, or carcinoma *in situ* [130] while invasive breast cancers are characterized by the dissemination of cancer cells outside of the TDLUs, into the surrounding tissue [131]. The dissemination of metastatic cancer cells throughout the body, as well as other important events in cancer progression including the EMT, have recently been linked to TACAs and by extension to the underlying metabolic reprogramming that determines glycosylation profiles and TACA expression [132].

Mechanistically, connections between metabolism and cancer-driving TACAs are enabled by glucose-driven flux through the hexosamine biosynthetic pathway (HBP), which increases production of UDP-GlcNAc. As brief background, Figure 4 summarizes how a proportion of glucose that enters a cell (typically ~2 to 5%) is diverted into the HBP [133] resulting in increased levels of UDP-GlcNAc (and its epimer, UDP-GalNAc) in cancer cells as a consequence of their characteristically high levels of glucose. UDP-GlcNAc and UDP-GalNAc are critical feedstocks for many facets of TACA biosynthesis but in most cases the increased levels of these nucleotide sugars is not expected to necessarily promote TACA biosynthesis because the majority of glycosyltransferases that employ these substrates have K_m 's in the low micromolar range (e.g., often < 20 μ M). These enzymes, therefore, are expected to be affected only minimally by the Warburg effect because baseline levels of UDP-GlcNAc of ~100 μ M are sufficient for maximal transferase activity in most cases. A

critical exception lies in the activity of two GlcNAc transferases (MGAT4/5, also known as GnT4/5) that together work to produce highly-branched (i.e., tri- and tetra-antennary) N-glycans because their K_m values are in 5 to 11 mM range [35, 108]). As a result, Warburg effect driven flux into UDP-GlcNAc production is required for robust activity of these enzymes, which produce highly-branched glycan structures implicated in aspects of malignant progression [41, 134] ranging from the promotion of oncogenic signaling [35] to metastasis [20].

Our interest in links between TACAs and cell surface sialylation was piqued by two recent observations made by our team of investigators. First, in unpublished work, we found that inhibiting Warburg effect-driven flux through the HBP resulted in an unexpectedly complex pattern of changes to cell surface N-glycans. Specifically, instead of changes being limited to a targeted reduction in highly-branched N-glycans due to the expected selective impact of lower UDP-GlcNAc levels on MGAT4/5 activity, dozens of additional glycan structures were affected with about half increasing in abundance; a reduction in UDP-GlcNAc could not have this effect based on any currently-known mechanism. Second, in recently-published studies we discovered links between metabolism and sialylation that also were not easily explained by direct interplay between metabolic intermediates and biosynthetic enzymes [76, 101]. In particular, cell surface sialylation was rapidly diminished upon induced nutrient deprivation of breast cancer cells evaluated *in vitro*, which based on the metabolic networks diagrammed in Figure 4, initially appears reasonable because UDP-GlcNAc is the metabolic feedstock for sialylation. However as discussed earlier sialylation puts only modest demands on the UDP-GlcNAc pool in cells because feedback inhibition of GNE activity occurs when CMP-Neu5Ac levels exceed $\sim 5 \mu\text{M}$; typically, this metabolic demand requires only a minor fraction of the UDP-GlcNAc present in cells. Sialylation is therefore not expected to be highly sensitive to nutrient deprivation but, in contrast, our results in breast cancer cells showed that sialylation was exceptionally sensitive to nutrient deprivation.

A possible explanation for these initially puzzling observations was that UDP-GlcNAc levels were modulating *O*-GlcNAc transferase (OGT) (Figure 4) activity. As a brief background, OGT is an enzyme that installs a single GlcNAc on thousands of intracellular proteins to make the “*O*-GlcNAc” post-translational modification originally described to have a yin-yang relation with phosphorylation [135]; it is now known that *O*-GlcNAc modification of proteins extends well beyond cross-talk with phosphorylation [133]). Of interest to nutrient-deprived breast cancer cells, the emergence of *O*-GlcNAc as a master regulator of epigenetics [136, 137] suggests that changes to flux through the HBP modulate a bevy of epigenetic-driven effects ranging from (1) direct changes to the chromatin (e.g., through changes to the methylation of promoter regions), to (2) indirect regulation via microRNAs that modulate glycosylation [138] and, (3) finally, to the expression of sialyltransferases, consistent with our finding that both UDP-levels and the these enzymes were highly affected by nutrient deprivation in breast cancer cells [76]. The *O*-GlcNAc protein modification ties oncogenesis to metabolism and illustrates how cancer progression does not rely solely on genetic mutations or even epigenetic mechanisms but extends to the dynamic regulation of the proteome. For example, *O*-GlcNAc modification stabilizes c-MYC, a well-known oncogene [139] that helps maintain the Warburg effect in the presence of oxygen [140]. In a complementary manner, *O*-GlcNAc modification of PFK-1 increases flux into the pentose

phosphate pathway (PPP) [141], which supplies cells with NADPH as well as metabolites needed for cell growth and proliferation.

4. Towards exploiting cancer metabolism for diagnosis and therapy

4.1 Clinical translation of “glyco-therapies” remains in its infancy

The information provided above hopefully conveyed the message that glycosylation is a defining feature of malignant disease and also provides a rudimentary outline of the role of glycosylation in cancer in general and breast cancer more specifically. In a nutshell, it is puzzling (at best) or even distressing that glycosylation aberrations that were first discovered at least four decades ago are not being used to treat – or at least diagnose – cancer patients but on the other hand, the challenges inherent in developing “glyco” therapies are immense (as discussed in several reviews [18, 142]). More positively, in recent years the idea that sugar-based compounds are viable drug candidates have been supported by the idea that monosaccharides are attractive scaffolds for drug development [143] and ‘real world’ examples such as the success of Glycomimetics in developing a monosaccharide-inspired drug for sickle cell disease [144]. More specific for breast cancer where mucins such as MUC-1 play a defining role, approaches taken by the Boons group to develop “cancer vaccines” that target the combined peptide backbone and attached TACA are very promising [145]. We believe that encouraging approaches such as these will be augmented in the foreseeable future by explicitly incorporating the contributions of the unique features of cancer cell metabolism (e.g., the Warburg effect and/or nutrient deprivation) into therapeutic and diagnostic strategies. Although there are many potential avenues to exploit the interplay between energy metabolism and glycosylation, we have recently uncovered compelling suggestions that nutrient deprivation – which comprises a repeated challenge faced by cancer cells (as described next in **Section 4.2**) – can be combined with “metabolic glycoengineering” approaches to empower new approaches for cancer diagnosis and treatment.

4.2 Nutrient deprivation promotes aggressive cancer phenotypes

Nutrient deprivation within a tumor microenvironment affects the metabolism of individual cells in different ways that contribute to intratumor heterogeneity; for example, cells within a growing tumor larger than a millimeter are able to starve themselves to acquire necessary nutrients before angiogenesis [146–149]. Even after initial vascularization, cancer cells face dynamic oscillations in nutrient availability during oncogenic progression as cells within a growing tumor repeatedly face nutrient limitations in transiently (or in some cases, long-lived) poorly-vascularized regions. As a consequence, cancer cells at various stages of oncogenic progression retain and exploit diverse metabolic strategies to bypass unfavorable conditions and nutrient deprivation in a manner that confers a selective advantage for aggressive cancer phenotypes [150–158].

One mechanism exploited by nutrient-deprived cancer cells is a reliance on opportunistic nutrient acquisition systems to survive and proliferate in metabolically-poor conditions. In particular, these cells have an ability to withstand nutrient deprivation by increasing their capacity to import nutrients from the surrounding environment by recovering pre-made

intracellular macromolecules and fuse whole organelles within the cell lysosomes [159]; influencing the integration of appropriate nutrients into abnormal metabolic pathways that contribute to the establishment and maintenance of the tumorigenic state; and by exerting long-range effects that contribute to cell fate transitions through conferring their specific-function signals that include regulating cell proliferation and survival, subverting adaptive immune responses, altering responses to hormones, and promoting angiogenesis and metastasis [160–162]. Particularly relevant to breast cancer where most tumor cells are derived from the epithelium, emergent cancer cells can grow faster than healthy endothelial cells during energetically stressful circumstances and nutrient deprivation actually promotes the survival of highly aggressive transformed cells by manipulating the surrounding nutrient-poor environment into actively assisting cellular viability and the inadequacies of the tumor vascular supply [129, 163, 164].

In addition to sugars such as glucose and similar N-acetyl-modified monosaccharides (e.g., GlcNAc and GalNAc) obtained by glycosaminoglycan remodelling and salvage used for energy production, sialic acid can be being scavenged from the microenvironment of a cancer cell and utilized by salvage pathways, as noted in Figure 4. In part, the recovery of sialic acids released during lysosomal degradation or digestion of macromolecules helps maintain cellular glycan sialylation capacity [99]. There are also several reports that described the efficient uptake of non-conjugate bound sialic acid (e.g., the monosaccharide from Neu5Ac or Neu5Gc) by various cell types including cancer cells [165–168]. Up to very recently, these studies have been conducted under nutrient-rich conditions where only modest changes in overall levels of cell surface sialic acids were typically observed, although in some cases certain TACAs such as PSA increased by 300% or more [169]. By comparison, the impact of supplementation in nutrient-deprived breast cancer cells – where a distinguishing feature was dramatically-reduced cell surface sialylation – was considerably more dramatic insofar surface sialylation was rapidly rescued by supplying the cells with exogenous sialic acid [75].

We have already characterized the restoration of surface sialylation in nutrient-deprived breast cancer cells upon exogenous supplementation at a rudimentary biochemical level [75, 76, 101, 170]. These studies discounted the possibility that Neu5Ac was routed for energy production by showing that ATP levels did not increase upon supplementation but instead dramatically decreased in certain cell lines. Instead of being used for energy, exogenously-supplied Neu5Ac was routed into CMP-Neu5Ac production as evidenced by increased levels of this nucleotide sugar that ultimately – in concert with increased expression of sialyltransferases – resulted in the recovery of cell surface sialylation. Interestingly, exogenously-supplied Neu5Ac was also routed into UDP-GlcNAc biosynthesis (as outlined in Figure 4). This large increase in UDP-GlcNAc, which also is “high energy” compound that required multiple inputs of ATP during its biosynthesis, offers an explanation for the energy drain experience by cell lines supplemented with sialic acid insofar as cellular stores of ATP were depleted by the resulting synthesis of both CMP-Neu5Ac and UDP-GlcNAc. The large pools of UDP-GlcNAc in Neu5Ac supplemented cells also provide a testable hypothesis for the increased sialyltransferase expression in these cells; specifically their increased expression could be a consequence of epigenetic control of “glyco genes”

mediated through cross-talk between *O*-GlcNAc protein modification and epigenetic control of glyco genes [171].

4.3 Nutrient deprivation powers sialic acid-based diagnostic and therapeutic strategies

Ultimately, our goal is to exploit glycan signatures characteristic of the metabolic reprogramming of breast cancer cells – which are strongly influenced by nutrient deprivation for sialylation as described above (and more generally for glycosylation in many studies [172–180]) – for the discovery of novel biomarkers and eventually, for the implementation of new therapeutic approaches. These efforts are based on consolidating evidence that sialylation provides a read-out of the physiological state of the cell that is exacerbated during nutrient deprivation. The ability to witness such changes by monitoring easily-accessible cell surface glycans in the context of nutrient deprivation augments our understanding of breast cancer biology and potentially provides new clinical tools for disease diagnosis. As a caveat, the general idea of using TACAs for the diagnosis or treatment of breast cancer is not new [16, 181] but relatively paltry progress has been made in translating these biomarkers into the clinic. We believe that the metabolic signatures of cancer cells under nutrient deprivation that can now be unravelled using new tools and approaches that are providing immense benefits across many aspects of the “glycosciences” provide unparalleled opportunities to discover new TACAs, or to utilize known TACAs in more skillful and meaningful ways. For example, because cell surface glycans offer a surprisingly comprehensive snapshot into the internal metabolic status of a cell and by cataloging these changes under various states of nutrient availability [101, 103, 182–186], TACA profiles that indicate the “energetics” status of cancer cell could be used to assess the stage of cancer and then to suggest a treatment strategy. In particular, we focus on nutrient-deprived breast cancer cells that efficiently take up sialic acids used to restore cell surface display of this sugar [75, 76, 101].

Sialic acid uptake in the nutrient-deprived cells presumably utilizes the mechanisms used *in vivo* and described for a variety of cell types under normal cell culture. First, sialic acids can be salvaged from the recycling macromolecules found in the ECM of a cancer cell’s microenvironment or through the partial or complete digestion of surrounding cells. In a diagnostic or therapeutic setting, the ability of cancer cells – and in particular nutrient-deprived breast cancer cells – to salvage sialic acids can be exploited by supplementation with natural or even non-natural forms of this sugar [76, 187–189]. Exogenously-obtained sialic acids taken up by cells can experience one of several biochemical fates [190–192] based on the salvage pathways outlined in Figures 2 & 4. For example, they can be recycled and re-used for glycan sialylation fairly directed by conversion to the CMP-sialic acids required as substrates for sialyltransferases (Figure 2). Furthermore, pathways exist by which ManNAc can be converted to GlcNAc (Figure 4) [193], which in turn can be salvaged into the HBP to augment UDP-GlcNAc biosynthesis; our results showing high levels of UDP-GlcNAc in sialic acid-supplemented cells [76] indicate that breast cancer cells are able to robustly exploit this metabolic route. Alternately, a biochemical pathway exists that can route GlcNAc towards glycolysis [194], making it plausible that exogenously-supplied sialic acids could be used to replenish cell bioenergetics; this possibility, however, was discounted for nutrient-deprived breast cancer cells where ATP levels were not restored by sialic acid supplementation [76].

Two observed metabolic fates of sialic acid supplementation, which are restoration of surface sialylation and augmentation of UDP-GlcNAc levels, have important implications. For example, increased UDP-GlcNAc levels can lead to epigenetic re-programming of glyco gene expression, which is consistent with our observation of increased sialyltransferase levels [76] upon glucose (energy) supplementation. Looking forward to clinical translation, the putative epigenetic effects hold promise as biomarkers for diagnosis or to monitor treatment efficacy. For example, diagnostic methods to monitor DNA methylation status [195–197] can be used to identify promoter regions for specific glyco genes or their controlling microRNAs (miR) that are modulated by the Warburg effect. A second implication is that by efficiently salvaging exogenous sialic acids for cell surface display, nutrient-deprived breast cancer cells provide exceptional candidates for metabolic glycoengineering intervention, a topic that is described next in more detail below.

4.4. Sialic acid supplementation and metabolic glycoengineering (MGE)

Increased glucose uptake in cancer cells already has been exploited for the imaging of cancer, providing precedent for the idea that metabolic nuances of cancer cells have potential clinical utility. This concept is exemplified by fluorodeoxyglucose positron emission tomography (FDG-PET), and, more recently, by ^{18}F -labeled glutamine or hyperpolarized ^{13}C -labeled metabolite tracers that, in one context, have been used for the imaging of breast metastatic cancers that are localized to sites of heavy glucose utilization, such as the brain [198]. In a conceptually-similar manner, we expect that labeled sialic acid precursors - which are taken up preferentially by breast cancer cells at certain stages of malignant progression – could be used as a diagnostic tool to detect primary and metastasizing cancer within the body and also to help determine cancer grade. As “added value,” we propose that the metabolic responses that breast cancer cells acquire and/or exploit to deal with periodic nutrient deprivation render them particularly susceptible to non-natural nutrient mimetics, such as sialic acid analogues used in metabolic glycoengineering (MGE) thereby opening the door to a plethora of “next-generation” theranostic possibilities.

To provide a brief background on MGE, the “nonhuman” N-glycolyl form of sialic acid (Neu5Gc, see **Section 2** and Figure 4) which is introduced into the human body through dietary sources including “red meat” and dairy products, provided early evidence that the biosynthetic machinery for sialic acids are permissive to non-natural sialic acid analogues and that these forms of sialic acid are preferentially utilized by cancer cells. Based on this precedent, pioneering studies by Werner Reutter’s group in the early 1990s extended this technology (which had previously been explored on cell-free and *in vitro* systems [199–201]) into the *in vivo* milieu [202]. These studies demonstrated the feasibility of using non-natural sialic acids to label cellular glycans by using N-propanoyl-D-mannosamine (ManNProp), which has one additional carbon atom to the N-acyl group of ManNAc, [124]. In subsequent work, non-natural modifications to the N-acyl position of ManNAc were extended to include longer N-acyl groups (e.g., ManNBut and ManNPent [85, 202]); ketones (ManNLev [203], ManNHomoLev and ManNOxoHex [85]); alkenes (ManNCrot); the “N-glycolyl” hydroxy group (ManNGc [204]). Unlike “natural” high-flux ManNAc analogs discussed earlier that increase the free monosaccharide levels of sialic acid by orders of magnitude in a cell, many (or most) of these non-natural analogs do not increase production

of this sugar even measurably within a cell (an example is provided by Ac₄ManNLev [85]) but nevertheless are presented on the cell surface in millions of copies (e.g., as 2 to 20 million of the ~100 million sialic acids found on Jurkat cells) [60].

The MGE approach has now been extended to include several bio-orthogonal functional groups (i.e., chemical functional groups that do not naturally occur in glycans in general or TACAs in specific); these include the early example of the azide group [205], the follow-up example of the alkyne group [206], which is the other “classic” click chemistry coupling partner, as well as photo-activated functional groups such as phenyl azides [207] or diazarines [208] and thiol groups [209, 210]. These advances in bioorthogonal chemical coupling reactions [211] that target cell surface TACAs bearing non-natural TACAs installed using MGE have spurred interest in using these methods for cancer diagnosis [61] or therapy [203]. By using the appropriate sialic acid supplementation conjugates and nutrient deprived conditions, a growing assortment of functional groups can be exploited to selectively tag “glycoengineered” sialic acids with fluorescent probes as summarized in Figure 5). For example, it was shown almost 20 years ago that Sia5Lev (a ketone-containing sialic acid installed on “ManNLev” treated cancer cells), can undergo highly chemoselective ligation with hydrazide or hydroxylamine derivatives to selectively label cancer cells with imaging agents or kill them with toxins based on the level of cell surface ketone display [60, 203]. In a similar manner, azide-containing sialosides (Sia5Az) were developed that enable specific conjugations reaction with phosphine-activated reagents in a modified Staudinger ligation [205]; these experiments set the stage for the widespread incorporation of functional groups exemplified by the “click chemistry” [212] into MGE strategies. In particular, azide- or alkyne-containing monosaccharide analogues provide attractive opportunities for using bioorthogonal chemical reactions to remodel the cell surface [213]. These strategies – which rely on the facile incorporation of “non-human” sialic acids into cell surface glycans – are supported conceptually by our recent publications [75, 76, 101, 170] that provide provocative indications that MGE strategies could be exceptionally valuable for treating cancer because nutrient deprivation enables cancer cells to rapidly and robustly incorporate exogenously-supplied sialic acids thereby providing a therapeutic window to minimize harm to healthy cells.

4.5 Diagnostic and therapeutic exploitation of MGE strategies

The MGE strategies outlined above set the stage for a remarkably broad range of therapeutic and diagnostic possibilities. First – although Figures 5 and 6 emphasize how bioorthogonal conjugation reactions can be used in a two step process to tag cells with diagnostics or therapeutics – in some cases sialic acid metabolic glycoengineering holds promise without the need for a second agent. For example, installation of fluorinated sialic acids into cancer cell glycans was inspired by the hope that these “Teflon-like” moieties would reduce metastasis by making it difficult for cell traveling through the body to re-attach to the ECM and initiate expansion into a secondary tumor [168, 214]. In a second example, we found that increased sialylation achieved by treating cells with the “high flux” analogue 1,3,4-*O*-Bu₃ManNAc [82, 83, 215] restored sensitivity of drug-resistant cancer cells to tyrosine kinase inhibitors (TKIs) [90] by altering cell surface receptor trafficking, internalization, and recycling [91]. Finally, efforts are underway to increase the immunogenicity of TACAs by

installation of non-natural sialosides such as “Neu5Prop” (which has an elongated N-acyl group) or “Neu5Ph” (which has a phenyl ring attached to the N-acyl group) [216, 217].

In many cases the value of MGE for diagnosis or treatment of cancer can be enhanced through a two-step process where remodeling of the cell surface (Figure 6a) sets the stage for follow-up intervention. For example, efforts to increase the immunogenicity of TACAs via MGE [216, 217] can be augmented by a passive immunotherapy approach where antibodies to the targeted epitope (e.g., glycoengineered GM3) are introduced into the host, thereby enhancing the tumor-fighting immune response [218] (Figure 6b); the success of cancer vaccine efforts mentioned above [145] could also benefit from this approach. Novel MGE-based diagnostic strategies are now being developed based on imaging modalities that can be used to visualize glycans tagged with “click” functional groups [219, 220] (Figure 6c); similarly, efforts are being devoted to directing MRI contrast agents to tumors [61] to enable earlier detection and diagnosis (Figure 6d). As illustrated in a rodent study by Neves and co-authors [221], these approaches show promising tumor-specificity, thereby setting the stage not only for diagnostic but also for therapeutic intervention. Therapeutic approaches typically rely on the delivery of a drug with a bioorthogonal functional group to a cancer cell that displays the complementary group; early examples of this strategy involved targeting hydrazide-modified ricin to ketonized sialic acids installed on cancer cells through MGE [203] (Figure 6e) as well as small molecule drugs such as doxorubicin [92] (Figure 6f). An interesting aspect of drug delivery is that over a period of two to six hours, metabolically glycoengineered sialoglycoconjugates can cycle between the cell surface – where they can pick up a drug cargo – and internal compartments – where they can deliver the drug and then be again directed to the cell surface to repeat the process [92].

Over the past few years the flourishing field of nanotechnology has provided many new opportunities for biomedicine and we envision that that application of MGE-based strategies to breast cancer exemplifies this point by enabling theranostic approaches (Figure 6g). A technical foundation for combining diagnostic and therapeutic modalities into a single nano-sized construct is illustrated by the MGE analogue 1,3,4-*O*-Bu₃ManNAc that can be formulated into sebacic acid (SA) – polyethylene glycol (PEG) nanoparticles for controlled release [222] thereby increasing potency for applications such as sensitizing drug resistant cells to TKIs [90, 91]. Similarly, monosaccharide analogues used in MGE can be formulated into biodegradable poly(lactic-co-glycolic acid) (PLGA) constructs for controlled release *in vivo* [223]. Towards the development of theranostics, we have tagged drug-laden SA-PEG nanoparticles (of the type used to deliver 1,3,4-*O*-Bu₃ManNAc[222]) with radiolabeled iodine and demonstrated *in vivo* imaging [224], in essence demonstrating potential theranostic approach. Moving forward, a PLGA-based platform offers opportunities for advanced theranostic approaches [225–227] that cover multiple aspects of an MGE strategy designed to treat breast cancer. For example, the surface of a drug-laden, fluorescently-tagged nanoparticle (Figure 6g) could be derivatized with chemical functional groups designed for selective conjugation with a bioorthogonal coupling partner installed via MGE (Figure 5).

4.6 Looking forward: Implementation of MGE for “real world” cancer theranostics

On a practical level, the implementation of MGE for breast cancer diagnosis (or cancer diagnosis and therapy in general) has been hindered by concerns that -- because virtually all tissues in the human body express sialic acid -- off-target effect could severely compromise diagnostic imaging or therapeutic strategies. Although there have been counterevidence to this concern -- for example the aforementioned ability of cancer cells to highly over-express non-human “Neu5Gc” scavenged from the diet and more recently, an *in vivo* study showing strong signal in tumors compared to healthy tissues [221] -- the underlying biological basis of these observations have been unclear. Therefore, as discussed above, we believe that unique adaptations of cancer cells gained from nutrient deprivation during their development, provide them with a unique ability to use exogenously-supplied metabolic intermediates to supplement sialic acid biosynthesis with high efficiency compared to healthy tissues -- thus providing an attractive therapeutic window; moreover, this feature provides both a conceptual and biochemical foundation for the clinical translation of MGE strategies to treat cancer.

Clinical translation of MGE could take several directions; at present, it is not obvious what direction will be the most productive or lead to “real world” use most rapidly (in part, we hope that this article will inspire investigators to pursue various approaches that we propose, or to come up with complementary strategies not described in this article). One option is inspired by isotope-labeled (deoxyfluoro)glucose already employed in cancer diagnosis that we believe positions corresponding isotope-labeled metabolic intermediates of sialic acid biosynthesis as tools for identifying specific stages of cancer progression. As a caveat, the *in vivo* supplementation of nutrient-deficient tumors with sialic acid pathway intermediates could be problematic because the delivery of these agents to cancer cells requires a mechanism to traffic the compound to the tumor, such as already “in place” vascularization. Therefore, although the clinical translation of MGE-based strategies may not be applicable to the very earliest stage tumors, this strategy nevertheless could be used to identify and characterize the proportion of cells still harboring a “nutrient deprivation” geno-or phenotype in newly-vascularized tumors. In the case of biopsies, patient-derived cell lines could be cataloged with respect to the stage of cancer progression based on the tumor’s preexisting “glyco-profile” as well as the ability of the cells to incorporate various MGE analogs into their TACAs. These efforts to obtain a more sophisticated glycoprofile of breast cancer cells offer many opportunities to refine and tune efforts to use TACAs as biomarkers for breast cancer and ultimately, to develop new individualized “personal medicine” therapies.

5. Concluding comments

The location and widespread occurrence of sialylated glycans provide these molecules with a central role in several aspects of biomarker discovery for breast cancer. The glycan sialylation adaptations and nutrient deprivation conditions that arise during tumorigenesis open the door to advance increasingly sophisticated strategies based on the emerging understanding of how breast cancer metabolism relies on increased glucose utilization characteristic of the Warburg effect but -- often at the same time -- is sensitized to MGE

strategies by nutrient deprivation. The idea that although glycan sialylation metabolism is itself a unifying feature of breast cancer cells in general, specific glycan sialylation changes within a given “nutrient deprivation” metabolic signature seems to be better biomarker strategy. Accordingly, glycan sialylation changes and its correlation to nutrient deprived metabolic signature have now been added to the list of biological targets amenable to yield promising diagnostic, therapeutic, and theranostic utilities.

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GlycoMantra, Inc. filed a non-provisional full patent entitled “Exploiting nutrient deprivation for modulation of glycosylation – research, diagnostic, therapeutic, and biotechnology applications” on August 16, 2016 with a priority date August 17, 2015. H.A., H.A.B., and K.J.Y. were listed as inventors.

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Figure 1. Representative breast cancer tumor-associated carbohydrate antigens (TACAs)
(a) Mucin type *O*-glycans are initiated by the attachment of a single GalNAc to a serine or threonine residue of a protein (in breast cancer, often MUC1) that – in healthy cells – typically is elongated into one of 8 “core” structures that are further elaborated into *O*-linked glycans that range in size from 3 to 8 monosaccharides. In cancer, over-expression of sialyltransferases prematurely “caps” a nascent *O*-linked glycan with a terminal sialic acid moiety, preventing further elongation and thus resulting in truncated glycans that are a distinguishing feature of this disease. **(b)** Lewis antigens are another distinguishing feature

of breast cancer, the capping of “Lewis X” with sialic acid (shown in shades of purple) forms “Sialyl Lewis X” (sLe^X), which is TACA strongly associated with breast cancer and many other types of malignant disease. (c) Ganglioside-based TACAs (a ganglioside is a sialic acid-modified glycolipid) illustrate the ability of sialic acid to tune biological activity. The simplest and most abundant ganglioside is GM3, which is produced by the addition of an α 2,3-linked sialic acid to lactosyl ceramide (LacCer); in cells that express the appropriate α 2,8-sialyltransferase GM3 can be further elaborated to GD3. Over the past 20 years, both GM3 and GD3 have been linked to cancer progression although in an enigmatic manner where both protective and disease-exacerbating roles have been described; more recently it has become more evident that the oncogenic properties of these gangliosides emanate from further modification of GD3 – e.g., by C8-O-acetylation or the addition of a GalNAc moiety to form GD2.

Figure 2. Outline of sialoglycan biosynthesis highlighting the role of sialyltransferases

The human genome has the capability to produce 20 sialyltransferases that can link sialic acids to an underlying glycans (that in turn are attached to proteins or glycosphingolipids) through α 2,3- (magenta), α 2,6- (blue), or α 2,8-linkages as well as least 5 recycling enzymes (the sialidase NEU1-4 and Klotho) [228]. Typically only a subset of these genes are expressed in human cells and the neo-expression of certain of these enzymes – as highlighted – contribute to oncogenesis. For example ST6GALNAC1 is implicated in sTn (Figure 1a) production in breast cancer [229], ST3GAL4 has been linked to sLe^x expression (Figure 1b) in cancer [230], and ST3GAL5 produces ganglioside GM3, which can be further elaborated into GD3 by ST8SIA1, an enzyme that adds an α 2,8-linked sialic acid to GME and is also known as GD3 synthase; ST8SIA2 and ST8SIA4 are additional cancer-associated

sialyltransferases (also referred to as STX and PST, respectively) that produce polysialic acid, a TACA discussed in the main text. This figure also diagrams the incorporation of CMP-sialic acids (Neu5Ac and Neu5Gc) into the Golgi and the recycling of sialic acids from salvages sialoglycans; additional details for how cells obtain and metabolize these compounds is given in Figure 4.

Figure 3. The sialic acid template

The term “sialic acid” refers to over 50 naturally-occurring forms of this sugar, several of which are shown in this diagram that is intended to illustrate how almost all of the heteroatoms of this sugar can be modified. In addition to these many naturally-occurring sialic acids, many non-natural forms of this sugar can be used by biological systems; most of these are modified at the C5 position with examples provided in Figure 5.

in this report are highlighted in yellow (additional details on the relevant enzymes involved and supporting literature references are provided in the main text).

Figure 5. Overview of bioorthogonal labeling strategies enabled by metabolic glycoengineering (MGE)

In vivo use of MGE was pioneered with "R₁"-modified ManNAc analogs (panel **a**) that were at first limited to extended alkyl chains [85, 202] but now include a bevy of versatile chemical functional groups including (**b**) ketones [85, 203], (**c**) azides [205], (**d**) thiols [209, 231], and even (**e**) carbene-generating analogues [208, 232] (additional options also exist, as discussed elsewhere [228, 233, 234]). This figure, as indicated at the bottom, describes the "first step" in an MGE strategy enabled by metabolic features of cancer cells that prime them to preferentially take up and incorporate ManNAc and sialic acid analogues into their cell surface glycans, thus setting the stage for the second step – which is the selective delivery of immunologic, diagnostic, or therapeutic agents to cancer cells (specific examples are provided in Figure 6).

Figure 6. Examples of MGE-enabled therapies

Panel (a) depicts the use of MGE to modify cell surface glycans with epitopes selectively incorporated due to the unique metabolic features of cancer cells (e.g., primarily nutrient deprivation); examples of specific “R₁” functional groups and bioorthogonal ligation reactions are illustrated in Figure 5. Once incorporated onto the cell surface, the “R₁” groups can (b) directly serve as epitopes for antibody dependent cellular cytotoxicity (ADCC) or other immunotherapy strategies (such as cancer vaccines [145]). Alternately, the R₁ groups can serve as “chemical tags” for secondary delivery of (c) fluorophores for imaging [221], (d) MRI contrast agents [61], (e) cancer-killing toxins [203], (f) small-molecule cancer drugs [92], or – in the future – for (g) theranostic nanoparticles constructed out of polymers such as PLGA that encapsulate drugs and imaging agents (such particles typically have a surface coating with poly(ethylene glycol) or a similar agent that reduces non-specific adhesion).