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Carbohydrate Engineered Cells for Regenerative Medicine

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Abstract

Carbohydrates are integral components of the stem cell niche on several levels; proteoglycans are a major constituent of the extracellular matrix (ECM) surrounding a cell, glycosaminoglycans (GAGs) help link cells to the ECM and the neighboring cells, and small but informationally-rich oligosaccharides provide a “sugar code” that identifies each cell and provides it with unique functions. This article samples roles that glycans play in development and then describes how metabolic glycoengineering – a technique where monosaccharide analogs are introduced into the metabolic pathways of a cell and are biosynthetically incorporated into the glycocalyx – is overcoming many of the long-standing barriers to manipulating carbohydrates in living cells and tissues and is becoming an intriguing new tool for tissue engineering and regenerative medicine.

Keywords

metabolic glycoengineering; glycan and oligosaccharide engineering; sialic acid; tissue engineering; ManNAc analogs; glucosamine; hexosamine-based drugs

1. Introduction/Overview of the chapter

Carbohydrates contribute to the welfare of cells found in a multicellular organism in several ways, in essence providing them with food, housing, and clothing. “Food” is a well known attribute of sugars as this class of molecules is ubiquitously regarded as an energy source. “Housing” refers to the structural role of large carbohydrates – primarily glycosaminoglycans (GAGs) – that largely comprise the proteoglycans component of the extracellular matrix (ECM) in forming the microenvironment (or “niche”) in which most cells in the body reside (Figure 1). Finally, sugars coat the surfaces of virtually all mammalian cells and, much like clothing, signify the function of a cell and specify how it interacts with its surroundings.

This article first (in Section 2) outlines how carbohydrates contribute to the stem cell niche, with a focus on their role as embryonic markers and emerging evidence that they play many active roles in determining cell fate. Next (in Section 3) difficulties of manipulating sugars in living cells are discussed, with an emphasis on a solution to this problem – metabolic glycoengineering – that is providing the tissue engineer with new experimental tools. Finally (in Section 4), challenges in translating metabolic glycoengineering from the laboratory into

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clinical practice are outlined along with new opportunities arising from efforts to improve the pharmacologic properties of the sugar analogs used in this technique.

2. Glycans in stem cell biology, tissue engineering, and regenerative medicine

2.1 Carbohydrates dominate the stem cell niche

Stem cells reside in niches that regulate their self-renewal, activation and differentiation [1]. Efforts to reproduce a stem cell niche in the laboratory, either to study the underlying biology of these cells or in practical attempts to engineer tissues for regenerative medicine applications, require detailed knowledge of the composition of the niche. For example, stem cell niches are maintained through signaling contributions from somatic cells, require a basement membrane for cell anchoring, and the presence of extracellular matrices (ECM) that modulate adhesion and the activity of signaling molecules [2]. As depicted in Figure 1, and outlined below, sugars are optimally positioned to help the stem cell communicate with its microenvironment and interact with its niche, thereby making crucial contributions to all of these functions.

2.2 Carbohydrates are important components of the ECM

Proteoglycans are major components of the ECM that consist of proteins linked to glycosaminoglycans (GAGs). While each has a distinctive molecular composition, GAGs typically are composed of repeating disaccharide units consisting of an amino sugar (GlcNAc or GalNAc) and an uronic acid (glucuronate or iduronate), and include polymers such as heparin, heparan sulfate, chondroitin, keratin and dermatan [3]. GAGs are a minor component of the ECM by mass (usually $\leq 5\%$) but when hydrated become hydrogels that constitute 70% or more of the volume of the ECM [4].

In addition to forming a hospitable physical home for cells, GAGs also provide an interface between cells and their surrounding microenvironment. Many cell adhesion molecules (CAMs) as well as almost all the ECM-resident adhesive glycoproteins [5], including laminin and fibronectin, have heparan sulfate-binding sites and heparan sulfate proteoglycans (HSPGs) consist of a defining core protein (such as syndecan, glypican, or perlecan) anchored in the plasma membrane and extracellularly decorated with highly sulfated GAG side-chains of heparan sulfate (HS) [6]. The resulting sugar-dominated boundary between the cell surface and ECM can be up to 4 μm thick in chondrocytes; an exceptional case where hyaluronic acid – a GAG of up to 50,000 monosaccharide units in size (most GAGs consist of dozens to a few hundred monosaccharides) – constitutes an significant proportion of the surface associated glycans [7].

2.3 Cell surface glycans

2.3.1 Cell surface sugars constitute a glycan signature for stem cells—The surfaces of all mammalian cells are dominated by the glycocalyx, a layer of carbohydrates that range in thickness from about 8 nm in leukocytes to the aforementioned several microns in chondrocytes. Most commonly, cell surface glycans are modest in size, consisting of 20 or fewer monosaccharide residues ranging up to a maximum size of 37 [8]; their modest size, however, masks an incredible potential for complexity. The total number of hexamer structures, for example, that can be built with an alphabet of 20 letters (i.e., the α and β anomers of the 10 common mammalian monosaccharides) reaches the staggering figure of $\sim 1.93 \times 10^{11}$ [8]. Exacerbating the diversity of individual glycans, each carbohydrate structure can be attached to protein in various ways (e.g., as *O*- or *N*-linked glycans; as

reviewed elsewhere [9]), to glycosphingolipids [10], or to GPI anchor structures [11] to create added complexity.

Significantly, glycans afford a versatile means to build biochemical signals – dubbed the “sugar code” [12] – as they greatly surpass other classes of biomolecules in coding capacity. Once a glycan code is present on a cell surface, it is useful only if it can be decoded; this function is fulfilled by lectins, a generic name for carbohydrate-binding proteins that recognize diverse sugar structures. Families of lectins – many derived from plants and crustaceans – have evolved to have various degrees of binding stringency and to elicit various downstream effects on signaling pathways [12,14], in part by exploiting specific intermolecular recognition through hydrogen/co-ordination bonds and for C-H/ π -interactions [13]. Lectins have been extensively used to identify and characterize cell surface glycosylation patterns [15-17] and have proven valuable for delineating embryologic developmental stages through the identification of cell types based on presentation of specific cell surface carbohydrates [18]. As discussed next, these features have established glycans as markers for defining stages of embryogenesis and led to a growing appreciation that sugars actively contribute to developmental outcomes.

2.3.2 From lectins to antibodies – stem cell markers—Lectin studies – which now include sophisticated array approaches [19-21] – indicate that the large numbers of glycoproteins and carbohydrates resident on the stem cell surface constitute “glycan signatures” for stem cells at various developmental stages [22]. The finicky binding characteristics of lectins, however, which often rely on the cluster glycoside effect and multivalency to establish both specificity and avidity [23,24], has hampered their use in the characterization of stem cells. Fortunately, although not always well appreciated, many carbohydrate structures are sufficiently immunogenic ([25,26]) to generate antibodies during hybridoma screens. Consequently, the combination of antigenicity and lineage-specific signatures fully establish glycan structures as potent tools for the identification and purification of stem cells [18] and numerous sugar-based stem cell markers – which are expressed in specific combinations by undifferentiated pluripotent cells, i.e., embryonic stem (ES) cells [27], induced pluripotent stem (iPS) cells [28], primordial germ (PG) cells, and embryonic germ (EG) cells [12] – are now known [18].

The biosynthesis of membrane-bound proteins, wherein *N*-glycans are added to ensure quality control during folding [29], ensures that virtually every surface marker, including those exploited as stem markers, is a glycoconjugate. Delving deeper, in many cases sugars are not simply incidental baggage of biosynthesis but instead dominate the properties of the marker. For example, the CD34 antigen (Figure 2A), a pioneering marker of primitive stem and progenitor cells [30,31] observed during hematopoietic reconstitution and blood formation [28,29] is a transmembrane glycoprotein that belongs to the sialomucin family of molecules. CD34 is ~75 to 80% carbohydrate by mass; in fact much of its extracellular peptide chain does not have higher orders of structure typical of proteins but instead exists as an extended polymer that serves as a scaffold for glycan display [32].

The tumor-rejection antigens (TRA) are another family of widely used embryonic markers that are highly expressed in undifferentiated human embryonal carcinoma cells but are almost absent in cells derived from pluripotent embryonal carcinoma (EC) cells, xenograft tumors, and germ cell tumor cell lines [33]. TRA antigens exemplify the rather artificial distinction between the “glyco” and “protein” parts of glycoprotein surface markers. For example, TRA-1-60 and TRA-1-81, which are expressed by human [34] and non-human primate ES cells [35-37], human iPS cells [28] and cultured human EG cells [38], can be monitored using the monoclonal antibody GCTM-2 [39] that detects a peptide epitope on the protein core of a keratan sulfate/chondroitin sulfate pericellular matrix proteoglycan [40].

Alternately, TRA-1-60 can be recognized by antibodies that bind to keratan-sulfated proteoglycan (KSPG) in neuraminidase-sensitive fashion [41], thereby illustrating that stem cell markers can directly consist of carbohydrate structures.

The widely used stage specific embryonic antigens SSEA-1, -3, and -4 represent additional carbohydrate-based embryonic stem cell markers: SSEA-1 consists of the Lewis X (Le^X) trisaccharide structure (Figure 2B) and SSEA-3 and SSEA-4 consist of five to six monosaccharides attached to a ceramide lipid tail, forming the globoseries glycosphingolipids GL-5 and GL-7 [27, 42] (Figure 2C). These antigens can distinguish types (e.g., ES v. EG) of embryonic cells in species-specific manner; for example, mouse ES cells express the SSEA-1 antigen [27] and EG cells from human [38], chicken [43], and goat [44], and primordial germ cells in turkey embryos [45] also express this marker. By contrast, undifferentiated human [34], rhesus macaque [35], and common marmoset ES cells [17], as well as human iPS cells [28], express SSEA-3 and SSEA-4, but not SSEA-1 while human EG cells express SSEA-3 only very weakly and inconsistently but strongly express SSEA-1 and SSEA-4 [38]. In general, stage specific embryonic antigens decrease rapidly upon differentiation, making them useful markers of pluripotency. In an interesting nuance, one study showed that the SSEA-1 antigen could be “unmasked” by sialidase in certain later stage cells during turkey embryogenesis [45] suggesting that the Le^X trisaccharide structure was still being made but was converted to sialyl Lewis X (sLe^X) as cells progressed beyond the embryonic stage (Figure 2B). The role of sLe^X in leukocyte homing and cancer metastasis makes it tempting to speculate that the production of this selectin ligand during embryogenesis facilitates the movement of cells throughout the body – a premise that is gaining experimental support [46] – leading to the next topic, namely that glycans are active determinants of stem cell fate and tissue development.

2.4. Glycans are active determinants of stem cell fate

To be philosophical for a moment, one might wonder why Nature provides the scientist with carbohydrate-based stem cell markers; a very unlikely explanation is that millions of years of mammalian evolution anticipated today’s moment in time when these molecules would be useful for biomedical science. A more reasonable possibility is that they are merely a byproduct or artifact of some other cellular process (e.g., the *N*-linked glycans involved in quality control during the folding of secreted and membrane bound proteins [29]) and it is just a lucky happenstance that they now can be exploited for stem cell characterization. A third – and most intriguing possibility – is that glycans are deliberately added to increase or alter the function of the host protein; this option provides a logical explanation for the installation of *O*-glycans on surface proteins as well as elaborations to *N*-glycans – such as the addition of sialic acid – that occur post-synthetically after the folding process is finished.

To build on the premise that glycan-based stem cell markers are not the passive entities that the word “marker” implies, but rather active participants in cellular function, it is instructive that most cellular glycans are on the outer surface of the plasma membrane in a position to modulate or mediate cell-cell [47,48], cell-matrix [49], and cell-molecule interactions critical to the development and function of complex multicellular organisms [50-52]. In addition, intracellular glycans – exemplified by the *O*-GlcNAc protein modification [53] – are abundant within the nucleus and cytoplasm where they can serve as regulatory switches [54]. Specific examples where glycans play important roles in maintaining stem cell pools in different organs [55] include FGF2, Notch, and Wnt signaling. FGF2 and Notch, as detailed next, illustrate the contributions of large ECM-associated and small receptor-associated glycans respectively. Interestingly, Wnt signaling appears to straddle both mechanisms as Wnt proteins interact with heparan sulfate proteoglycans in ways that modulate signaling [56,57] and metabolic glycoengineering experiments (described in more detail in Section

3.4) implicate sialylated cell surface glycans in differentiation processes control by Wnt [58].

2.4.1 FGF2 signaling, an example of the regulatory functions of GAGs—A long-held view of GAGs was that they primarily provided a structural framework for the stem cell niche but compelling evidence now implicates these macromolecules as active participants in the regulation of stem cell fate and tissue morphogenesis [59]. This concept is illustrated by the interaction between heparan sulfate GAGs (HSGAGs) and the fibroblast growth factor (FGF) family (specifically FGF-1 and FGF-2). These mitogens, which are key players in regulating self-renewal and proliferation of stem cells, bind to cell surface HSGAGs that function as co-receptors to facilitate FGF oligomerization and binding of FGF to its tyrosine kinase receptors (FGFRs) on the cell surface, leading to FGFR oligomerization and the consequent multimeric assembly of FGF-FGFR complexes that subsequently activates of MAP kinase signaling [60] (Figure 3A). More broadly, GAGs can serve as reservoirs of FGF and other growth factors within the ECM, which in turn can guide tissue development patterns and regeneration after injury [61].

2.4.2 Notch signaling, an example of the regulatory functions of small glycans—Moving from the large carbohydrates of the ECM to smaller cell surface associated glycans, Notch signaling provides an outstanding example of a sugar-based mechanism that controls an extraordinarily broad spectrum of cell fates and developmental processes through local cell interactions. Notch receptor proteins are modified by unusual *O*-linked fucosylated glycans within their numerous (up to 36) epidermal growth factor (EGF) domains [62] (Figure 3B). Initially, a protein *O*-fucosyltransferase enzyme adds fucose directly to the hydroxyl group of serine/threonine residues in certain Notch EGF repeats [63] and the fucosylated receptor then becomes a substrate for Fringe, an *N*-acetylglucosaminyltransferase, which adds a GlcNAc residue [64]. These disaccharides mediate interactions of Notch with proteins on neighboring cells and transmit signals to the nuclei that trigger cell fate decisions in several tissue types [62].

3. Metabolic glycoengineering: An emerging method for modulating embryonic cell fate

Based on consolidating evidence that glycans play many roles in the maintenance and maturation of embryonic cells, manipulation of these complex molecules holds promise in directing stem cell fate in tissue engineering or ameliorating cellular defects in regenerative medicine. To evaluate prospects for achieving these objectives, we next discuss the complexities of glycosylation, describe why conventional approaches have been largely refractory to substantial progress (Section 3.1), and then outline “metabolic glycoengineering” methods that facilitate the manipulation of glycans in living cells and animals (Section 3.2). Then, in Section 3.3, we provide pioneering examples and pilot experiments establishing the relevance of metabolic glycoengineering for regenerative medicine. Finally, we discuss how this seemingly straightforward technique actually engages a broad spectrum of regulatory and signaling mechanisms and leads to a many-layered set of overlapping responses (Section 3.4).

3.1 The incredible complexity of glycosylation hinders experimental intervention

As discussed above, the diversity of glycan structures is immense with literally trillions of oligosaccharide structures theoretically possible [8]). When restricted to the glycosidic linkages capable of being made by the biosynthetic enzymes found within a single cell, however, computer models predict a much small number of glycan structures, in the hundreds of thousands or millions [65]. This predicted diversity nevertheless remains

dramatically higher than the (usually) dozens of different glycans present in measurable quantities in any particular cell or tissue type (including human ES cells [66]). The disconnection between predicted and empirical diversity suggests that a potent controlling mechanism governs glycan biosynthesis, restricting structures to a narrow “glycan signature” diagnostic of tissue type or developmental status. Glycan biosynthesis, however, is not template based and thus is governed in a way that differs dramatically from nucleic acids and proteins; consequently, there is no straightforward way to predict the repertoire of glycan sequences made by a cell.

A major limitation of a lack of a template for glycans is that there is no equivalent of PCR that allows amplification of low copy number structures that may be present (for example, those predicted by computer models) but that remain invisible to even very sensitive mass spectrometry analysis. Equally significantly, the lack of a template means that genetic tools, such as recombinant DNA or RNA inhibition methods widely employed for manipulating nucleic acids and subsequent protein expression do not apply to glycans and oligosaccharides remain largely refractory to experimental intervention in living cells. Moreover, chemical and pharmaceutical strategies, such as the development of small molecule inhibitors of enzymes or transporters involved in the biosynthetic process, or genetic manipulation of these players, remain hindered by the lack of knowledge of what element of the glycosylation machinery to target [21] (although the latter pitfall is now being solved by computer models that can describe with considerable precision the effects of up- or down-regulation of particular enzymes [65]).

3.2 Metabolic glycoengineering – a chemical biology method to manipulate glycosylation

Metabolic glycoengineering (Figure 4) provides an increasingly attractive alternative to conventional chemical and biological approaches for manipulating carbohydrates. This nascent technology has greatly matured since Reutter and his colleagues first exploited the remarkable substrate permissivity of the sialic acid biosynthetic pathway to modify the cell surface by using a set of ManNAc analogs with elongated *N*-acyl groups [67,68] (Figure 4D). In the past decade, ManNAc analog-based sialic acid engineering efforts have blossomed, growing from the initial alkyl chain extensions to now include chemical functional groups such as thiols [69], ketones [70], and azides [71] (Figure 4E) normally absent from the glycocalyx. Furthermore, additional glycosylation pathways beyond sialic acid (e.g., GalNAc, GlcNAc, and fucose) have now been successfully exploited [72]. Because detailed descriptions of metabolic glycoengineering are provided elsewhere for readers who seek a broad perspective [72-76], in this article we will restrict our focus to elements of this technology germane to tissue engineering and regenerative medicine; specifically, the use of ManNAc analogs for the manipulation of neural cells and GlcNAc analogs for connective tissue health as discussed in the next two sections, respectively.

3.3 Pioneering metabolic glycoengineering experiments

3.3.1 ManNAc analogs – agents for neural cell engineering?—The idea that metabolic glycoengineering might be a useful tool in tissue engineering and regenerative medicine originated a decade ago when ManNAc analogs with simple *N*-acyl modifications (for example, *N*-propyl, butyl, or pentyl groups, Figure 4D) were found to alter the developmental fate of embryonic neural cells [77]. As an aside, neural tissue is a rather obvious starting point for developing practical applications for sialic acid glycoengineering because of the plentiful expression and many critical roles of this family of sugars in the nervous system (in fact, the alternate name of sialic acid – “neuraminic acid” – is derived from the initial isolation of these sugars from neural tissue [78]). ManNAc analogs have now been found to modulate several aspects of neural cell development including axonal

and neurite outgrowths [79, 80]; conceptually, these experiments hold promise for nerve regrowth in disease or spinal cord regeneration after injury.

Indications that metabolic glycoengineering extends beyond neural tissue were provided by the affects of ManNAc analogs on the adhesive properties of blood cells [81] and, broadening the playing field even more by moving to a different sugar vehicle, glucosamine (GlcN) engages an entirely different set of disease states and can in some respects be considered a natural glycoengineering experiment.

3.3.2 Glucosamine - a “natural” metabolic glycoengineering experiment?—

Glucosamine (GlcN) is an amino sugar abundant in connective tissues that serves as a metabolic precursor for the biosynthesis of glycosaminoglycans (GAGs), the building blocks of the large aggregating proteoglycans of the cartilage ECM [82,83]. Although GlcN has been touted as a chondroprotective nutraceutical for decades and certain clinical studies have shown modest efficacy of GlcN in alleviating symptoms of osteoarthritis (OA) [84-87], in part through anti-inflammatory activity that slows disease progression [82,88,89], its impact on *in vivo* cartilage regeneration remains highly controversial [90]. *In vitro* studies have been more clear cut in showing benefits for GlcN. For example, treatment of human OA chondrocytes reduced levels of cartilage catabolic mediators such nitric oxide (NO) and prostaglandin E₂ while enhancing aggrecan protein levels [91,92]; these results suggested that GlcN controls inflammatory mediators and stimulates proteoglycan synthesis in response to cartilage damage. As well as enhancing cartilage repair in this manner [89], GlcN also can direct the differentiation and tissue formation of ES cells [93]. Interestingly, a non-natural form of GlcN – “GlcNbu” bearing a butyl group attached to the amino position reminiscent of ManNbu (Figure 4D) – spurs the production of ECM components by chondrocytes [94].

3.4 Unraveling mechanism

To build on these pilot experiments, several obstacles must be overcome before metabolic engineering can be widely used in regenerative medicine. For one, the practical matter of *in vivo* delivery of monosaccharide analogs – a class of “undruggable” compounds [95] where poor oral availability has been a major factor bedeviling attempts to establish the efficacy of GlcN – must be solved (this matter is addressed in Section 4). A second necessity is to gain a more complete mechanistic understanding of metabolic glycoengineering so that the field can progress from its current trial and error phase to a solid hypothesis-driven footing. To understand the challenges faced in gaining a thorough understanding of analogs used in metabolic glycoengineering, these compounds can elicit cellular responses by one, or a combination, of mechanisms (Figure 5). These include changes to the rate of natural flux through a biosynthetic pathway (sec 3.4.1), changes to classes of surface glycans (3.4.2), changes to specific surface glycans (3.4.3), and finally, changes in the way the modified glycocalyx engages its microenvironment through adhesive interactions (3.4.4).

3.4.1 Flux: sugars as “signaling molecules”—The lac operon of prokaryotes familiarized the idea that simple monosaccharides can function as signaling molecules. Recently, based in part on the aforementioned ability of ManNAc analogs to alter the developmental fate of neuronal cells, this notion was extended to mammalian systems [96,97]. Speculatively, these analogs were posited to elicit signaling responses by engaging the transcriptional apparatus during the steps where the sialic acid biosynthetic pathway transits the nucleus (Figure 5A). Clearly, this explanation does not apply to sugars (e.g., GlcN, GalNAc, or Fuc) that employ metabolic pathways that do not alter flux through the nucleus.

Broadening the search for an explanation of how sugar analogs modulate cell signaling and gene expression can be intimidating; GlcN, for example, induces multiple biochemical and cellular effects including depletion of cellular nucleotide pools, inhibition of protein, RNA and DNA synthesis, and alterations of plasma and intracellular membranes [98]. Even focusing on carbohydrate metabolism can be unexpectedly complex because simple metabolites can alter glycan production in unanticipated ways (ammonia, for example, impinges on sialic acid biosynthesis [99,100]). The two examples of GlcN and ammonia are mentioned as caveats before we turn (in the following two sections) to explanations based on changes to cell surface glycans; the reader should be aware that although elegant explanations based on surface effects can be put forward, many mysteries remain within the metabolic processes of a cell.

3.4.2 Changes to classes of glycans—As illustrated in Figure 5B, sugar analogs can alter cell fate through the non-specific modification of “classes of similar molecules;” for example, one way that GlcN (or GlcNAc) functions is by increasing flux into the hexosamine biosynthetic pathway thereby shifting production of *N*-glycans towards highly branched structures that modulate cell signaling (e.g., TGF- β 1 [101, 102]) via the galectin lattice [103]. Similarly, ManNAc analogs that alter flux through the sialic acid pathway have the potential to alter ganglioside composition (albeit indirectly through changes to the enzymes involved in sialylation [104-106]). Gangliosides, which are sialic acid bearing glycosphingolipids, are key players in the “glycosynapse” (a name given to lipid raft structures that exist at the intersection of cell adhesion and signaling [107]) and have been described to “lubricate cell signaling” [108]. In essence, both mechanisms alter biophysical properties of the cell surface membrane – either on the micron (via the galectin lattice) or nanometer scales (via the glycosynapse) – thereby modulating signaling pathways.

3.4.3 The “sugar code” – specific changes to cell surface glycan composition—In a second explanation for how metabolic glycoengineering impacts surface glycans and downstream signaling, monosaccharide analogs alter specific epitopes of the sugar code and thus impinge on subsequent processes mediated by lectin-based recognition. This mechanism is made possible by the exquisite ability of lectins to discriminate between subtle structural differences of the core sialic acid molecule, exemplified by the Neu5Ac and the non-human Neu5Gc forms of sialic acid, which differ by a single oxygen at the *N*-acyl group (see Figure 4C). This small structural difference modulates the functioning of the immune system through siglec interactions [109], influences influenza virus binding and infectivity [110], and is speculated to played a role in the evolution of human intelligence [111]. Clearly, metabolic glycoengineering – which focuses on modifying this exact position with specificity that has been likened to submolecular microsurgery [73] – has the potential to affect all of these, as well as many additional, cellular processes that involve sialic acid, GalNAc, or fucose (Figure 5C), the three surface displayed monosaccharides impacted by metabolic glycoengineering.

3.4.4 Bringing cell adhesion into the equation—Another layer of complexity arises when surface sugars modified by metabolic glycoengineering engage adhesion processes. Adhesion in general constitutes a nexus between cell attachment to its microenvironment and downstream signaling that governs cell fate; this is particularly true for the stem cell niche where the adhesion to the microenvironment maintains stem cell functions. Attachment of stem cells to matrix or other cells is essential for viability – individual cells do not survive in suspension [112] – as well as the exchange of molecular signals that maintain the unique characteristics (e.g. pluripotency and self-renewal) of the stem cells [113]. Sialic acid is involved in cell-cell and cell-substrate adhesion in numerous ways. Well known examples include the impact of α 2,8-linked polysialic acid on the neural cell

adhesion molecule (NCAM), the importance of the α 2,3-linked sialic acid of sLe^X in leukocyte tethering and rolling, and the role of α 2,6-linked sialic acids in integrin function [72]. Therefore it is not surprising that the use of ManNAc analogs to alter sialic acid display can affect cell adhesion (e.g., treatment of HL-60 neutrophils with ManNProp enhanced the activation of β ₁-integrins and subsequently increased cell adhesion to the extracellular protein fibronectin [81]) and lead to subsequent changes in cell fate (e.g., stimulated axonal growth [79]).

Mechanistically, incorporation of analogs plausibly can alter adhesion molecule function “naturally” by changing properties (to evoke examples discussed above) of the galectin lattice or glycosynapse. An added wrinkle is the use of non-natural sugars to introduce novel chemical functionalities into the glycocalyx where – particularly in the case of sialic acid that is situated at the outer periphery of oligosaccharides – the newly installed functional groups are ideally situated for attaching cells to scaffolds with complementary chemical features. Sialic acid displaying ketones, for example, have been used to attach cells to hydrazide-derivatized scaffolds; a potential application of this technology is the removal of cancerous cells from the blood based on the over-expression of sialosides in the transformed cells [114-116].

In an example from our laboratory, a complementary co-engineered binding interface was specifically designed to control stem cell fate (Figure 5D). Briefly, human embryonic cells [117] were incubated with Ac₅ManNTGc to install thiols into surface displayed sialic acids. Cells bearing increased expression of cell surface thiols were grown on a gold surface, which led to neuron-like morphology and the accumulation of β -catenin [58], a key player in the Wnt signaling pathway involved in neuronal differentiation. These results highlight the importance of selective cell adhesion achieved via high affinity thiol-gold linkages between the metabolically glycoengineered sialic acids and growth substrate in cell fate determination. By comparison, weaker, non-specific thiol-glass interactions did not lead to changes in cell morphology or biochemical markers indicative of neural differentiation [58]. As a result, Ac₅ManNTGc was established as a representative of a new class of small-molecule glycosylation-based tools for tissue engineering [58].

4. Overcoming obstacles and exploiting new opportunities

Although significant effort remains to fully flesh out mechanistic details to precisely exploit metabolic glycoengineering for tissue engineering and regenerative medicine ends, sufficient progress has been made to contemplate the translation of this technology from *in vitro* experiments into animal models and even clinical testing. Two potential hurdles do remain, however; these two topics – first safety and second, the notoriously poor pharmacologic properties or sugar-based drugs – are discussed next.

4.1 Safety

Thoughts of using engineered glycans therapeutically – for example, by implanting tissue engineering constructs made with metabolic glycoengineering input – immediately raises the red flag of immunogenicity by evoking two well known examples of antigenic carbohydrates: blood type antigens and the “ α -gal” epitope [25]. Fears that metabolic glycoengineering could be similarly dangerous were engendered by the presence of the non-human Neu5Gc form of sialic acid on embryonic stem cells prepared using xenogenic culture methodology [118]. Because people have circulating antibodies for Neu5Gc [119], engineered tissues displaying this or other non-naturally sialylated antigens could compromise transplantation success by triggering hyperacute immune rejection. Even if this obstacle is avoided, the involvement of sialic acids in self-recognition events could lead to

unexpected impairments of cell function and tissue development for tissues displaying non-human sialic acids [118].

The concern that metabolic glycoengineering – which impacts the *N*-acyl chain of sialic acid that comprises the immunogenic structural epitope of Neu5Gc – would elicit prohibitive immune responses has been allayed by several lines of evidence. First, a re-evaluation of the danger posed by the presence of Neu5Gc on ES cells has diminished initial concerns [120,121]. Second, a survey of the immunogenicity of various *N*-acyl groups used in metabolic glycoengineering revealed that relatively few – in one study, only propionyl and phenyl groups [122,123] – are potent immunogens leaving an adequate selection of non-immunogenic analogs available for practical applications. Third, removal of the non-natural analog from the culture system results in the replacement of non-natural sialic acids with their natural counterparts over several days [58], a process that can be facilitated by the addition of natural ManNAc that increases natural flux through the pathway and hastens the restoration of the natural sialosides to the cell surface [124]. In essence, sugar analogs can be used as a vehicle to induce enduring changes – such as the use of Ac₅ManNGTc to trigger neuronal differentiation (Figure 5D) – and then the engineered tissue construct can be transplanted after the potentially immunogenic sugar epitopes have been metabolically replaced with their natural counterparts.

4.2 Increasing efficiency – challenges and new opportunities

4.2.1 Efficiency of replacing natural glycans on the cell surface—There are a couple of ways to evaluate the efficiency of installing non-natural glycans onto the cells surface – the first of these is to consider the relative level of replacement of natural sugar epitopes. In the absence of competitive flux into the sialic acid pathway, as occurs in GNE-knockout cells, ManNProp (see Figure 4D) resulted in the replacement of 85% of the natural sialic acid found on the cell surface with “Sia5Prop” [125]. *In vivo*, faced with endogenous competition (GNE-null animals are not viable) levels of substitution are lower and vary dramatically between tissue and organ types ranging from < 1% in the brain to ~ 70% in the heart [126]. As the *N*-acyl chain deviates more and more from the natural *N*-acetyl group, metabolic flux through sialic acid pathway is successively reduced (as described by Kim and coworkers [127]) until the ketone-bearing levinyol group actually inhibits metabolites to below endogenous levels. As we discuss in more detail elsewhere [72, 76] this reduced flux can be partially overcome through the use of sialic acid or CMP-sialic acid analogs, which feed into the pathway downstream of sialic acid synthase, an enzyme that comprises a “bottleneck” for *N*-acyl modified ManNAc.

In absolute numbers, there are typically hundreds to tens of thousands of copies any particular cluster of differentiation (CD) marker on the cell surface [128]; by comparison, the number of any particular sugar residue is much higher. For example, there are ~10⁹ copies of glycoconjugate bound sialic acid per cell [129] and, presumably, the majority of these sialosides are either in the secretory pathway (e.g., the Golgi) or plasma membrane. Considering that the relative areas of the Golgi and plasma membranes are ~14,000 mm² and 2,400 mm² [130], approximately 15% of cellular sialoglycoconjugates, or ~150 million, are expected to be on the cell surface. At the upper bounds of metabolic incorporation, if 70% of the natural sialic acids were replaced with an abiotic analog, there would be over 100 million neoglycans on the cell surface. Even in low flux situations, when only a few percent of natural monosaccharide residues are replaced metabolically, there would still be a several million non-natural glycans on the cell surface, which has been confirmed by Scatchard analysis for ManNLev incorporation in Jurkat cells [124]. Therefore, even when the rate of metabolic incorporation for a particular analog is low, the display of engineered

binding epitopes (e.g., in the millions) greatly exceed the number naturally-occurring adhesion molecule.

4.2.2 Improving the efficiency of analog utilization—Another way to measure analog efficiency is to consider how many molecules of analog must be administered to install a single non-natural sugar on the cell surface. For ManNAc analogs such as ManNLev, the answer is a dismal “approximately one million” [124,129]. Efforts to increase the efficiency of mono- and disaccharides metabolism began by constructing precursors with improved membrane permeability by exploiting the age old strategy utilized by aspirin [131] where ester-linked short chain fatty acids (SCFAs) increase the lipophilicity of hydrophilic drug candidates [76]. Our laboratory extended this concept from acetates to longer SCFA such as propionate and *n*-butyrate (e.g., Pr₄ManNAc & Bu₄ManNAc) based on the premise that further increases in lipophilicity would enhance membrane permeability. Correctly, we found the expected trend where analog utilization increased with SCFA length leading to enhanced metabolic efficiencies of ~600, 1,800-, and 2,100-fold for acetate, propionate, and *n*-butyrate, respectively [127].

4.2.3 SCFA-monosaccharide analogs provide new opportunities—The enhanced efficiency of peracylated ManNAc analogs in cell culture models led us to consider routes of *in vivo* administration and, thinking in this vein, we noted that tributanoylated hexosamines had attractive Lipinski Rule-of-5 properties for oral availability [95]. Subsequent testing of tributanoylated ManNAc analogs led to the discovery of intriguing structure activity relationships (SAR) that overturned the long held assumption that biological responses to these compounds emanated from the esterase-catalyzed hydrolysis products (e.g., *n*-butyrate and ManNAc). To elaborate briefly, any tributanoylated isomer of ManNAc was expected to exhibit similar bioactivity because they each provided three equivalents of *n*-butyrate for each monosaccharide upon hydrolysis. Experimental testing, however, showed that 3,4,6-*O*-Bu₃ManNAc elicited dramatically different cellular responses than 1,3,4-*O*-Bu₃ManNAc [132]; 1,3,4-*O*-Bu₃ManNAc lacked the cytotoxic and growth inhibitory effects of Bu₄ManNAc while 3,4,6-*O*-Bu₃ManNAc showed enhanced toxicity and also inhibited pro-invasive oncogenes [133]. The latter responses, which are attractive for cancer therapy, are linked to suppression of NF-κB-driven gene expression for analogs bearing a SCFA at the C6 position [133,134].

Overall, the SAR that separate the “anti-cancer” properties of SCFA-derivatized ManNAc analogs from “high-flux” properties useful in tissue engineering extend to – and in fact dominate – glycosylation and HDACi responses that emanate from the hydrolysis products of the analogs. In particular, the presence or absence of a SCFA group at the C1 or C6 position extend to “Lev” core sugars (e.g., ManNLev, Figure 4E) where the 1,3,4-*O*-isomer lacks the toxicity of Ac₄ManNLev [132] while 3,4,6-*O*-Bu₃ManNLev elicits exacerbated anti-cancer responses compared to perbutanoylated ManNLev. This newfound ability to separate flux from toxicity by leaving the C6-OH group of ManNAc analogs unprotected promises to enhance the safety of labeling and glycomic and regenerative medicine efforts. Moreover, the exquisite SAR made possible by “mix and matching” substituents of hexosamines (Figure 6) has opened the door for greatly enhanced control of the biological effects – both intended and auxiliary – or metabolic glycoengineering experiments.

5. Summary and Conclusions

The increasing ability to manipulate carbohydrates on multiple levels, ranging from synthetic efforts to reproduce that GAG component of the ECM [61] to the metabolic glycoengineering strategy described in this report, offers bright prospects for engaging sugars in stem cell manipulation, tissue engineering efforts, and regenerative medicine. The

use of monosaccharide analogs is on the cutting edge of multiple disciplines – for example, the use of small molecules to control stem cell biology is just getting underway [135,136] and the notion that monosaccharides are suitable templates for pharmacologic intervention is also fairly novel [134,137]. Consequently, efforts to translate metabolic glycoengineering into the clinic will no doubt experience growing pains but the numerous compelling roles of glycans in many aspects of stem cell biology, coupled with increasing evidence that manipulation of these molecules can regulate cell fate, ensures that robust efforts will continue and these efforts will ultimately reach fruition.

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Figure 1. Landscape of the cell surface

The cell surface is shown illustrating representative carbohydrates found in the ECM and attached to cell surface protein and lipids (note that the various components of the cell:cell and cell:matrix interface are not to scale).

Figure 2. Glycan-based stem cell markers

Examples of stem cell markers comprised of, or dominated by, carbohydrates include (A) the CD34 antigen, a highly glycosylated sialomucin, consisting of up to 80% carbohydrate by mass [32], (B) SSEA-1 consists of the (shaded) Lewis X (Le^X) trisaccharide, which can be found on CD34 as well as many other surface elements, that can be "masked" with sialic acid (to create the sLe^X tetrasaccharide) [45], and (C) SSEA-3 and SSEA-4 consist of similar glycolipid structures but with different antigenic saccharide structures [18].

Figure 3. Functional roles of carbohydrates

(A) Growth factors (e.g., FGF) are sequestered in the proteoglycan components of the ECM and also associate with GAGs attached to surface proteins to spatially organize receptors.

(B) Notch signaling depends on *O*-fucosylated glycans to transmit signals from one cell type to another during development, thereby regulating the developmental fate of the target cell (additional details are provided elsewhere [138]).

Figure 4. Overview of metabolic glycoengineering

(A) Normally monosaccharides are obtained from the diet or salvage processes and metabolically assembled into the complex glycan structures that comprise the glycocalyx of mammalian cells. (B) Non-natural monosaccharides (e.g., a ManNAc analog, shown) used in metabolic glycoengineering intercept the glycosylation pathways of a cell and substitute for the natural metabolites, ultimately replacing the native surface sugars (e.g., the Neu5Ac form of sialic acid prevalent on human cells). Types of ManNAc used in metabolic engineering include analogs used to install (C) natural mammalian sialosides Neu5Ac and the “non-human” Neu5Gc sialoside, (D) the first generation analogs developed by Reutter’s group, and (E) analogs with various chemical functional groups not naturally found in the glycocalyx.

Figure 5. Multiple, overlapping mechanisms monosaccharide analogs

Cellular responses emanating from metabolic glycoengineering can arise from one or more factors acting separately or in combination. These include (A) increased flux through glycosylation pathways that has been proposed to engage transcriptional mechanisms [96] (e.g., by activating transcription factors, TF); (B) changes to cell surface glycans such as increased *N*-glycan branching that facilitates formation of the galectin lattice [103]; (C) installation of abiotic sialic acid, fucose, or GalNAc residues into surface glycans that alter lectin binding (illustrated by the reduced binding of viruses to sialic acids with modified *N*-acetyl groups) [72], and (D) new chemical functionalities, such as thiolated sialic acids, that enable new modes of cell attachment to chemically compatible growth substrates, such as gold, that can trigger changes to cell fate, illustrated by neuronal differentiation of human embryonic cells [58].

Figure 6. The hexosamine as a template for drug discovery

(A) ManNAc (as well as GlcNAc and GalNAc, B) provides a versatile template for modulation of biological activity; by “mix and matching” groups at each of the R_1 - R_5 positions (additional R_1 groups are shown in Figure 4 and provided in other publications [72,76]), tens of thousands of compounds can be generated that have specific biological properties and pharmacologic characteristics [134].