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Exploiting metabolic glycoengineering to advance healthcare

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Abstract

Metabolic glycoengineering (MGE) is a technique for manipulating cellular metabolism to modulate glycosylation. MGE is used to increase the levels of natural glycans and, more importantly, to install non-natural monosaccharides into glycoconjugates. In this Review, we summarize the chemistry underlying MGE that has been developed over the past three decades and highlight several recent advances that have set the stage for clinical translation. In anticipation of near-term application to human healthcare, we describe emerging efforts to deploy MGE in diverse applications, ranging from the glycoengineering of biotherapeutic proteins and the diagnosis and treatment of complex diseases such as cancer to the development of new immunotherapies.

The pivotal roles that carbohydrates have in cell proliferation, signalling, structure and morphology, as well as in many diseases, have inspired numerous efforts to develop glycan-based therapies. Indeed, early efforts to develop cancer therapies focused on inhibiting glycolysis because cancer cells characteristically rely on enhanced glucose metabolism1,2. For decades, these efforts rarely yielded clinically relevant therapies, owing to technical difficulties in manipulating glycans and the intrinsic complexity of glycosylation, as reflected in articles such as ‘The bittersweet promise of glycobiology’3 and ‘The sweet and sour of cancer: glycans as novel therapeutic targets’4. The prospect of glycan-based therapies now appears brighter, in part due to advances in metabolic glycoengineering (MGE), a technique that emerged from chemoenzymatic glycan labelling efforts in the late 1980s and is used to exploit the substrate promiscuity of certain glycosylation pathways to incorporate non-natural monosaccharides into cellular glycans. Over the past three decades, MGE has evolved to become a far-reaching strategy to manipulate (that is, to understand,

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control and exploit) glycosylation in living organisms (FIG. 1). This Review focuses on the sialic acid pathway (FIG. 1), which dominates current efforts to develop biomedical applications of MGE; however, this technology also applies to other glycosylation pathways, including fucose, N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc)5, which provide future opportunities to expand clinical translation.

In this Review, we describe how developments in MGE have situated this technology to advance healthcare on multiple fronts. We begin with a brief history of MGE (FIG. 2), emphasizing facets of this technology that we consider relevant for clinical translation (a broader description of MGE can be found elsewhere[5–9]). We then discuss ways that MGE promises to benefit healthcare, including applications to biopharmaceuticals and the ex vivo use of MGE to improve cell-based therapies. Ex vivo implementation of MGE sidesteps a major hurdle to clinical translation — the poor pharmacological properties of monosaccharide-based drug candidates. The recent design of monosaccharides with improved pharmacokinetics coupled with new delivery strategies and promising in vivo tests have set the stage for the clinical translation of MGE. In particular, we highlight diagnostic applications of MGE, with an emphasis on cancer detection, and, finally, outline emerging MGE-based treatment strategies, with a focus on immunotherapy.

Chemical biology foundations of MGE

Early development of MGE

Today’s MGE technology originates from the seminal work of Brossmer, Gross and colleagues in the late 1980s and their demonstration that the remarkable substrate promiscuity of sialyltransferases could be exploited to incorporate diverse chemical functional groups into biological glycans[10–13]. Unexpectedly, even large functional groups, including fluorophores (FIG. 2Aa) and photoreactive moieties (FIG. 2Ab), attached to the C9–OH group of cytidine monophosphate-sialic acids (CMP-sialic acids) were found to be substrates for several sialyltransferases[11]. However, a limitation of these early efforts to functionalize glycans was that CMP-sialic acids were difficult and expensive to synthesize and could not be used in living cells; instead, cells had to be fixed and permeabilized to enable the non-natural sialic acid analogues to enter the lumen of the Golgi where sialyltransferases are localized. These pitfalls precluded cell-based and in vivo testing and, ultimately, clinical translation.

The approach developed by Brossmer, Gross and colleagues more than 30 years ago typifies contemporary chemoenzymatic glycan labelling strategies, in which enzymes are used in cell-free settings to create activated nucleotide sugars from monosaccharide analogues and then transfer these sugars to acceptor glycans. An advantage of the chemoenzymatic glycan labelling approach is that enzymes from different species can be ‘mixed-and-matched’[14] to exploit their different substrate promiscuities to accommodate various non-natural substituents (FIG. 2). For example, *N*-acetylhexosamine 1-kinase can be used to phosphorylate GlcNAc analogues[15]. An *N*-acetylglucosamine 1-phosphate uridyltransferase[16] or UDP–GlcNAc pyrophosphatase[17] is then used to synthesize an activated UDP–GlcNAc nucleotide sugar analogue. Once an activated nucleotide sugar has been created, an appropriate glycosyltransferase, many of which are now available in

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soluble, recombinant forms, is used to attach the monosaccharide to a glycoconjugate. Applications of chemoenzymatic glycan labelling range from constructing glycan arrays with increased structural diversity[18], modulating receptor signalling and ion channel function by in situ glycan editing[19] to detecting β-linked GlcNAc (O-GlcNAc)[20,21].

In contrast to chemoenzymatic glycan labelling, which depends on the ex vivo availability of monosaccharide-processing enzymes, MGE relies on a cell's endogenous complement of enzymes to manipulate glycosylation, which enables this strategy to be deployed in living cells and animals[22]. An important advance in MGE was the development of *N*-acetylmannosamine (ManNAc) analogues that replace the natural form of this sugar at an early stage of the sialic acid biosynthetic pathway. These compounds (FIG. 2Ba) are simpler and, thus, synthetically more amenable to structural modification compared with the CMP-sialic acid analogues used in chemoenzymatic glycan labelling. A trade-off, however, is that ManNAc analogues must be processed by several enzymes and overcome metabolic bottlenecks in the sialic acid biosynthetic pathway[23]. This constraint means that the ManNAc analogues amenable for MGE are limited to derivatives with relatively short *N*-acyl substituents (namely, propanoyl, butanoyl and pentanoyl; FIG. 2Ba), which are less bulky than the groups accommodated by chemoenzymatic glycan labelling (FIG. 2A).

Despite only modest alterations to the chemical structures, the ManNAc analogues with longer alkyl chains on the *N*-acyl groups exhibited intriguing activity, which, in some cases, could be explained by straightforward changes to biological recognition. For example, the infectivity of viruses that use sialic acid as a cell-surface receptor[24,25] can be reduced by the incorporation of a ManNAc analogue into their binding epitopes. Steric crowding resulting from the installation of ManNAc analogues with elongated *N*-acyl groups, such as *N*-propylmannosamine (ManNPr; FIG. 2Ba) or *N*-butylmannosamine (ManNBu), into sialic acids in the confined influenza haemagglutinin binding pocket[26] reduces the binding of the virus to mammalian cells. The ability of these analogues to modulate cell fate, especially in neural progenitor cells[27,28], is less well understood but has been traced to various mechanisms, including intracellular calcium fluctuations[29] and the alteration of specific receptor–ligand interactions by *N*-glycolylmannosamine pentaacetate (Ac_5ManNGc)[30].

Incorporating bioorthogonal chemical functionality

Building on the chemically inert *N*-alkyl-modified monosaccharides (FIG. 2Ba), reactive functional groups were subsequently introduced into ManNAc analogues used in MGE (BOX 1). For example, the addition of a ketone to the alkyl group of *N*-pentylmannosamine (ManNPe) to give *N*-levulinoylmannosamine (ManNLev; FIG. 2Bb) introduced a new chemical functionality onto the cell surface[31]. The ketone groups serve as functional handles to install larger moieties such as hydrazide-conjugated or hydrazone-conjugated biotin or fluorophores on the cell surface through chemoselective ligation reactions with bioorthogonal functional groups. These functionalized analogues, similar to their inert alkyl-modified counterparts, were compatible with living cells and animals. For example, in early studies, toxins and magnetic resonance imaging contrast agents were directed to glycan-displayed ketone groups expressed on cancer cells to either kill[31] or image them[32], respectively. These seminal experiments provided a foundation for both increased chemical

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diversity in analogue design and approaches to target abnormal glycosylation associated with disease.

Following the demonstration of the chemoselective ligation reaction strategy with ManNLev, several additional bioorthogonal functional groups (FIG. 2Bc,Bd) were installed into glycoconjugates through MGE (see part **a** of the figure, BOX 1). Examples of these functional groups include azides[33] and alkynes[34], which are typically absent from mammalian cells but are the classic click-chemistry reaction partners. Bioorthogonal click chemistry has evolved from copper-catalysed cycloadditions to copper-free methods exemplified by the cyclooctyne-based strain-promoted alkyne–azide cycloaddition (SPAAC; see parts **b** and **c** of the figure, BOX 1) and strain-promoted alkyne–nitrone cycloaddition. Other bioorthogonal functional groups include thiols, which are bioorthogonal in the context of glycans and have been used to direct stem cell fate and study ion channel activity[35,36]; light-activated functional groups such as phenylazides[37] and diazirines[38], which have been used to study protein–protein interactions in situ; terminal alkenes[39,40] and cyclopropenes[41,42], which have been used as chemical reporters; and, finally, isonitrile, which has been used in dual-labelling strategies[43].

Increasing efficiency

The potential clinical applications of MGE have been apparent since its early days. For example, the ability of *N*-alkyl-modified ManNAc analogues to inhibit the binding and infectivity of viruses[6] positioned these compounds as a new class of antiviral drugs. A stumbling block to clinical translation, however, is the inherently poor cellular uptake and rapid serum clearance of monosaccharide-based drug candidates[44]. For example, the poor cellular uptake of ManNAc analogues means that concentrations of ~5–50 mM are required for in vitro cell culture experiments[45]. These concentrations correspond to implausibly high doses (tens of grams per day) to treat human patients. Early efforts to increase the potency of MGE analogues focused on peracetylation to increase the lipophilicity and concomitant membrane permeability of the compounds[46,47] (FIG. 2Ca). This approach increased cellular uptake, reducing the millimolar concentrations required for cell culture experiments to the micromolar range[32]. Encouragingly, there are numerous reports that peracetylated MGE analogues are effective in vivo, usually through intraperitoneal or intravenous administration, and one report wherein a rare muscle disease (GNE myopathy) was treated upon oral delivery[48].

Although the peracetylation of monosaccharide analogues expedites their membrane diffusion and cellular uptake, the uptake can be further enhanced by including longer short-chain acyl groups, such as propionate and *n*-butyrate[49] (FIG. 2Ca). In addition to enhanced metabolic efficiency, certain acylated analogues, especially the *n*-butyrate derivatives, exhibit activities not expected from simply altering flux through a glycosylation pathway[50–52]. These ‘off-target’ activities depend on positions of the acyl groups on the core monosaccharide[53]. Specifically, tributanoylation of the OH groups at the C1, C3 and C4 positions of mammalian hexosamines (while leaving the C6–OH group unmodified; FIG. 2Cb) results in high flux through the targeted biosynthetic pathway without cytotoxicity[54], whereas the 3,4,6-trisubstituted regioisomer has enhanced cytotoxicity towards cancer cells

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and is anti-inflammatory[52]; both properties result from so-called whole-molecule effects, that is, structure-activity properties elicited by the parent compound[55]. These structure-activity relationships provide additional control over MGE applications; for example, high-flux analogues such as tributanoylated ManNAc (1,3,4-*O*-Bu_3ManNAc) that lack the detrimental (for example, pro-apoptotic) off-target activity of the 3,4,6-regioisomer facilitate biotherapeutic protein production[56–58], whereas anti-inflammatory analogues such as tributanoylated GalNAc (3,4,6-*O*-Bu_3GalNAc) show promising, disease-reversing potential for treating osteoarthritis[59–62].

Delivery strategies

A complementary strategy to further improve in vivo efficacy is to encapsulate the monosaccharide analogues in biomaterials to overcome the poor pharmacological properties of monosaccharide-based drug candidates. For example, the incorporation of butanoylated hexosamines into biodegradable polymers formulated into nanoparticles or microscale wafers prevents rapid serum clearance. This strategy extends the in vivo clearance time from 4 hours for the soluble drug[48] to one day for the polymer-embedded nanoparticles and to 2 weeks for the larger wafers[63]. The in vivo benefits of controlled release over extended time periods have been demonstrated using drug-laden electrospun nanofibres to promote cartilage regeneration over a period of several weeks[62]. In general, analogue-laden biocompatible materials (for example, 3,4,6-*O*-Bu_3ManNAc embedded in sebacic acid:polyethylene glycol[63] or *N*-azidoacetylmannosamine tetraacetate (Ac_4ManNAz) encapsulated in glycol chitosan[64]) can be formulated into nanoparticles or encapsulated in liposomes[65] to passively or actively target diseased tissue. Passive targeting commonly exploits the enhanced permeability and retention (EPR) effect, which is due to defective fenestrated blood vessels and a lack of lymphatic drainage[66,67]. The EPR effect leads to the selective accumulation of appropriately sized nanoparticles (for example, ~150–200 nm in tumours) in tissues associated with several pathologies, including cancer[68], infection[69], inflammation[70] and heart failure[71].

In addition to passive delivery approaches, increasingly complex active strategies have been developed to target diagnostics or therapeutics to cancer cells[72]. In its most basic form, active targeting of tumours involves decorating nanoparticles with antibodies that recognize cancer-associated antigens or conjugating the particles with folic acid to engage the folate receptor, which is overexpressed on many types of cancer. A more elaborate, two-step tumour-targeting approach that involves MGE and click chemistry (BOX 1) combines active and passive strategies[64]. In the first step, a tumour-bearing mouse is injected with nanoparticles loaded with Ac_4ManNAz, which passively accumulates in the tumour through the EPR effect. Metabolic conversion of the Ac_4ManNAz released in the tumour microenvironment to azido-sialic acids followed by their incorporation into cell-surface sialoglycoconjugates creates artificial azido receptors on the tumour. In a subsequent step, the mouse is injected with drug-containing nanoparticles decorated with dibenzocyclooctyne (DBCO) groups that actively target cancer cells by undergoing [3+2] cycloadditions with the metabolically installed azido groups.

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Improving biopharmaceuticals

Biotherapeutic protein manufacturing

We anticipate that the fastest way for MGE to have an impact on healthcare is by improving the safety, serum half-life and efficacy of biotherapeutic proteins. This class of drugs includes immunotherapeutics such as hormones, cytokines, antibodies, enzymes and immune cells, which have sales projected to be in the hundreds of billions of US dollars in the next few years[73]. Glycosylation is central to the safety, stability, serum half-life, immunogenicity, effector functions and receptor-binding affinity of biotherapeutic proteins[74–76]; accordingly, control of this parameter is crucial to the design and production of these drugs[77–81]. Supplementation with natural monosaccharides is already used to control glycosylation. For example, galactose supplementation of murine GS-NS0 melanoma cells during recombinant protein production increases the content of this monosaccharide in humanized immunoglobulin G (IgG)[82]. This increased galactosylation can, in turn, increase sialylation, which influences the bioactivity and serum half-life of therapeutic proteins[83]. As a second example, glucosamine and GlcNAc increase flux through the hexosamine biosynthetic pathway, thereby increasing the production of triantennary and tetraantennary *N*-glycans[84,85]. By creating additional acceptor sites for sialic acid (most *N*-glycans are biantennary), enhanced *N*-glycan branching can increase sialylation, as exemplified by a 32% increase in sialylation of interferon-γ[83]. Enhanced branching and sialylation increases the size of glycoproteins, often improving the physicochemical and pharmacological properties, as demonstrated by the enhanced solubility and in vivo activity of glycosylated leptin[86] and darbepoetin alfa, a variant of erythropoietin with increased glycosylation[87].

The predominant natural monosaccharide used to metabolically glycoengineer therapeutic proteins is ManNAc, which increases sialylation[83,88,89]. Terminal sialic acid masks the underlying galactose and GlcNAc residues of circulating serum glycoproteins from Ashwell-Morell (hepatocyte asialoglycoprotein) receptors, thereby inhibiting endocytosis and slowing clearance from systemic circulation[90,91]. Moreover, the net negative charge of sialic acid under physiological pH further prolongs the serum half-life of a protein by decreasing proteolytic degradation and renal clearance by the kidneys, owing to electrostatic repulsion from the negatively charged glomerulus[92–94].

The chemical structure of natural ManNAc can be modified to optimize the biomanufacturing of therapeutic proteins; in particular, the inclusion of three *n*-butyrate groups in the high-flux 1,3,4-isomer[53,54] (FIG. 2Cb) increases efficiency and enhances overall levels of sialylation. For example, ~100 times less 1,3,4-*O*-Bu_3ManNAc than natural ManNAc is required to enhance sialylation of erythropoietin and IgG antibodies[56], which is important given the high cost of ManNAc. In addition, the *n*-butyrate released during metabolic processing of ManNAc (FIG. 2Ca) enhances recombinant protein production while improving glycan quality[57,95]; this is a notable improvement over past efforts to increase recombinant protein production using sodium butyrate, which resulted in decreased glycan quality[57,96].

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Design of antibody–drug conjugates

In addition to increased productivity and enhanced glycan quality achieved during biomanufacturing, MGE can provide therapeutic glycoproteins with new features. This idea is illustrated by antibody–drug conjugates, for which MGE has been used to covalently link drugs to antibodies through the single *N*-glycan in the crystallizable fragment (Fc) region of each heavy chain of IgG. This strategy ensures that the drug is not linked to the antibody's antigen-binding fragment (Fab) domain and, thus, the products retain immune recognition. As they have a specific site for drug conjugation, these antibody–drug conjugates are more uniform than those produced through random or semi-random conjugation strategies and, thus, have more predictable pharmacodynamic properties. In an early demonstration of this strategy, the fucose analogue Ac_5-6-thiol-fucose (FIG. 3a) was used to metabolically incorporate thiol functional groups into the core fucose of the glycans in the Fc region of an IgG monoclonal antibody. A maleimide-mediated reaction was subsequently used to crosslink a drug to the thiolated glycans[97]. However, a downside of targeting the core fucose is that the maximum drug-to-antibody ratio is 2, with an actual value of 1.3 reported in this study.

Although MGE using a chemical functional group not normally found in glycans provides a superior synthetic route to well-defined antibody–drug conjugates, thiol-based conjugation (FIG. 3a) is not completely bioorthogonal because the maleimide reagents can react with thiol groups that form upon reduction of disulphides naturally present in antibodies. Subsequent MGE-based syntheses of antibody–drug conjugates have used azido-modified ManNAc analogues[98] (FIG. 3b) rather than fucose analogues to take advantage of both the higher number of sialic acids in *N*-glycans and the increasingly diverse arsenal of azide-reactive reagents now available (BOX 1). Moreover, the azide-based conjugation strategy is fully abiotic, preventing nonspecific cross-reactivity associated with maleimide conjugation to thiols. Antibody–drug conjugates represent just one way that bioorthogonal ligation reactions are creating a foundation for clinical applications of MGE; several additional examples are discussed below.

Cell-based ex vivo therapies

Cell homing

The success of cell-based therapies in tissue engineering, regenerative medicine and cancer treatment depends on the ability to control cell migration, because, typically, as few as one or two percent of therapeutic cells home to the defective target tissue. In many cases (for example, leukocyte homing to sites of inflammation and lymph tissue as well as cancer metastasis[99,100]), innate cell homing is initiated by adhesive interactions between ligands such as sialyl Lewis X (sLe^X), a tetrasaccharide epitope that includes both sialic acid or fucose, and selectin receptors on the vascular epithelium of target tissues. This homing mechanism works inefficiently for therapeutic cells because, like almost all cells in the body, they typically do not express sLe^X. However, most therapeutic cells do express one of two trisaccharide precursors to sLe^X that lack either fucose or sialic acid. When fucose is lacking, fucosyltransferases expressed in the target cells or introduced extracellularly have been used to install sLe^X onto therapeutic cells[101]. Similarly, sialyltransferases can be used

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to install sialic acid when this monosaccharide is absent from the nascent sLe^X structure[102]. Both approaches to glycoengineer sLe^X onto the surfaces of therapeutic cells have successfully facilitated the homing of implanted therapeutic cells to tissues such as bone marrow[103,104], mouse calvarium[101] and the central nervous system in an experimental autoimmune encephalomyelitis rodent model[105], as well as regulatory T cells to sites of inflammation[106].

In addition to genetic manipulation, MGE can be used to increase sLe^X expression to increase cell tropism to target tissues. For example, unlike most therapeutic cells, the SW1990 pancreatic cancer line expresses the necessary sialyltransferases needed to produce sLe^X; nevertheless, treatment with 1,3,4-*O*-Bu_3ManNAc, which increases flux through the sialic acid biosynthetic pathway, increased sLe^X expression in SW1990 cells by ~50%[107]. Similarly, treatment of Chinese hamster ovary cells, which overexpress sialyltransferase, with 1,3,4-*O*-Bu_3ManNAc further increased sialylation[56,58]. These results indicate that, even when therapeutic cells that lack sLe^X expression are provided with the requisite sialyltransferase[102], full expression of this epitope is not achieved unless flux through the sialic acid pathway is concomitantly increased through ManNAc analogue supplementation. MGE can also enhance sLe^X expression in ways that do not directly depend on increased metabolic flux. For example, in a study using human promyelocytic leukemia (HL60) cells, both ManNAc and ManNPr supplementation increased sLe^X production[108]. However, ManNPr increased the abundance of sLe^X more strongly than ManNAc, despite supporting a smaller increase in flux through the sialic acid biosynthetic pathway[49]. Moreover, the response elicited by ManNPr was selective to sLe^X, preferentially increasing its expression compared with that of most other cellular sialoglycans, possibly by changing the affinities of the activated sialic acids for different sets of sialyltransferases[108]. In a follow-up study, ManNPr also increased sLe^X expression on mesenchymal stromal cells (MSCs, also known as mesenchymal stem cells)[109], enhancing the in vivo homing of these cells (FIG. 4a). The ability to control cell homing has medical applications ranging from enhancing human cord blood engraftment[110] and increasing osteotropism of MSCs for bone repair[101] to augmenting the neurotropism of neural stem cells in multiple sclerosis[105].

Tumour-infiltrating cells

Tumour-infiltrating cells are heralded as a novel cancer therapy owing to their ability to lyse or otherwise combat cancer cells, leading to tumour regression[111]. A study has shown that pre-labelling of tumour-infiltrating MSCs with metabolically glycoengineered azide groups (FIG. 4b) does not interfere with the innate ability of the MSCs to selectivity home to lung and metastatic ovarian tumour microenvironments. Instead, this strategy facilitated bioaccumulation of the azide tags carried into the tumour microenvironment by the MSCs. Once localized in a tumour, the azide-bearing MSCs can be imaged after ligation with a Cy5.5-conjugated DBCO fluorophore[112]; the MSCs thus serve as a proxy to locate the tumours. This study also illustrated the close ties between MGE-based diagnostics and therapeutics by showing that the tumour-infiltrating MSCs can also be targeted with paclitaxel-loaded, DBCO-functionalized nanoparticles to reduce tumour growth and concomitantly enhance survival[112].

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Control of cell fate

The ability to modulate cell adhesion[107], signalling[113] and differentiation[29] creates a niche for MGE in tissue engineering, whereby cells are combined with biomaterials outside the body to create functional tissues for reimplantation. MGE appears to be particularly promising for neural tissue regeneration, with studies on the early *N*-alkyl-modified ManNAc analogues (FIG. 2Ba) showing that they regulate the differentiation of rodent neonatal neuronal cells[27,29]. In human cells (an embryoid, body-derived, lymphatic, vascular, endothelial cell line), a peracetylated, *N*-thiolated ManNAc analogue (Ac_5ManNTGc) stimulated cell adhesion to complementary gold or maleimide-derivatized scaffolds and induced changes in the morphology, cell signalling (for example, β-catenin pathway activation) and epigenetics (for example, changes to the expression of genes such as nestin) that are consistent with differentiation to a neural cell lineage[35,114]. Using a hydroxyl variant of the thiol analogue, Ac_5ManNGc, the resulting *N*-glycolyl sialic acids installed on neural cells inhibited myelin-associated glycoprotein binding, promoting axonal outgrowth and spinal cord regeneration[30] (FIG. 4c). On the basis of the challenges inherent in effectively controlling cell fate in tissue engineering and regenerative medicine, the ability to control differentiation and other attributes of cell function through MGE will substantially benefit healthcare.

MGE-based diagnostics

We posit that biotherapeutic proteins and ex vivo cell-based strategies offer streamlined routes for clinical translation because deploying MGE outside of the body sidesteps the poor oral availability and rapid serum clearance of monosaccharide analogues. Notwithstanding challenges in using MGE in vivo, ongoing advances (for example, the development of ester-derivatized analogues for enhanced cellular uptake; FIG. 2C) provide hope for clinical translation in the near future. In particular, abiotic functional groups incorporated into glycoconjugates through MGE can serve as disease-selective chemical tags, that is, artificial markers for diagnosis. The tumour-infiltrating MSCs[112] discussed above provide an example of this strategy; in this case, the cells were treated with MGE analogues ex vivo to install artificial receptors for imaging. As an alternative to cell-based approaches, which are promising but prohibitively expensive at present, an attractive option is to install artificial markers selectively in diseased tissue through direct in vivo administration of MGE analogues. Once metabolically incorporated into tissue, the non-natural, functionalized glycans enable new bioimaging modalities (FIG. 5).

Artificial markers for imaging and biomarker discovery

The ability to distinguish healthy from defective tissue is a fundamental requirement of efforts to detect, treat and monitor disease. Cancer exemplifies the difficult nature of this task because differences in the surface markers of healthy and diseased cells are often marginal. Moreover, variations between patients, as well as the heterogeneous nature of a single tumour, make it difficult to target cells of interest (for example, cancer stem cells[115]) with precision. Considering the inherent difficulty in exploiting endogenous markers, researchers are engineering artificial markers to detect diseased cells. The development of bioorthogonal, copper-free click chemistry combined with MGE-based bioimaging in living

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animals exemplifies this paradigm. This two-step strategy first involves injecting live animals with an azido-modified MGE analogue, which is metabolized and expressed on the surface of the target cells as an artificial receptor. In a subsequent step, the animals are injected with a fluorophore conjugated to a ring-strained cycloalkyne, which undergoes bioorthogonal ligation to the glycoengineered azido receptors, thereby enabling bioimaging of the target cells or tissues. In an early proof-of-concept experiment, this copper-free bioorthogonal imaging strategy was used to image membrane glycans in zebrafish[116], laying the foundation for MGE bioimaging in rodents[117] (FIG. 5a).

To date, MGE-based imaging in mice and rats has focused on cancer diagnosis. For example, murine Lewis lung carcinoma and T cell lymphoma were selectively imaged in mice treated with $Ac_4ManNAz$ followed by conjugation with a biotinylated phosphine[118]. The tumours were then stained with an avidin-conjugated far-red fluorophore and visualized using optical imaging or probed with a radioisotope by single-photon-emission computed tomography[118]. In a variation of this approach, tumour-bearing mice were treated with $Ac_4ManNAz$ and then probed with DBCO-conjugated liposomes laden with a Cy5 fluorophore[119]. This approach also enabled the selective imaging of human adenocarcinomic alveolar basal epithelial cells used in a murine tumour model[119]. Since these initial reports, MGE-based in vivo imaging modalities have proliferated, ranging from iron-oxide nanoparticles imaged by magnetic resonance and gold nanoparticles detected by computed tomography[64] to aggregation-induced emission (AIE) dots based on a polyyne-bridged, red-emissive, AIE fluorogen, 2TPE-4E[120].

In addition to imaging tumours in living animals, MGE also holds promise for cancer diagnosis through biomarker discovery based on the capture and analysis of bioorthogonally modified glycoconjugates[121]. To date, this approach has focused on azido-modified ManNAc analogues to metabolically label cancer cells as a first step in biomarker discovery. The azido-modified glycoconjugates are subsequently biotinylated using bioorthogonal modified Staudinger or click-chemistry ligation reactions[33,122], enriched through capture on streptavidin-derivatized beads or resin and then characterized through glycoproteomics analysis[33,122]. In a pilot demonstration of this approach to identify glycosylation sites[123], ~55 glycosites in SW1990 pancreatic cancer cells treated with a tributanoylated ManNAz (1,3,4-*O*-$Bu_3ManNAz$) were identified, including several glycoproteins implicated in pancreatic cancer and identified in primary tumour biopsy samples[122]. In a similar mass-spectrometry-based study, an expanded set of 954 metabolically labelled potential biomarkers were identified in three pancreatic ductal adenocarcinoma lines[124].

Although in their early stages, increasingly sophisticated MGE-based biomarker discovery approaches[125] provide new, metabolism-based strategies to diagnose cancer. In particular, the ability of MGE labelling to install chemical tags into putative biomarkers offers two advantages over current methods. First, the presence of the chemical tags provides a means to enrich low-abundance glycoprotein markers. In theory, this ability enables the detection of vanishingly small quantities of glycoproteins from serum that would not be possible without MGE-based enrichment, thereby enhancing the detection of early-stage cancer. Second, this method takes advantage of the unique, carbohydrate-based metabolitic features of cancer cells[126] to selectively label glycoconjugates produced in tumours over those in healthy

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tissue. The success of burgeoning MGE-based biomarker discovery efforts relies on tumour-selective metabolic incorporation of non-natural bioorthogonal analogues, necessitating an increase in specificity.

Increasing tumour selectivity through MGE

Heterogeneity within and between tumours coupled with often subtle differences between key biomarkers in healthy and diseased tissues makes precise diagnostic or therapeutic targeting difficult using conventional methods[115]. MGE provides an attractive option to increase the precision of cancer targeting, thus enabling bioimaging or biomarker discovery based on the selective partitioning of non-natural monosaccharides into tumours and tumour-associated carbohydrate antigens (TACAs). Even in healthy animals, MGE analogues selectively partition into a subset of cell and tissue types, as demonstrated in organisms ranging from zebrafish[116] to rodents[127] (FIG. 5b, left). In theory, tumours (FIG. 5b, second from left) can be diagnosed using MGE provided that the labelling patterns of healthy tissues are known and areas of strong background labelling are distinct from the locations of the tumours. Conceptual precedent for this approach is provided by tumour diagnosis using ^{18}F-labelled 2-deoxyglucose, which is a clinically established method[128]. Nevertheless, diagnostic strategies designed to detect diffuse and metastasized tumours that rely on the partitioning of a metabolic probe into tissues characterized by a low endogenous background signal can be challenging, especially if the tumours are located in areas where the probe is incorporated into healthy tissue at measureable levels (FIG. 5b, second from right). Accordingly, increasing the selectivity of the incorporation of MGE labelling reagents into tumours is needed to increase diagnostic sensitivity and specificity (FIG. 5b, right).

The tumour specificity of MGE-based metabolic labelling and imaging is determined at several levels by a complex interplay of physiological, genetic and metabolic factors[129]. At the physiological level, tumour-selective metabolic labelling is facilitated by an abundant supply of the MGE analogue to a tumour microenvironment; in many cases, the robust vascularization of tumours helps direct these compounds to the tumours. In addition, the EPR effect can be exploited for disease-specific delivery of MGE analogues by embedding these compounds in nanoparticles; indeed, several cancer-targeting, MGE-based strategies already use nanoparticles[65,112,119,120,130,131]. MGE-relevant TACAs are often abundant in cancer because of upregulated expression of tumour-associated genes that encode enzymes and transporter proteins that regulate glycosylation. For example, sialylated epitopes[132] that are susceptible to replacement by ManNAc analogues are produced by sialyltransferases such as ST6Gal1 (REF[133]) or polysialyltransferases[134], which are both overexpressed in cancer cells. sLe^X exemplifies a sialylated TACA for which MGE can be used to increase the abundance[107] (FiG. 4a). Another cancer marker influenced by MGE is ganglioside GM3, which can be metabolically glycoengineered using ManNPr and *N*-phenylacetylmannosamine (ManNPhAc) to enhance immunotherapy (discussed further below).

An emerging tactic that provides even greater selectivity for tumour labelling involves caging the MGE analogue by conjugation to a cleavage substrate to restrict the uptake or metabolism of the precursor until initiated by cleavage mediated by an enzyme that is

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specific to the cancer cells. This general strategy was developed to target prodrugs and imaging probes to tumours[135–137] and later adapted for MGE. In a proof-of-concept investigation, a caged Ac_4ManNAz analogue was designed for selective metabolism by Chinese hamster ovary and prostate cancer cells that overexpress the prostate-specific antigen (PSA)[138]. Specifically, a PSA substrate (PSA is a serine protease) was conjugated to the C6 position of ManNAz, rendering the analogue non-metabolizable until PSA secreted from the cancer cells cleaves the substrate to enable cell uptake and metabolism[138]. One pitfall of this strategy is that active ManNAz is produced extracellularly, which potentially limits the selectivity of this approach because secreted PSA in the circulation (with globally elevated levels in cancer) could uncage the substrate at a distance from the target tumour.

Cathepsins, which are cysteine proteases overexpressed in the cytoplasm of various cancer cells[139,140], provide another option to uncage metabolic substrates. Importantly, because cathepsins are intracellular, cancer cells can be targeted with increased precision. In one study, cathepsin B was used to uncage KGRR-conjugated Ac_3ManNAz (where KGRR (Lys–Gly–Arg–Arg) is a cleavable peptide), increasing the display of azide-tagged glycans on cancer cells and aiding targeted bioimaging[141]. In a second study, cathepsin L was used with a second enzyme (histone deacetylase) to achieve even greater cancer selectivity (FIG. 5c). In this case, the C1 position of peracetylated ManNAz was caged by substituting the anomeric acetyl group with an ether linkage to inhibit extracellular hydrolysis. Histone deacetylase and cathepsin L sequentially uncaged the C1 position, selectively releasing 3,4,6-Ac_3ManNAz inside cancer cells[142].

Designing MGE-based therapies

An alluring goal of MGE in healthcare is the use of glycosylation-modifying monosaccharides as drugs. In some cases, as discussed below, MGE analogues hold therapeutic potential as drug candidates that directly modulate biological activity. More commonly, MGE therapies follow the two (or more)-step paradigm described above for diagnosis, wherein the first step involves targeting diseased tissue with a metabolic probe and the second step involves the delivery of the diagnostic agent. In many cases, bioorthogonal conjugation strategies used to target an azide-laden tumour with a fluorophore or other imaging agent for diagnosis can be adapted to deliver therapeutics. The concept of exploiting MGE to kill cancer cells dates back to the initial use of ManNLev to install bioorthogonal chemical functionality into cell-surface glycans. In this early work, lymphoma cells were eradicated by directing a ricin conjugate to cells expressing sialic-acid-displayed ketones[31]. The therapeutic potential of MGE extends beyond directing toxins to cancer cells to encompass cutting-edge immunotherapy and cell-based therapies (FIG. 6).

Direct-*a*cting and multistep MGE drugs

In some cases, MGE promises direct therapeutic benefits upon administration. One example is the administration of Ac_4ManNAc to ameliorate GNE myopathy[48,143], which results from genetic loss of GNE (the primary enzyme that produces ManNAc in mammals[144]), by compensating levels of ManNAc. Increased metabolic flux through the sialic pathway may also offer therapeutic benefits. For example, upon treatment with 1,3,4-*O*-Bu_3ManNAc, the

increased sialylation of pancreatic cancer cells dampens oncogenic epidermal growth factor receptor signalling[113]. Direct administration of MGE analogues may also be an effective treatment for osteoarthritis[59–62]. In osteoarthritis, therapeutic activity is likely a combination of the whole-molecule, anti-inflammatory activity of the parent 3,4,6-tributanoylated hexosamine[51,52,55], epigenetic effects of the released *n*-butyrate (which is a histone deacetylase inhibitor, a class of compounds that have therapeutic activity against osteoarthritis[145]) and the effects of increased flux through the hexosamine biosynthetic pathway[59,60].

Although MGE administration can have direct therapeutic effects, in other cases, MGE intervention alone is not effective. Indeed, despite some of the examples discussed above, MGE is often regarded as a 'silent' labelling technique that has minimal impact on biological structures, processes and functions. Azide-modified analogues exemplify this idea, because the *N*-azide group has not been reported to modulate biological recognition events. In other cases, such as the treatment of SW1990 pancreatic cancer cells with 1,3,4-*O*-Bu_3ManNAc to increase surface sialylation[107,113], measurable changes to cell motility, adhesion[107] and signalling[113] were observed, but there was no convincing antitumour effect (for example, the induction of apoptosis) in cell culture experiments that would justify in vivo testing. However, 1,3,4-*O*-Bu_3ManNAc treatment re-sensitized the drug-resistant SW1990 line to tyrosine kinase inhibitor drugs erlotinib and gefitinib[146]. If this finding holds in vivo and the effectiveness of erlotinib and gefitinib observed in this pilot study is restored in human patients, this will be an important clinical advance and provide precedent for the synergistic use of MGE with other classes of drugs.

Passive immunotherapy

Coupling MGE with an auxiliary input provides a platform for the development of cancer-vaccine-based immunotherapy. The incorporation of non-natural monosaccharides into cellular glycans is almost always weakly if at all immunogenic; this general lack of immunogenicity is helpful because it enables the safe in vivo translation of MGE. ManNAc analogues, however, do exhibit immunogenicity in certain cases; for example, ManNPhAc metabolically replaces the natural sialic acid moiety of ganglioside GM3 with *N*-phenylacetylated sialic acid to form *N*-phenylacetyl GM3 (GM3NPhAc)[147–150]. The tumour-selective, cell-surface expression of GM3NPhAc observed in various types of cancer, including melanoma, leukemia, breast cancer, pulmonary cancer and prostate cancer, has spurred the development of cancer vaccines.

Although immunogenic, ManNPhAc typically elicits a weak tumour-fighting response as a stand-alone therapy. Instead, antitumour efficacy requires a passive immunotherapy approach in which the host is supplemented with synthetic immune-system components to boost response. For example, keyhole limpet hemocyanin conjugated to GM3NPhAc (KLH–GM3NPhAc) triggers a strong immune response and can be used as a cancer vaccine to boost the antitumour efficacy of ManNPhAc[150] (FIG. 6a). In this two-step strategy, a tumour-bearing mouse was first inoculated with either sera containing anti-GM3NPhAc antibodies or the antigenic KLH–GM3NPhAc construct, which triggered the development of anti-GM3NPhAc antibodies in the immunized mouse. In the second step, the animal is

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treated with ManNPhAc to stimulate tumours to express GM3NPhAc. The cancer-vaccine-elicited antibodies then selectively recognize and coat the GM3NPhAc-expressing tumours, leading to tumour eradication through antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC) or antibody-dependent cellular phagocytosis (ADCP), thus increasing survival[150].

In a variation of this approach, the KLH–GM3NPhAc construct was not used directly as a cancer vaccine. Instead, it was loaded onto dendritic cells, which subsequently served as a more potent cancer vaccine than KLH–GM3NPhAc alone. The cells loaded with KLH–GM3NPhAc elicited GM3NPhAc-specific T cell-dependent immunity in vivo against melanoma and leukemia in animals that were also treated with ManNPhAc. This combinatorial approach decreased tumour growth, reduced metastasis and prolonged survival times[151].

Applying MGE to active immunotherapy

Elegant, multistep strategies that incorporate MGE into immunotherapy have been recently reported. For example, a drug consisting of a caged ManNAz analogue was selectively directed for uptake by cancer cells by appending a folic-acid moiety that engages the folate receptors overexpressed on many tumours[152] (FIG. 6b). Once inside a cell, the linker to the folic acid is cleaved by glutathione (or another free thiol), resulting in the selective release of 1,3,4-*O*-Ac$_3$ManNAz in the tumour cells, facilitating tumour-specific surface glycan display of azido-labelled sialic acids. The animals were subsequently treated with a DBCO-L-rhamnose conjugate, which reacted with the azido-modified glycans selectively displayed on the tumour. Because L-rhamnose is an immunogenic, non-mammalian monosaccharide abundant in the gut biome, host animals had high levels of pre-existing circulating antibodies to this antigen, resulting in tumour eradication through ADCC, CDC or ADCP[152] by macrophages and cytotoxic T cells. Thus, by using L-rhamnose as the antigen, the cumbersome passive immunization strategy required for ManNPhAc treatment (FIG. 6a) was sidestepped.

Outlook

MGE has grown from its roots in chemical biology three decades ago to be positioned today at the threshold of clinical translation. It is poised to make contributions to healthcare on multiple fronts, including facilitating the design and production of biotherapeutic proteins, enabling cell-based therapies, developing novel cancer diagnostics and providing new immunotherapy approaches. Progress in advancing MGE to the clinic has been slow, having been hindered by factors that range from the poor pharmacological properties of sugar analogues and the toxicity associated with copper-catalysed bioorthogonal click reactions to a limited understanding of the enzymatic processing of these compounds and challenges in conducting detailed structural analysis of the engineered glycans. As described above, many of these challenges have been, or are actively being, overcome. For example, the poor cellular uptake of MGE analogues has been improved through the addition of ester-linked, short-chain fatty acids and their short in vivo half-life has been extended through polymer-based delivery. Similarly, advances in copper-free SPAAC ligation reactions have enabled

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click chemistry to be used in in vivo applications. In short, substantial advances in the chemistry underlying MGE over the past three decades have brought the field to the cusp of clinical translation.

To realize the full potential of MGE, it will be necessary to take advantage of as-of-yet untapped opportunities. For example, until now, the most enticing advances have been directed towards cancer, with other applications (such as biotherapeutic protein production, treatment of muscle disorders and arthritis, and tissue-engineering applications) remaining at very early stages of development. In a second direction, owing to its origin from cell-free chemoenzymatic glycan labelling, MGE has been dominated by ManNAc analogues that target the sialic acid biosynthetic pathway. We believe, however, that extending MGE to other glycosylation pathways greatly increases the biomedical potential of this technique. As an example, the application of MGE to antibody–drug conjugates was pioneered using fucose analogues[97]. Considering a different pathway, azido-tagged hexosamine analogues can label O-GlcNAcylated proteins[153,154], which have numerous roles in disease[155]. Finally, azido-modified GalNAc analogues have been used to label T cells to increase their affinity for bicyclo[6.1.0]nonyne-modified tumour cells[156]. These three examples illustrate how MGE extends beyond sialic acid to at least four (of the ten) monosaccharides found in human glycans, creating many new opportunities to apply this strategy to healthcare applications.

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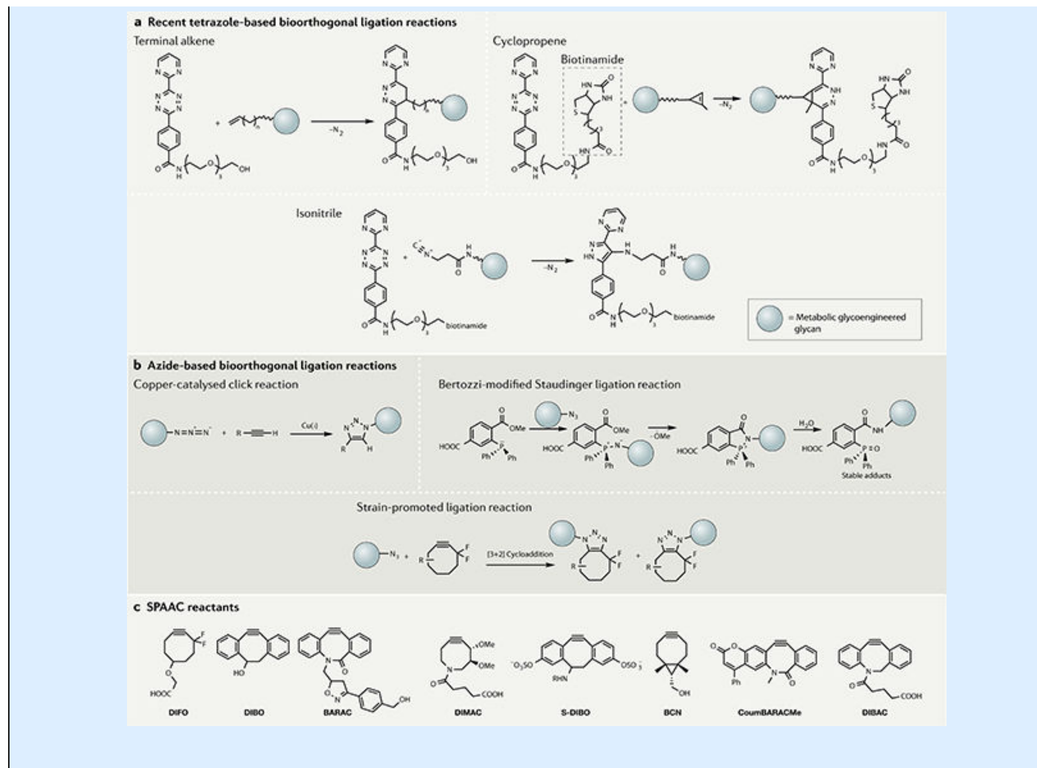
Box 1 | Bioorthogonal ligation reactions used in MGE

Bioorthogonal ligation reactions can be used to install new functional groups into monosaccharide analogues that are compatible with metabolic glycoengineering (MGE). For example, the chemoselectivity of tetrazines in inverse electron–demand Diels–alder reactions has been exploited to ligate tetrazines to monosaccharides functionalized with terminal alkenes[39,40], cyclopropenes[41,42] and isonitriles[43] (see part **a** of the figure). Moreover, the tetrazine can be linked to biotin (see part **a** of the figure) or a fluorophore for imaging purposes.

At present, most translational MGE applications use azide-based ‘click’ reactions for bioorthogonal ligation. Copper-catalysed click reactions[157] (see part **b** of the figure) can be used for the ligation of glycan-displayed azide groups outside of living systems but are incongruent with the clinical translation of MGE owing to the toxicity of copper in cells. To overcome this pitfall, the Bertozzi group modified the Staudinger reaction for ligation of glycan-displayed azides under conditions compatible with the physiological milieu in living cells[33]. Another advance in copper-free, azide-based, chemoselective ligation was the development of click reactions based on difluorinated cyclooctynes (DIFOs)[158] (see part **b** of the figure). These reactions, driven by the strain released upon conversion of the cyclooctyne to a cycloalkene, have been termed strain-promoted alkyne–azide cycloaddition (SPAAC) reactions[159]. Additional cyclooctyne-based SPAAC reactants have been developed for MGE-based azide ligation reactions[160] (see part **c** of the figure), including several dibenzylcyclooctyne variants that have narrow and specific reactivity towards azide groups at room and physiological temperatures[161]. These reagents thus provide multiple options for cell-free ligation (for example, for the production of antibody–drug conjugates) and an increasing number of cell-based and in vivo diagnostic and therapeutic applications. BARAC, biarylazacyclooctynone; BCN, bicyclononyne; CoumBARACMe, coumarin-conjugated biarylazacyclooctynone; DIBAC, dibenzoazacyclooctyne; DIBO, dibenzocyclooctyne; DIMAC, dimethoxyazacyclooctyne; S-DIBO, sulfonylated DIBO.



a Recent tetrazole-based bioorthogonal ligation reactions
Terminal alkene
Cyclopropene
Biotinamide
Isonitrile
= Metabolic glycoengineered glycan
b Azide-based bioorthogonal ligation reactions
Copper-catalysed click reaction
Bertozzi-modified Staudinger ligation reaction
Stable adducts
Strain-promoted ligation reaction
[3+2] Cycloaddition
c SPAAC reactants
DIFO
DIBO
BARAC
DIMAC
S-DIBO
BCN
CoumBARACMe
DIBAC

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Glycan

A compound in which monosaccharides are glycosidically linked to each other or to other biological molecules, such as proteins or lipids.

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Chemoenzymatic glycan labelling

A technique in which cell-free systems, typically a glycosyltransferase and complementary nucleotide sugar, are used to introduce non-natural monosaccharides into glycoconjugates.

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Glycosylation

An enzymatic process in which a glycan is covalently attached to a non-carbohydrate molecule.

Immunotherapy

A cancer treatment designed to boost the body's natural immunity to detect and eradicate cancer cells.

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Glycoconjugate

A molecule consisting of one or more glycans covalently linked to a non-carbohydrate moiety.

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Chemoselective ligation reactions

Chemical reactions that are exclusive to two mutually specific functional groups.

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Bioorthogonal functional groups

Chemical functional groups that exclusively react with a specific ligation partner under physiological conditions in living systems without perturbing native biochemical processes.

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Whole-molecule effects

Biological activity derived from intact, ester-derivatized MCE analogues not observed in their monosaccharide or short-chain fatty acid metabolites.

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Biotherapeutic proteins

Proteins produced for pharmaceutical purposes.

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Sialylation

The enzymatic addition of sialic acid, which is an *N*-substituted or *O*-substituted derivative of neuraminic acid (a monosaccharide with a nine-carbon backbone), to a glycoconjugate.

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Glycoproteins

A class of proteins with one or more covalently conjugated glycans.

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Antibody–drug conjugates

Therapeutics that combine the antitumour activity of monoclonal antibodies with the (usually) cytotoxic activity of small-molecule drugs.

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Antibody-dependent cellular cytotoxicity

A cell-mediated immune defence whereby effector cells actively lyse a target cell after its surface antigens are recognized by specific antibodies.

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Complement-mediated cytotoxicity

Elimination of antibody-coated cells through the classical complement pathway, which leads to the formation of a membrane attack complex and cell lysis.

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Antibody-dependent cellular phagocytosis

Mechanism through which antibody-coated foreign entities, such as pathogenic bacteria or cancer cells, are eliminated.

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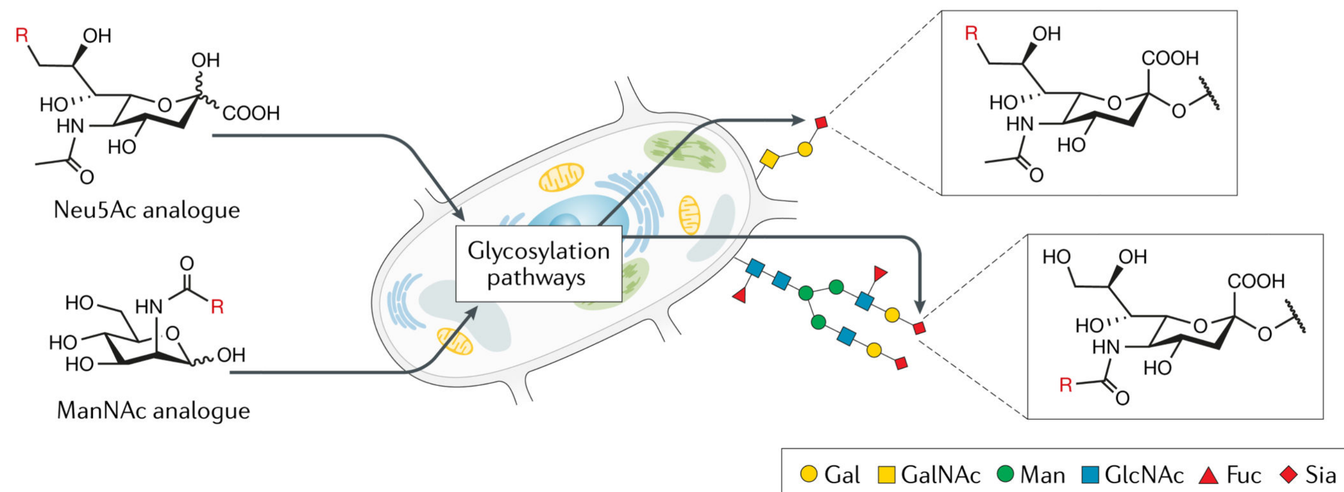

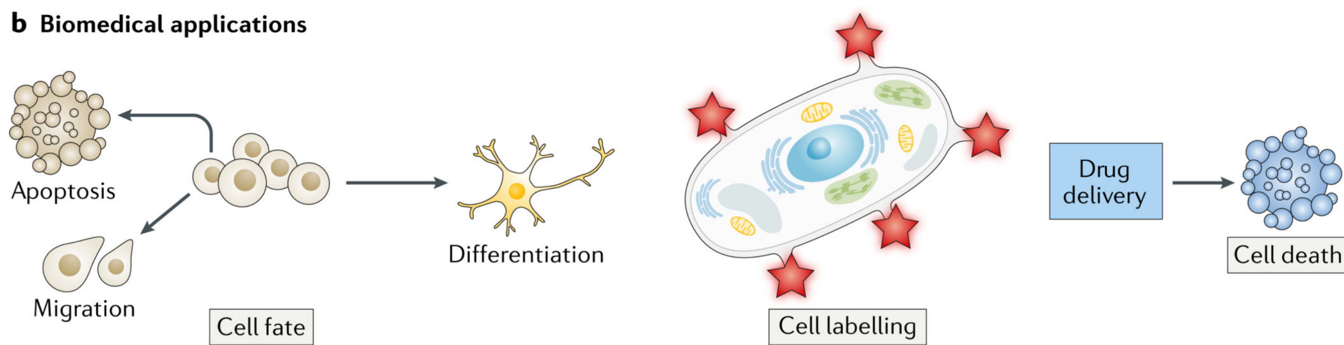

Fig. 1 |. Overview of MGE.

a | Metabolic glycoengineering (MGE) is based on chemically modified monosaccharides bearing non-natural R groups (specific examples are given in FIG. 2) that intercept the glycosylation pathways in mammalian cells, leading to the presentation of modified glycans on cell surfaces or their secretion as glycoconjugates[5,22]. Examples of modified monosaccharides include C9-modified sialic acids and *N*-acyl-modified *N*-acetylmannosamine (ManNAc) analogues; these compounds biosynthetically replace natural sialic acids in *N*-glycans, *O*-glycans and gangliosides. **b** | Biomedical applications of MGE include control of cell fate[27,28,35], namely, cell differentiation, migration and apoptosis; cell labelling for in vivo imaging[116,118,130]; drug delivery to promote healing[48,61]; or the delivery of agents to kill cancer cells[31,152]. Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Sia, sialic acid.

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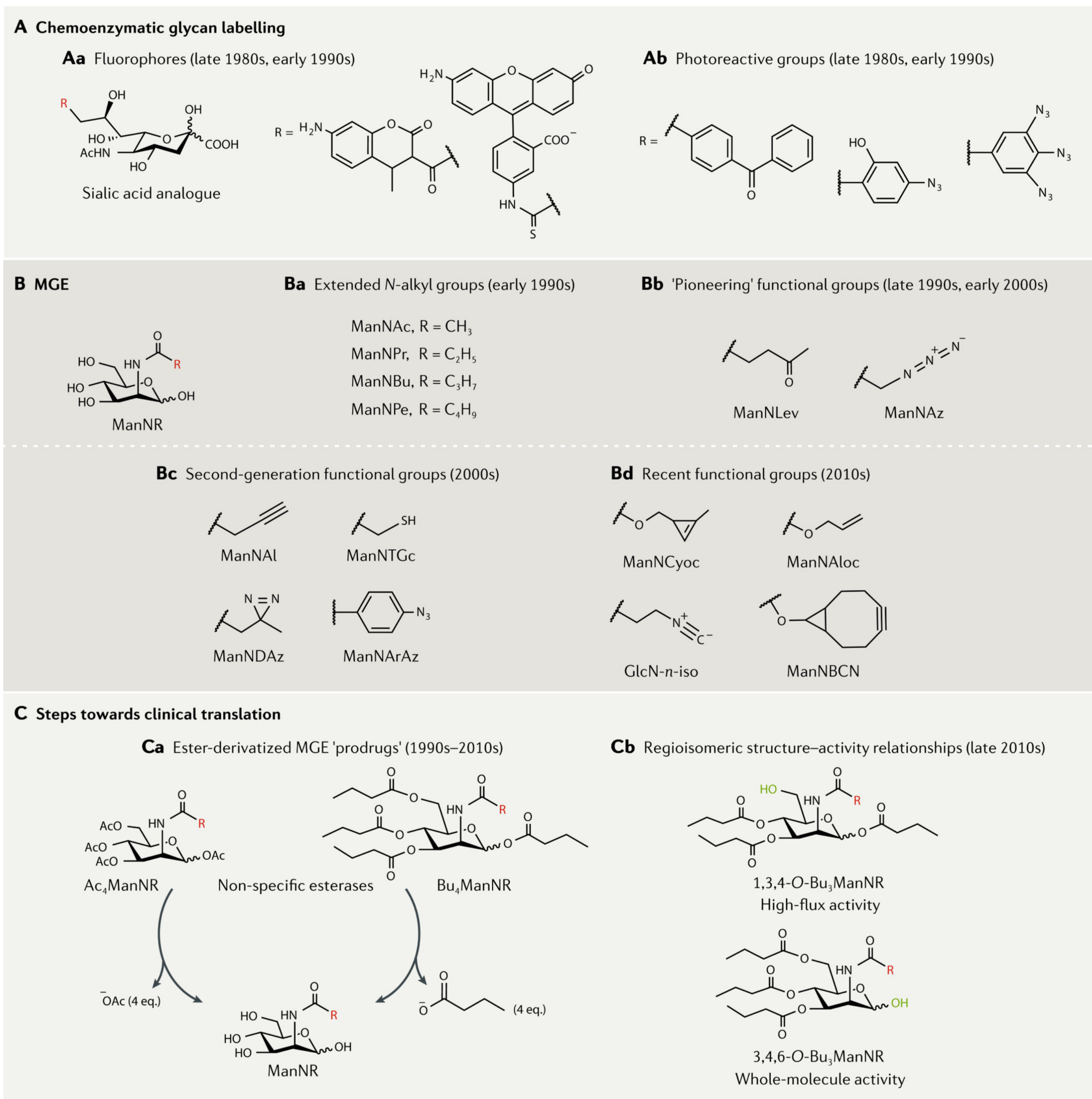

Fig. 2 |. The development of MGE.

Metabolic glycoengineering (MGE) arose from early chemoenzymatic glycan labelling technology, wherein cytidine monophosphate-sialic acid analogues that contained abiotic chemical functionalities such as fluorophores[10] (part **Aa**) and photoreactive groups[13] (part **Ab**) were used to manipulate glycosylation in fixed and permeabilized cells. Subsequently, MGE analogues based on *N*-acetylmannosamine (ManNAc) that are amenable for use in living cells were developed with extended *N*-alkyl groups[22] (part **Ba**) and bioorthogonal functional groups[31,33] (part **Bb**). The development of analogues has continued over the past

20 years, as indicated by the sampling of 'second-generation'[34,35,37,38] (part **Bc**) and more recently reported[39,41–43] (part **Bd**) labelling reagents. To complement these advances in chemical biology, efforts towards clinical translation include the development of efficient, high-potency, ester-derivatized analogues[47,49] (part **Ca**) and the exploitation of regioisomeric structure–activity relationships (part **Cb**) to either provide high levels of metabolic flux with minimal off-target activity or to increase anticancer or anti-inflammatory activity (for example, regioisomers of tributanoylated ManNR (Bu_3ManNR) exhibit unique bioactivity)[52,53]. GlcN-*n*-iso, *N*-isocyanoglucosamine; ManNAl, *N*-alkynylmannosamine; ManNAloc, *N*-allyloxycarbonylmannosamine; ManNArAz, *N*-arylazidomannosamine; ManNAz, *N*-azidoacetylmannosamine; ManNBCN, *N*-bicyclononynylmannosamine; ManNBu, *N*-butylmannosamine; ManNCyoc, *N*-cyclopropeneacetylmannosamine; ManNDAz, *N*-diazirinmannosamine; ManNLev, *N*-levulinoylmannosamine; ManNPe, *N*-pentylmannosamine; ManNPr, *N*-propylmannosamine; ManNTGc, *N*-thioglycolylmannosamine.

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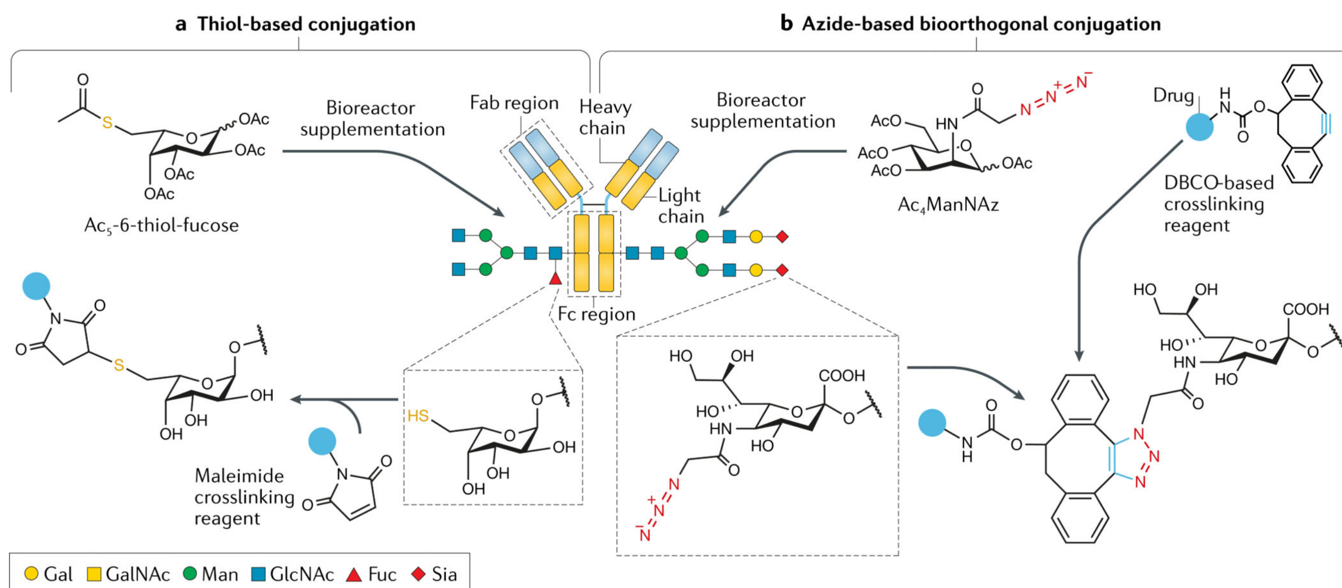

Fig. 3 |. MGE-enabled design of antibody–drug conjugates.

a | Through metabolic glycoengineering (MGE), thiol groups can be incorporated into the *N*-glycan in the Fc region of immunoglobulin G antibodies and subsequently covalently linked to drugs through maleimide conjugation[97]. **b** | The design of antibody–drug conjugates[98] has been advanced by using azide-functionalized *N*-acetylmannosamine (ManNAz) derivatives that undergo strain-promoted cycloadditions with dibenzocyclooctyne (DBCO)-based crosslinking agents. These azide-based ligation reactions are bioorthogonal, enabling the production of antibody–drug conjugates with high antibody-to-drug ratios and with drug conjugation restricted to the antibody's crystallizable fragment (Fc) region glycan by exploiting the growing number of available DBCO-based reactants (BOX 1). Fab, antigen-binding fragment; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; Sia, sialic acid.

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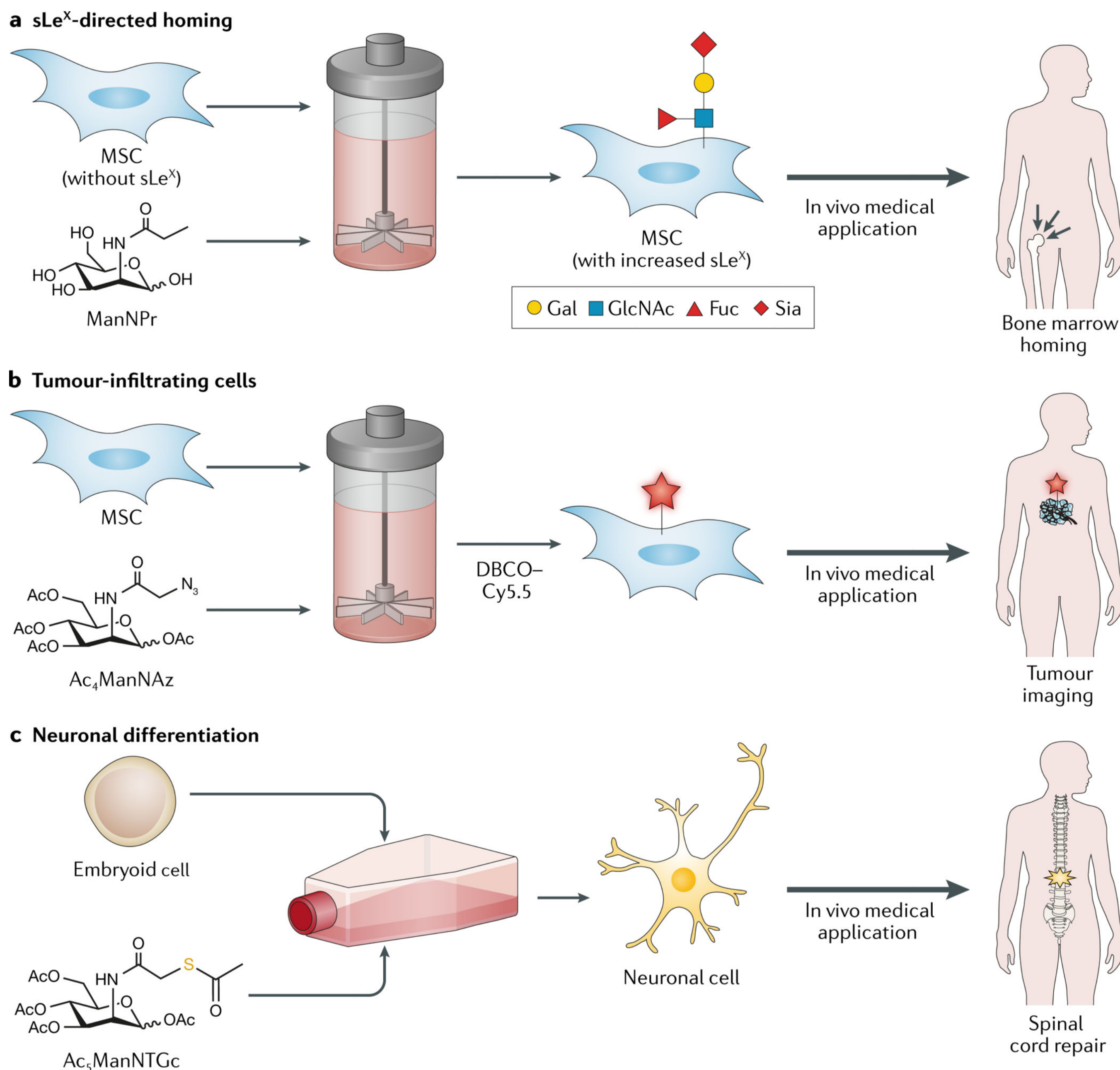

Fig. 4 |. Potential ex-vivo-based clinical applications of MGE.

Metabolic glycoengineering has potential applications in a range of cell-based therapies. **a** | Bioreactor-grown mesenchymal stromal cells (MSCs) incubated with *N*-propylmannosamine (ManNPr) show increased expression of sialyl Lewis X (sLe^X)[109], an epitope that promotes homing to bone marrow[101,103]. **b** | MSCs incubated with *N*-azidoacetylmannosamine tetraacetate (Ac_4ManNAz) retain their tumour-homing ability, which enables tumour imaging after labelling with a dibenzocyclooctyne (DBCO)–Cy5.5 conjugate (where Cy5.5 is a far-red fluorophore)[112]. **c** | Treatment of human embryoid stem cells with *N*-thioglycolylmannosamine pentaacetate (Ac_5ManNTGc) triggers neuronal differentiation[35],

which holds promise for neural engineering applications, such as spinal cord regeneration. Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Sia, sialic acid.

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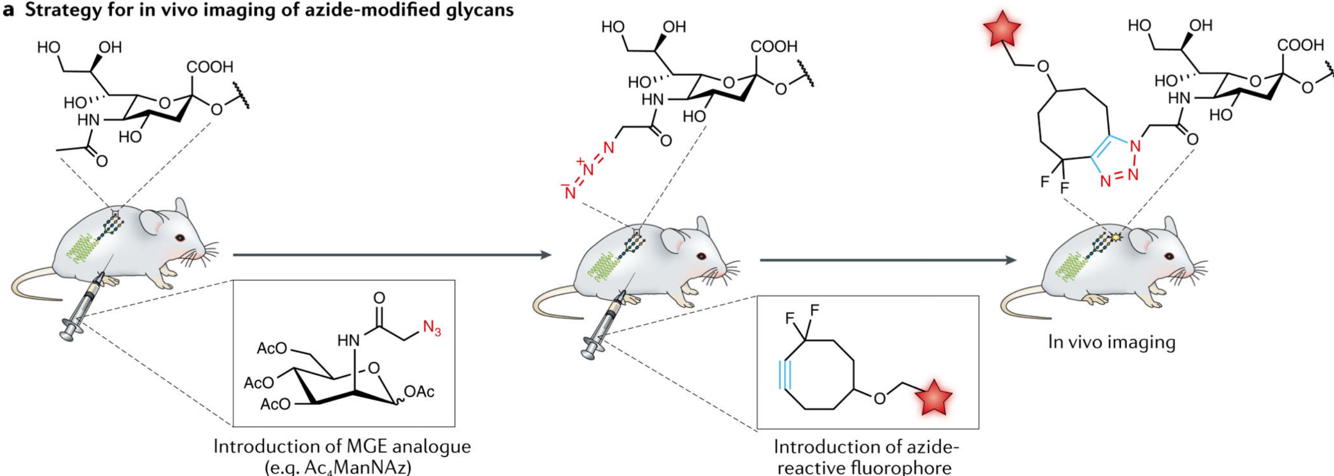

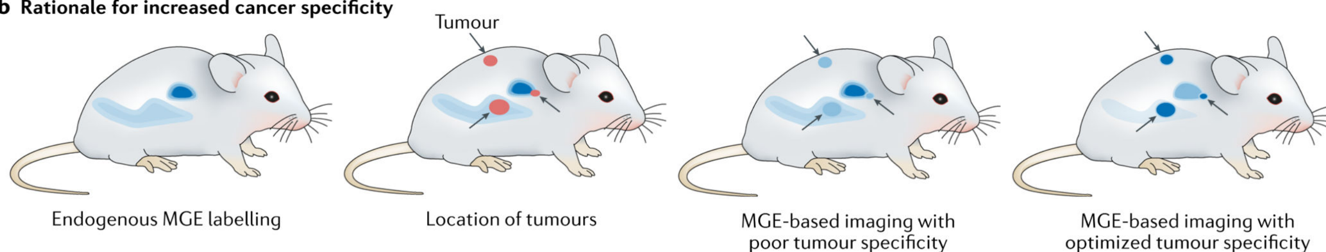

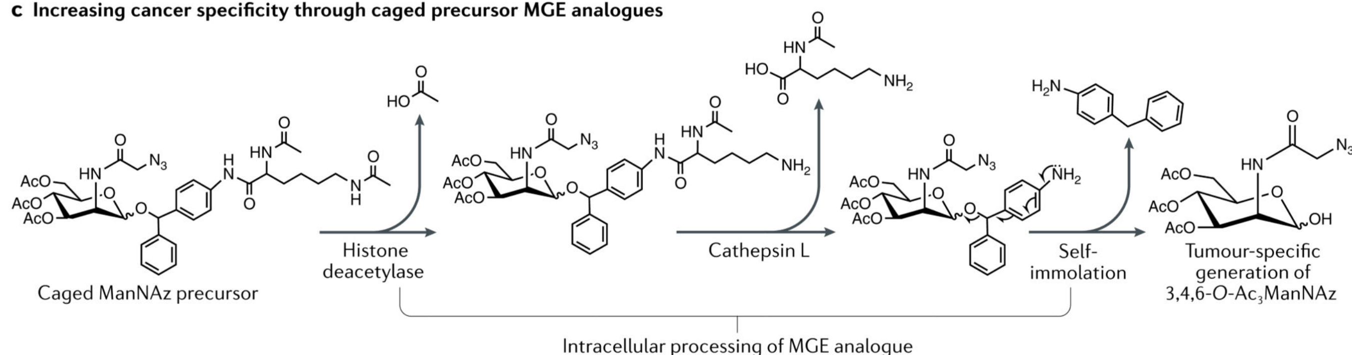

Fig. 5 |. Diagnostic applications of MGE.

Diagnostic applications of metabolic glycoengineering (MGE) are based on the incorporation of artificial labels into diseased tissue for bioimaging or biomarker discovery. **a** | For in vivo imaging, an animal is first treated with an MGE analogue such as *N*-azidoacetylmannosamine tetraacetate (Ac_4ManNAz) to selectively install azide groups in tumour-associated glycans, which can be detected through one of several imaging options, including conjugation to a fluorophore-bearing difluorinated cyclooctyne[117], a biotinylated phosphine probe visualized with avidin-conjugated fluorophores or radioisotopes[118], metallic nanoparticles[64] or aggregation-induced emission dots[120]. **b** | The need to increase the disease specificity of MGE labelling reagents is illustrated by cancer diagnosis. For successful diagnosis, the tumour-derived signal (the location of the tumours are indicated by arrows and shown in red in the mouse second from the left) must be distinct from, or quantitatively stronger than, the endogenous (background) signal (shown in blue, with a

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darker shade indicating a stronger signal). **c** | A method to increase the selectivity of MGE tumour labelling[142] exploits a caged *N*-acetylmannosamine (ManNAz) precursor that is selectively activated in cancer cells by the sequential activities of histone deacetylase and cathepsin L followed by self-immolation to produce 3,4,6-triacetyl-*N*-azidoacetylmannosamine (3,4,6-*O*-Ac_3ManNAz). As this analogue is generated selectively in cancer cells, the selectivity of tumour labelling is increased compared with Ac_4ManNAz administration (part **a**).

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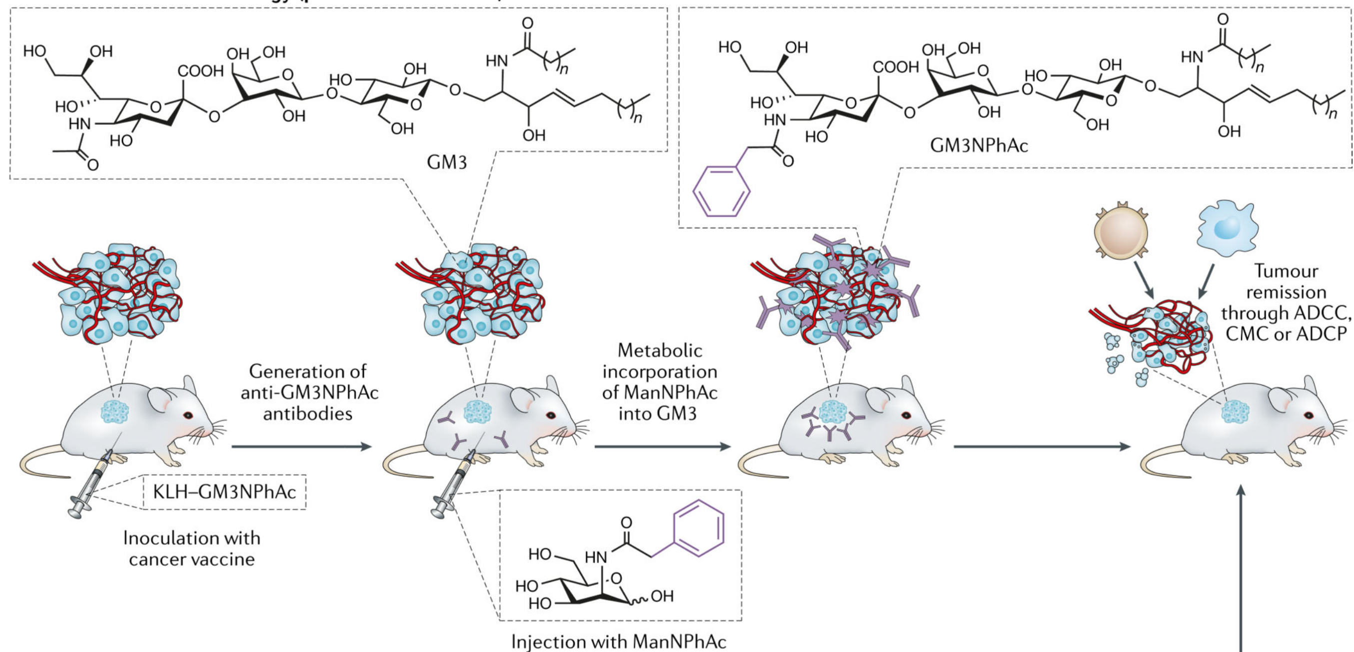

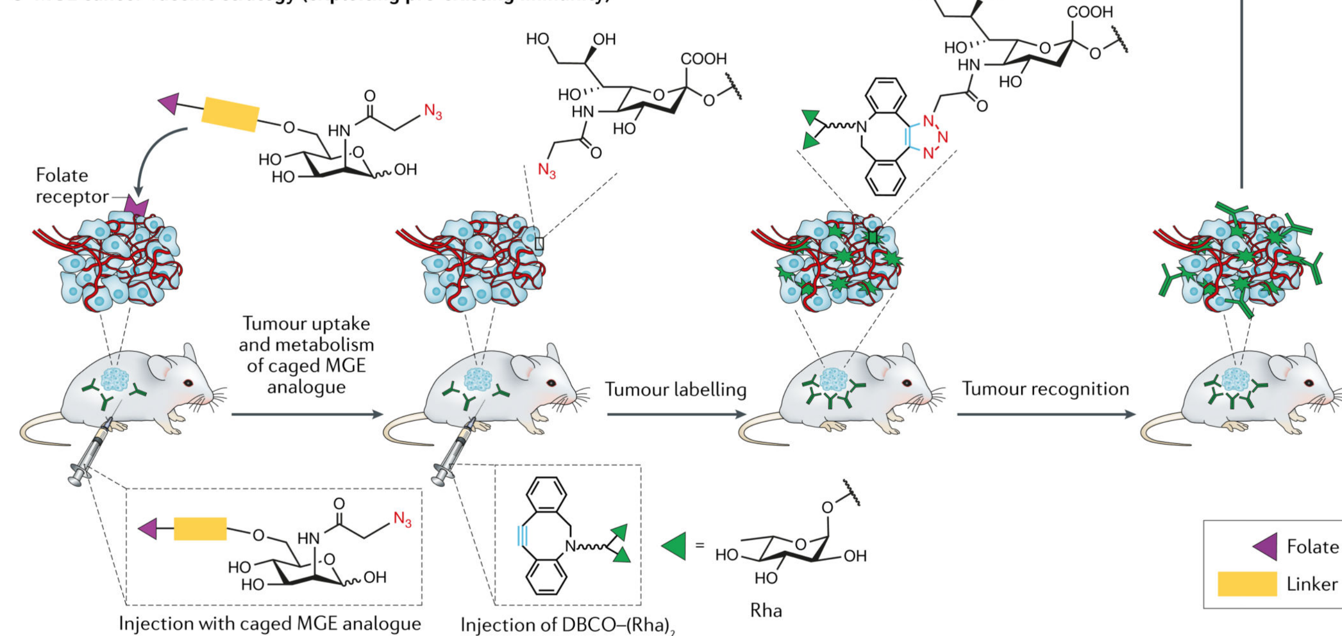

Fig. 6 |. MGE-based cancer immunotherapy.

Metabolic glycoengineering (MGE) is enabling new cancer-vaccine-based immunotherapies. **a** | A passive immunization approach[150] begins with inoculation of an animal with keyhole limpet hemocyanin (KLH) conjugated to GM3NPhAc, a form of ganglioside GM3 that contains the *N*-phenylacetido form of sialic acid. Subsequent injection with *N*-phenylacetylmannosamine (ManNPhAc) leads to selective metabolic incorporation of this analogue into cancer-cell-displayed GM3, sensitizing the tumour to antibodies generated to the vaccine. The binding of these antibodies leads to tumour remission through immune

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eradication (such as antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC) or antibody-dependent cellular phagocytosis (ADCP)). **b** | An active immunization approach that exploits pre-existing immunity[152] begins with injection of a caged *N*-azidoacetylmannosamine tetraacetate analogue that is conjugated to folic acid to target cancer cells through the folate receptor, which is overexpressed on many tumours. Upon tumour-selective display of the azide-modified glycans, dibenzocyclooctyne (DBCO)-conjugated rhamnose (Rha) is injected, which labels cancer cells through a [3+2] cycloaddition with the metabolically glycoengineered cell-surface azide groups. Subsequent binding of pre-existing anti-Rha antibodies leads to tumour remission through ADCC, CMC or ADCP.