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Review Article

Metabolic Glycoengineering: A Promising Strategy to Remodel Microenvironments for Regenerative Therapy

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Cell-based regenerative therapy utilizes the differentiation potential of stem cells to rejuvenate tissues. But the dynamic fate of stem cells is calling for precise control to optimize their therapeutic efficiency. Stem cell fate is regulated by specific conditions called "microenvironments." Among the various factors in the microenvironment, the cell-surface glycan acts as a mediator of cell-matrix and cell-cell interactions and manipulates the behavior of cells. Herein, metabolic glycoengineering (MGE) is an easy but powerful technology for remodeling the structure of glycan. By presenting unnatural glycans on the surface, MGE provides us an opportunity to reshape the microenvironment and evoke desired cellular responses. In this review, we firstly focused on the determining role of glycans on cellular activity; then, we introduced how MGE influences glycosylation and subsequently affects cell fate; at last, we outlined the application of MGE in regenerative therapy, especially in the musculoskeletal system, and the future direction of MGE is discussed.

1. Introduction

The need to regenerate or replace impaired tissues is rising nowadays, owing to the extended lifespan and the attendant degenerative diseases, as well as trauma and tumors [1]. Exogenous stem cells provide convenience to obtain as well as broad differentiation attributes. They not only can propagate under a static state but also can be induced to differentiate towards specialized cells or tissues with proper stimuli, aiming to rejuvenate the degenerated tissues back to their normal functional state [2, 3].

But the stem cells also bring us a question: how to maximize their regenerative efficiency after transplantation? The fate of stem cells can be more dynamic than we expected including adhesion [4], migration [5], proliferation, and apoptosis [6]. Besides, the survival rate of stem cells after transplantation also remained unsatisfactory.

The cellular microenvironment can be considered as a fundamental entry point for these obstacles above, because the decision of cell fate is highly regulated by matrix mechanical cues [7], biochemical factors [8], and other manners like intercellular crosstalk [9]. Researchers have endeavored to grasp the characteristics of the cellular microenvironment, in which cell-surface modification might be an ideal approach to regulate it.

In general, the cell membrane provides a platform carrying proteins or molecules that assist cells to conduct essential signals from external stimuli. Modification of cell membrane can display heterologous proteins on the surface, thereby inducing cellular responses and regulating biological behaviors [10].

The major technologies of cell-surface modification include hydrophobic membrane insertion [11], chemical conjugation [12], liposome fusion [13], metabolic pathways [14], enzymatic modification [15], and genetic engineering [16]. Among them, genetic engineering is the most widely used approach which incorporates materials into the genome of cells and then encodes target receptors onto the surface, such as cargocytes [17] and chimeric antigen receptor (CAR) T cells [18, 19].

Although genetic engineering could be a robust strategy to modify membranes, it is also associated with some drawbacks: (1) the process is time-consuming; (2) genetic transfection using viral vectors may cause unpredictable risks; (3) the irreversible modification may raise safety concerns for clinical applications; (4) moreover, not all cell types can adapt to genetic alteration without side effects, particularly in stem cells [20, 21].

In contrast, metabolic glycoengineering (MGE) is a safe and reversible strategy for membrane modification using nongenetic methods. Unlike nucleic acid or proteins, the cell-surface glycans are not directly encoded by genes. Biosynthesis of glycans (glycosylation) is determined by intra- or extracellular factors such as substrate transferases, signal transduction, and metabolic pathways [22, 23]. MGE technique is aimed at manipulating glycosylation and cellular metabolism to increase the expression levels of natural glycans and, more importantly, to install nonnatural monosaccharides into cell-surface glycoconjugates, such as ketone-, azide-, thiol-, or alkyne-modified glycans [24, 25].

Since MGE exploits the inherent natural metabolic pathway of cells, the process of modification barely interferes with other cellular functions—sort of like "silent" labeling [26]. Meanwhile, the MGE strategy also has other advantages as (1) easy but efficient process by simply coculturing cells with metabolic precursors; (2) exhibits noncytotoxicity even under high concentration of treatment; (3) applicable to almost all cell types; (4) nonpermanent modification that allows controlled reversal; and (5) the diversity of sugar analogs and bioorthogonal click chemistry endows MGE with numerous choices for membrane modification.

Therefore, by modifying surface glycans' structure or expression flux to decorate the cell membrane, MGE can circumvent the limitations associated with other strategies like genetic engineering.

In this review, (1) we firstly illustrated the significant role of cell-surface glycans in cell microenvironments; (2) then, we described how metabolic glycoengineering (MGE) influences cellular behaviors, and we exemplified MGE's effect on cell fate control; (3) finally, we outlined the application of MGE in regenerative medicine with a focus on the musculoskeletal system, and the future direction is also discussed.

2. Cellular Microenvironment, Extracellular Matrix, and Cell-Surface Glycans

2.1. Microenvironment and Extracellular Matrix: Shelters for Cells. Cell microenvironments are small zones around cells that can be defined as the intercellular substance containing dynamic body fluid components [27]. Particularly, stem cells are residing in specialized microenvironments that donated as "stem cell niches," which provide stem cells with static status and low-energy-consuming conditions to maintain their balance between self-renewal and differentiation [28].

The cell microenvironments consist of a set of elements that influence cellular activities (Figure 1), which can be mainly classified into the following types: extracellular matrix (ECM), adjacent cells (both homotypic or heterotypic), mechanical forces, proteolytic enzyme factors, and inflammatory cytokines [29]. And ECM represents the major component of the stem cell niche.

On the one hand, ECM provides cells with physical support, cytoskeletal structure, and transduction of physiological signals. The ECM components such as glycosaminoglycans (GAG) and adhesive molecules are maintaining the stability of cells, and the proper adhesion to ECM is essential for the survival of adjacent cells [30]. Partial geometric control of cell growth by spreading is also a basic mechanism for the developmental modulation of ECM [31].

On the other hand, microenvironments are rather dynamic than stuck in a rut. Cells remodel their microenvironments by altering the secretion of ECM components. Each kind of tissue creates a unique ECM composition, which is responsible for tissue-specific behaviors. The high affinity between stem cells and ECM can selectively influence the cell differentiation towards specific orientations, according to respective ECM components [32, 33] (e.g., fibroblasts to fibronectin, chondrocytes to type II collagen, or endothelial cells to laminin).

Conversely, without effective adhesion to ECM, cells tend to start the procedure of cell death such as apoptosis and necroptosis [34]. This phenomenon was initially reported in epithelial cells that the disrupted cell-matrix interaction will result in cell anoikis (a special type of apoptosis) [35]. However, the loss of function of the anoikis signals will also lead to another polarization, cancerous differentiation and metastasis [36], which reminds us about the injection of embryonic stem cells (ESCs) that generally results in teratoma formation [37].

Henceforth, considering the pluripotent of stem cells, it is critical to ensure desired interactions between cells and ECM. There could be many variations that we can precisely control for altering the specific characteristics of the microenvironment. And cell-surface glycan could be a major participant since sugars are ubiquitously present in all classes of cells, where they function as sources of energy, regulators of signaling, and participants of metabolic activity.

2.2. Cell-Surface Glycans: The Active Determinants of Microenvironment and Stem Cell Fate. Cellular glycans can be found in proteoglycans, glycoproteins, and glycolipids. Among all these glycoconjugates, cell-surface glycans (also called glycocalyx) are polysaccharides wrapping around the surfaces of all mammalian cells and participating in cell-cell [38] and cell-matrix [39] interactions (Figure 1). Like correspondences, these interactions mark the function of a cell, specify how it communicates with its surroundings, and also influence immune response [40].

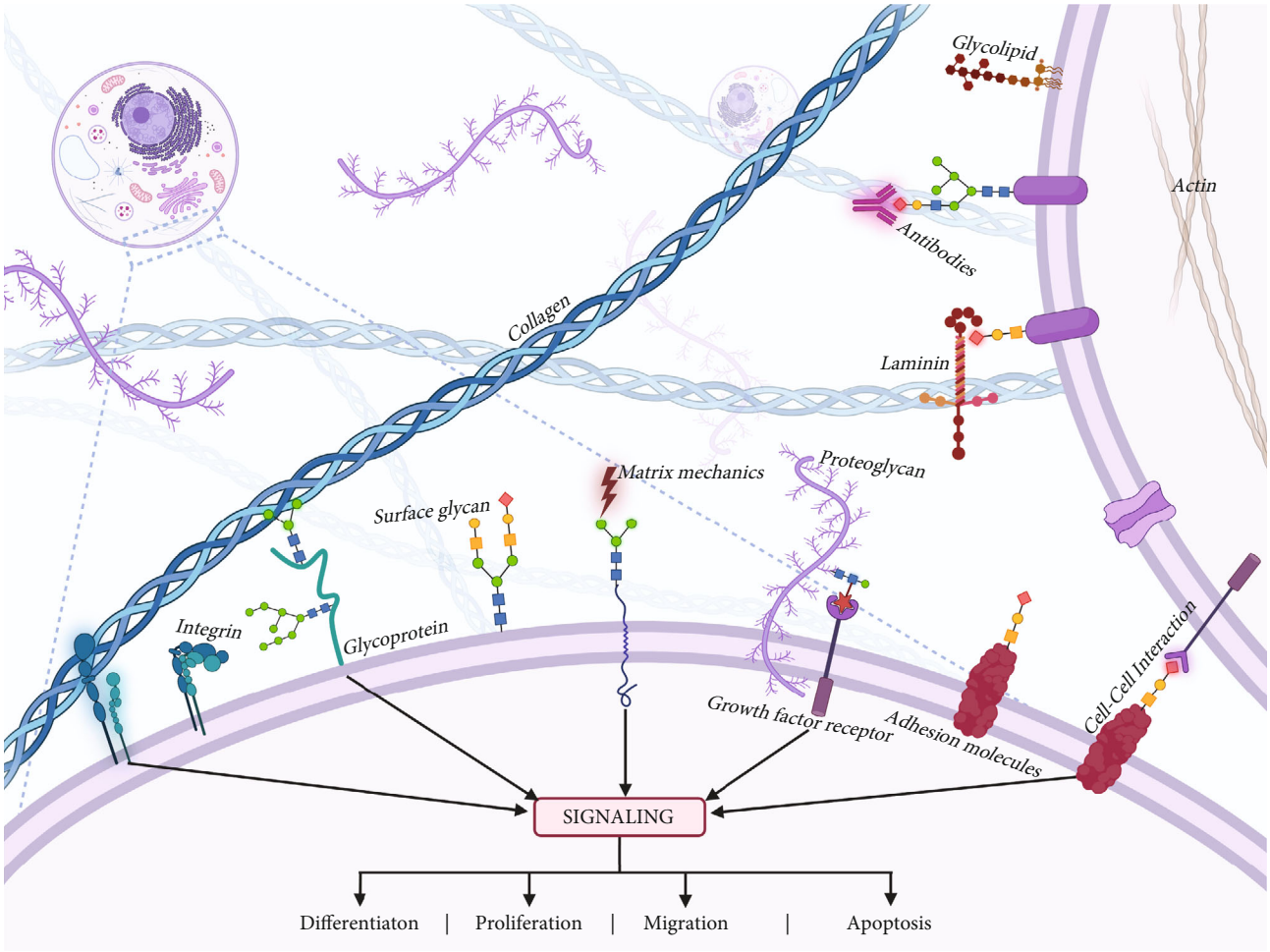

Figure 1: Interactions in the microenvironment. Cells reside in a dynamic environment consisting of ECM components, adjacent cells, mechanical forces, and various biological cues. The cell membranes offer platforms for cellular activities within their microenvironment, including molecule recognition, cell-cell interaction, and cell-matrix combination, which will, respectively, lead to different cell fates. Cell-surface glycans wrapped the membranes of cells and mediated those interactions in the microenvironment.

Glycosylation, the covalent attachment of glycans, is the most abundant posttranscriptional modification of proteins in nature [41]. O-Glycosylation and N-glycosylation are two major versions of protein glycosylation. O-Glycosylation involves the attachment of monosaccharides (N-acetylglucosamine) or polysaccharides (glycans) to threonine, serine, or tyrosine, while N-glycosylation added glycans to asparagine residues selectively [42]. Consequently, differences in structural composition conferred diversity of function to different glycosylated proteins.

For example, α-2-6-sialylated N-glycans, but not O-glycans, could be used as markers of the differential potential of mesenchymal stem cells [43]. Cell-surface N-glycans were also proved to influence the electrophysiological properties and differential fate of neural progenitor cells [44]. Moreover, the defect in glycosylation will lead to the disrupted adhesion of epithelial cells and then impair the cellular microenvironment [45]. The glycosylation can also influence cell fate directly. For example, both N- and O-glycosylation in cells functionally modulate the early steps of osteogenic differentiation of skeletal progenitor cells [46, 47].

Among the many glycosylation processes, sialylation is a significant one that happens on the terminal of mucin protein, mediated by sialyltransferases (ST) [48]. And cellular sialylation is essential in cell adhesion because the expression of integrin ligands is closely related to it [49, 50]. Integrin determines which ECM component cells would bind to during cell development and thus selectively affects the cell morphology [51]. Beyond integrins, the selectin family is another determinant cluster of adhesion that recognizes sialylation, while the sialylated Lewis X (sLeX) is the ligand for selectin [52, 53]. And some sialylated molecules also perform direct effects on the nervous system, with some gangliosides (GM3 and GD3) that control the early development by impacting cell growth and apoptosis [54]. Moreover, the precursors of sialyation were reported to act as signaling molecules that control the differentiation of neural cells [55]. In short, the function, stability, and metabolism of glycoproteins are dependent upon correct sialylation.

Cell-surface glycans are important factors that facilitate communication with the ECM and mediate signaling cascades and, consequently, make glycosylation or sialylation

an active determinant in the microenvironment, regulating cell fate in both direct and indirect manners.

3. Metabolic Glycoengineering: A "Silent" Method for Glycan Modification

The synthesis of cell-surface glycans is determined by substrate transferases and metabolic conditions. Herein, metabolic glycoengineering (MGE) is a nongenetic strategy for glycan modification based on metabolic precursors.

3.1. Overview of MGE. The main purpose of MGE is to increase the expression levels of natural glycans and install nonnatural monosaccharides into cell-surface glycoconjugates [24]. To put it in another way, MGE introduces various chemical groups into cellular glycan by artificially modified monosaccharides that bear unnatural functionalities (R-groups). While being incubated with mammalian cells, those monosaccharides can intercept the glycosylation pathways in cells, resulting in the submission of R-group-modified glycans on cell surfaces or secreted as glycoconjugate [56, 57].

Sialic acid, also known as neuraminic acid, is mainly located at the ends of the side chains of cell membrane glycoproteins, where it participates in numerous interactions between cell and microenvironment [25].

N-Acetylneuraminic acid (Neu5Ac) is the most common form of sialic acid in human cells while the N-acetyl-D-mannosamine (ManNAc) acts as the physiological precursor of all sialic acids. After ManNAc is absorbed into a cell as a precursor, it is converted to Neu5Ac with the help of specific sialyltransferases and will eventually be anchored to the residues of cell-surface sialic acid (Figure 2(a)).

The sialic acid pathway was the first glycosylation pathway to be utilized in MGE [58], and it is also the most commonly used pathway nowadays. The reason why the sialic acid pathway becomes a suitable choice for MGE is relevant to the remarkable substrate promiscuity of sialyltransferases [59, 60], which provides the possibility for the modified analogs to successfully intercept glycosylation pathways, resulting in the chemically modified sialic acid (Figure 2(a)). For example, N-propionyl-mannosamine (ManNProp) is an analog of ManNAc with a propionyl group on the N-acyl side chain (Figure 2(b)Ba), and the metabolism of ManNProp eventually submits N-propionyl-neuraminic acid (Neu5Prop) on the cell membrane surface [61].

3.2. Metabolic Precursors of Sialic Acid. Since Kayser et al. developed the 1st generation of ManNAc analogs (ManNProp, ManNBut, and ManNPent) in the 1990s [56], more than dozens of unnatural monosaccharides have been synthesized as appropriate precursors for MGE. Among all these ManNAc analogs, two major categories can be grouped: (i) aliphatic analogs and (ii) bioorthogonal analogs.

Aliphatic analogs are characterized by their N-acyl side chains which elongated with one or more methylene groups. Slight modifications of the sialic acid N-acyl side chain, such as the introduction of hydrophobic methylene, will cause significant impacts on specific cell-surface biological functionalities (Figure 2(b)Ba), including virus infection receptors [62], cell-surface differentiation markers [63], and cell proliferating regulation [64]. Thus, MGE based on aliphatic ManNAc analogs is aimed at bringing additional biological features to cells and then consequently influencing their behavior and fate.

Bioorthogonal analogs are synthesized with N-acyl side chains carrying reactive R-groups which are absent in biological systems but could be utilized for further chemical conjugations or reactions (Figure 2(b)Bb). Bioorthogonal chemistry enables the installation of artificial functionalities onto the cell surface, such as drugs [65], ligands [66], macromolecules [67], or fluorescent dyes [68]. For example, azide-functionalized N-acetylmannosamine (ManNAz) can produce azide groups on N-acyl side chains of sialic acid. Azide groups are absent from mammalian cells, but it holds bioorthogonal reactivity with most biofunctional groups. To be specific, based on the high efficiency of reactions between dibenzocyclooctyne (DBCO) and azide groups, certain DBCO-modified substances can be attached to these azide groups on cell surface, resulting in the combination that we expected. Theoretically, any functional substance of our interests can be installed onto sialic acid by using the two-step bioorthogonal reaction. This connecting process undergoes copper-free click chemistry, which is characterized by its linkage stability, biocompatibility, and noncytotoxicity.

Beyond sialic acid, other glycosylation pathways have also been exploited with the development of MGE, such as L-fucose [69], GlcNAc (N-acetylglucosamine) [70], and GalNAc (N-acetylgalactosamine) [71], which provide more opportunities for MGE's application in various fields. A comprehensive description of MGE can be seen in these review articles [26, 72, 73]. But the major effort in MGE continued to concentrate on the sialic acid pathway, due to its biological importance and the outstanding permissibility of sialyltransferases for nonnatural analogs.

In the next section, we will focus our sight on applications of MGE in inducing biological cellular response, particularly in cell fate control.

4. The Impact of MGE on Cell Fate

Considering the significance of glycocalyx in biological activities as well as the accessibility of sialic acid to be chemically modified, glycans can act as targets for controlling cell fate.

Evidence is adequate that sialic acid precursors can precisely influence cellular behaviors. For example, ManNBut could reversibly inhibit the expression of cell-surface polysialic acid (polySia) while ManNProp did not downregulate it, due to the shorter N-acyl side chain of ManNProp [74]. ManNProp and ManNBut only differ from 1 methylene unit (-CH3) in their terminal structure of the N-acyl side chain (Figure 2(b)Ba), but these two analogs tend to elicit different biological consequences.

Hence, in this section, we will summarize current approaches and applications of MGE analogs in modulating cell biological behaviors, including adhesion, differentiation, migration, homing, survival, and secretion, and the related mechanism is also summed up.

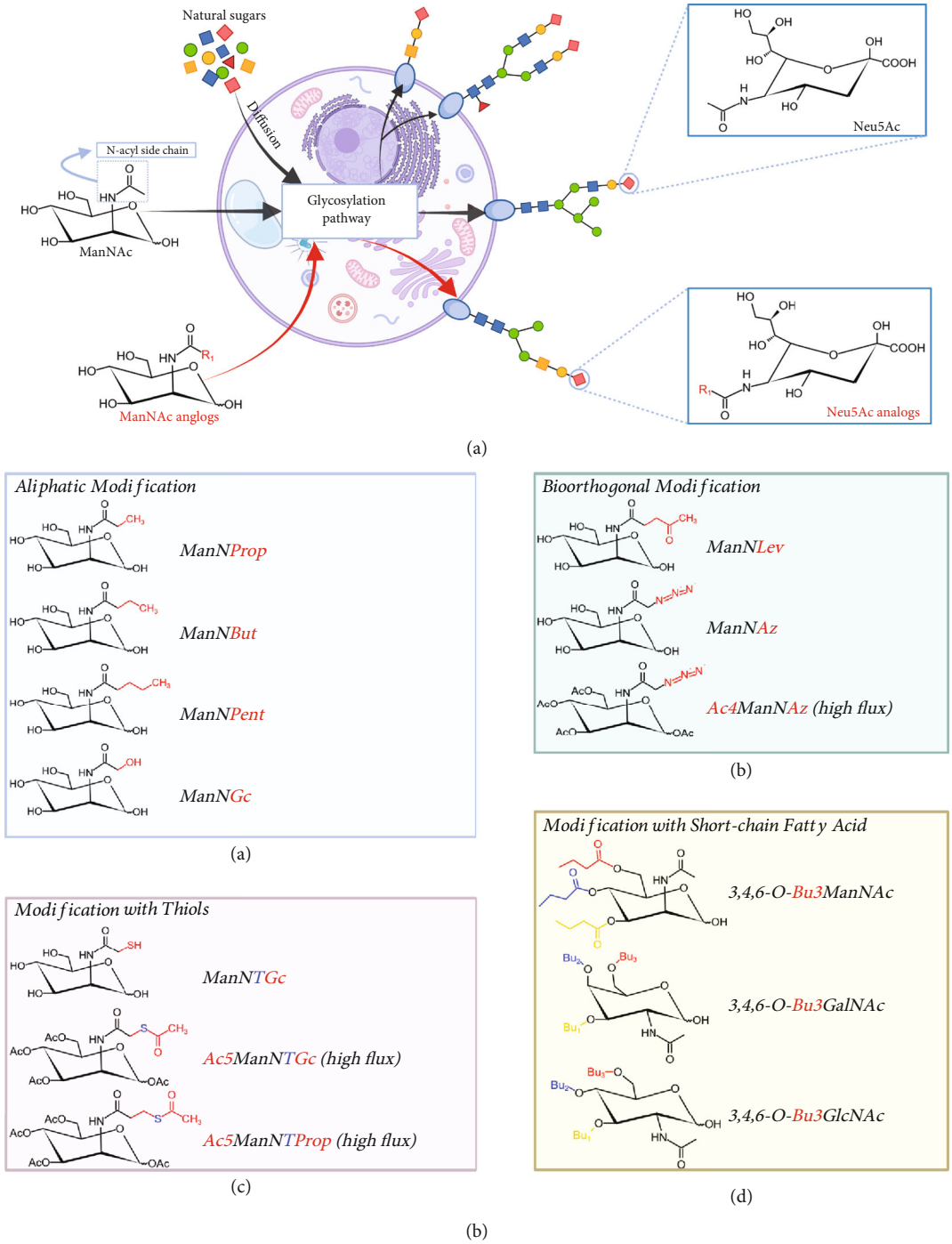

Figure 2: Overview of metabolic glycoengineering. (a) Illustration of MGE. Natural sugars are absorbed into cells and then metabolically assembled onto the cell surface as glycoconjugates (e.g., ManNAc into Neu5Ac). The analogs of metabolic precursors (ManNAc) can intercept the glycosylation pathways in cells, resulting in the submission of R-group-modified glycans and the modification of membranes. (b) Examples of MGE analogs. The analogs of MGE can be mainly classified into four catalogs: modification with aliphatic group, bioorthogonal group, thiol group, and short-chain fatty acid. The red marked text represents the R-group of different MGE analogs while the "high flux" indicates peracylated analogs with higher efficiency of modification.

4.1. Adhesion. Different chemical functional groups assembled on the surface can induce cell adhesion, and the strongest effect comes from methylene [75]. In 2002, Villavicencio-Lorini et al. reported that ManNProp stimulated the upregulation of intracellular β1-integrin receptors and eventually resulted in stronger adhesion between fibronectin and HL60 cells (the leukemia cell line) [76]. Although both natural and unnatural precursors of sialic acid can induce the upregulation of β1-integrins, the expression level induced by ManNProp (nonnatural) was twice than that of ManNAc (natural).

For the lack of the key enzyme of sialic acid pathway, HL60 cells do not express any sialylated molecules under normal conditions. But by incorporating Neu5Prop (Figure 2(a)) onto the glycans of HL60 cells, ManNProp dramatically increased the sialyl Lewis X (sLeX) biosynthesis, resulting in the promoted adhesion between HL60 cells and selectins [52]. The same effect of sLeX increasing was also detected in ManNProp-treated mesenchymal stromal cells (MSCs), which have been shown to target MSCs to bone marrow [77, 78].

Furthermore, the half-life time of some glycoproteins is modulated by terminal sialic acid. Thus, by altering the metabolic flux of sialic acid, MGE not only changes the glycan structure on the cell surface but can also affect the biostability of certain proteins. For example, ManNProp treatment extended the half-life of the sialylated molecule (CEACAM1) from 26 hours to 40 hours, which mediates cell-cell adhesion in PC12 cells (the rat pheochromocytoma cell line) [79].

Apart from methylene (-CH3), thiol is another kind of functionality that can promote cell adhesion.

Ac5ManNTGc is the hyperacetylated ManNAc analog with a thiol group on its N-acyl side chain (Figure 2(b)Bc). Jurkat cell is a T-lymphoma-derived cell line which possesses no adhesive property, but Ac5ManNTGc can incorporate thiols into the nonadhesive Jurkat cells [80] and then stimulate the additional cell adhesion to maleimide-functionalized surfaces [81]. These modified Jurkat cells also clustered spontaneously to produce numerous ECM components and upregulate their expression of β1-integrin, MMP-9, and CD44, which are involved in their attachment to ECM during T-lymphoma metastasis [82].

In these seminal explorations above, researchers generally use aliphatic analogs to modify membrane and elicit direct cell adhesion. But recently, bioorthogonal analogs (Figure 2(b)Bb) are also being exploited to enhance artificial adhesion since the click chemistry could endow cells to be chemically connected, building the "functional cell complexes." For example, Ac4ManNAz introduces azide groups into the cell surface as the first step; secondly, those azide groups are modified, respectively, with tetrazine (Tz) or *trans*-cyclooctene (TCO); thirdly, by mixing the modified cells, Tz-TCO click chemistry could produce intercellular adhesion [83, 84]. Click reaction between cyclodextrins (CDs) and adamantly (Adam) was also used to artificially combine A459 lung tumor cells and Jurkat cells, then triggered the activation of NK cells, and leads to the death of cancer cells [85].

In summary, the adhesion of cells to their microenvironment is indispensable because a cell cannot survive in the manner of an individual [3]. Because sialic acid participates in cell-cell and cell-matrix adhesion, it is worth wondering whether MGE can decide cell fate by introducing nonnatural glycans.

4.2. Differentiation and Proliferation. The earliest application of metabolic glycoengineering in regenerative medicine was conducted in neural cells by Schmidt et al. in 1998. ManNProp incubation in neural progenitor cells (NPC) induced the proliferation of astrocytes, microglia, and early-stage oligodendrocytes [86]. Buttner et al. also reported that ManNProp stimulated axonal outgrowth both in neuron cells and PC12 cells [87].

It has been demonstrated that ManNProp increased the calcium fluctuation in these cells [88]. Likewise, the adhesion effect of ManNProp in HL60 cells was also caused by intracellular calcium spiking, which thereby promoted the cell marker of monocytic differentiation [89]. And systemic administration of ManNProp significantly increased the axonal regeneration after sciatic nerves were transplanted into the mouse model [90], which proved to depend on the polysialyltransferase activity in the nerve graft [91].

It was suggested for the first time that ManNAc analogs can directly influence proliferation and axonal growth, and it is worth exploring for their influence on differentiation.

As described in Section 4.1, Ac5ManNTGc endows Jurkat cells with adhesive properties by introducing thiols. Moreover, when being applied in the human embryoid body–derived (hEBD) stem cells, Ac5ManNTGc was proved to stimulate neural lineage differentiation in the absence of Wnt signaling proteins [81], which are usually indispensable for neural differentiation [92].

In contrast, those cells treated with Ac5ManNGc—-which lacks thiol—showed only slight changes in the cytoskeleton, indicating no influence on differentiation. It can be inferred that the thiol group of the ManNAc analog is the key factor in enhancing cell adhesion and neural differentiation. But the upregulation of Wnt signal was only found on a gold-covered surface, indicating that the high-affinity bond of thiols was confined to the complementary scaffolds.

Du et al.'s group recently synthesized two novel thiol analogs—Ac5ManNTProp and Ac5ManNTBut (Figure 2(b)Bc)—which are claimed to install thiol on an elongated N-acyl side chain, thereby enhancing the ability of glycans to interact with other thiols, and overcome the necessity for complementary scaffolds [93]. When treated with human neural stem cells (hNSCs) and human adipose-derived stem cells (hADSCs), respectively, the stronger morphological responses were observed with Ac5ManNTBut, while Ac5ManNTProp exhibited better biocompatibility.

Among the thiol-treated hNSCs, the apparent neural differentiation was found as well as the upregulation of the Wnt signaling pathway. It was also demonstrated that the longer the N-acyl side chains were, the stronger the activation of Wnt signaling would be—raising from Ac5ManNTGc to Ac5ManNTProp and the strongest up to Ac5ManNTBut. According to the fact that the Wnt signaling also hinders adipogenesis [94], both Ac5ManNTProp and Ac5ManNTBut did inhibit the adipocyte differentiation in hADSCs [93]. The biosafety and scaffold-independent properties will make those ManNAc analogs be attractive tools in neural regenerative medicine.

In the previous studies, thiol-modified ManNAc analogs were usually used as bioorthogonal handles to link drugs to antibodies through maleimide conjugation [95]. But the work of Yarema et al. demonstrated that bioorthogonal analogs may also induce direct biological responses similar to those aliphatic analogs. And the outcome of thiol

modification could be changed by altering the length of the aliphatic side chain.

By introducing topological cues in the growth substrate and creating the glycoengineered binding interface, cell adhesion can be enhanced, gene expression can be regulated, and thus cell fate can be controlled, where the chemical composition of the cell surface is altered to promote carbohydrate-mediated interaction.

4.3. Migration and Homing. Beyond differentiation, cell migration and homing are regarded as the other two important activities that associated with sialic acid.

For instance, tumor cells usually synthesize polysialic acid (polySia) to regulate their ECM adhesion and migrate properties thereby becoming more metastatic [96]. Nagasundaram et al. reported a remarkable reduction of polySia in MCF7 breast cancer cells when treated with a series of ManNAc analogs (ManNProp, ManNBut, and ManNPent). Furthermore, the decreased level of natural polySia significantly suppressed adhesion and then inhibited breast cancer migration [97].

Conversely, compared with cancer cells, stem cells showed opposite responses. Incubation of ManNProp endows MSCs with a high expression level of sLeX which enhanced their osteotropism, also known as "homing" [77]. This homing effect of MSCs is determined by the interaction between sLeX and selectins, which is also involved in the neurotropism of NSCs [98].

In addition, supplementation with 3F-Neu5Ac was proved to increase migration and adhesion of MSCs and then promoted their survival rate in an ischemia model [63]. And the inhibition of osteogenic and adipogenic differentiation was also observed in those MSCs treated with 3F-Neu5Ac.

4.4. Apoptosis, Survival, and Secretion. The fact that sialic acid metabolism participates in cell apoptosis [54, 99] is also reflected in ManNAc analogs. By modulating metabolic flux of the sialic acid pathway, ManNAc analogs have the potential to either amplify or reduce cell apoptosis.

Research by Kim et al. demonstrated that ManNAc analogs can modulate cell apoptosis directly through N-acyl group effects, or indirectly via hydroxyl group effects. Especially, the ketone-bearing analog (Ac4ManNLev) possesses strong toxicity via inhibiting the sialic acid pathway [100]. Furthermore, the combination of 3,4,6-O-tributanoylation with the ManNLev (3,4,6-O-Bu3ManNLev) resulted in the most apoptotic ManNAc analog, which thus being a promising anticancer drug candidate [101]. Instead, 1,3,4-O-Bu3ManNAc increased the sialylation level of SW1990 cells (pancreatic cancer cells) up to 2-fold and in essence resensitize the SW1990 cells to anticancer drugs [102].

While the ketone groups are used to induce cell apoptosis, azide groups are promising in enhancing the survival rate of those cells seeded in biomaterials. Mao et al. fabricated DBCO-modified polymers as an MGE responsive platform and obtained azide-labeled macrophages through Ac4ManNAz. The bioorthogonal reaction between DBCO and azide accomplished fast in situ cellularizations and dramatically increased the selective capture and survival rate of the macrophages in the DBCO-modified scaffold [103].

Another intriguing application is the single-cell encapsulation via MGE. Oh et al. [104] utilized DBCO-azide conjugation to wrap every neural progenitor cell (NPC) with a layer of PEG polymer. The single-cell encapsulation with optimized stiffness changed the ADCY8-cAMP pathway due to the mechanical properties of polymers and enhanced the trophic factor secretion of NPCs, which reduced the required amounts of cells for therapy.

4.5. Brief Summary. All in all, the sialylation of N-glycans is tightly associated with the response to microenvironmental cues. Altering the structure of cell-surface glycans by metabolic glycoengineering (MGE) will certainly affect signaling pathways, no matter of incorporating either bioorthogonal or aliphatic modification.

From the analysis of the microarray data, up to a total of 14 pathways have been proved to be modulated via MGE products [105], including apoptosis, cell adhesion molecules, cell differentiation, leukocyte migration, and Wnt signaling as well as NF-κB signaling.

Taken together, these applications have established the MGE analogs as versatile tools for modulating biological activity such as cell adhesion, differentiation, migration, survival, or secretion, which may positively impact the therapeutic potential of the stem cells.

5. Applying MGE in Regenerative Medicine: With a Focus on Musculoskeletal System

Sialic acid possesses another name called "neuraminic acid," since it was initially isolated from neural tissues and highly expressed in the neural system. Hence, the earliest therapeutic exploration of MGE had mostly focused on neural lineage differentiation and neural tissue regeneration.

But sialic acid is widely existing in plentiful cells and tissues, rather than exclusively in neural systems. Along with the prospering development of MGE, this carbohydrate-based strategy also showed potential in therapy for the musculoskeletal system. In this section, we will introduce the application of MGE in regenerative medicine with a focus on the intervertebral disc and cartilage, which all tend to be ideal targets for metabolic glycoengineering.

5.1. Chondrogenic Differentiation and Cartilage Tissue Regeneration. The inflammatory environment in osteoarthritis (OA) joints disrupts the homogenesis of the articular microenvironment, thus reducing the ability of cartilage to regenerate and limiting the efficacy of OA therapeutics. To date, some carbohydrate-based molecules have shown potential in stem cell differentiation and chondrocyte regeneration.

5.1.1. Glucosamine-Metabolic Glycoengineering Produced by "Nature." Glucosamine (GlcN), a natural sugar that widely exists in cartilage, serves as the precursor for glycosaminoglycans (GAG), which are important components of the ECM secreted by chondrocytes and help sustain the

flexibility, toughness, and strength of this connective tissues [106]. Probably, we could define that glucosamine is kind of like a natural precursor of MGE since it helps with the supplement of ECM as many other analogs did.

It is well-known that GlcN has been regarded as a proper chondroprotective drug candidate for decades [107]. In 2007, Derfoul et al. demonstrated that the treatment of GlcN contributed to maintaining the chondrogenic phenotypes both in osteoarthritic chondrocytes and MSCs and promoted the secretion of ECM, as well as partially inhibited the expression of IL-1β and matrix metalloproteinase-13 (MMP-13) [108], which account for the clinical therapeutic effect of GlcN on OA, such as anti-inflammatory and chondroprotective.

In 2005, Khoo et al. reported the inducing effect of GlcN on the differentiation of embryonic stem cells (ESCs) [109]. By encapsulating GlcN in hydrogels, it was demonstrated that GlcN significantly enhanced the accumulation of chondrogenic ECM in the embryonic body (EB) [110]. Moreover, recent studies have shown that the treatment of GlcN also promoted the proliferation of chondrocytes via the Wnt/β-catenin signaling pathway [111], similar to the stem cells treated with ManNAc analogs [93].

The N-butyryl analog of glucosamine, "GlcNBut," was also found to stimulate normal chondrocytes to secrete ECM [112]. Poustie et al. cultured the normal chondrocytes with several glucosamine analogs, including GlcNAc, GlcNBut, and GlcNProp. The treatment of GlcNBut to chondrocytes increased the level of expression of mRNA of collagen-II and aggrecan, while GlcNAc and GlcNProp had no such influences. GlcNBut showed its potential to alleviate OA disease, but whether it could be applied *in vivo* to rejuvenize the senescent cartilage remained uncertain.

5.1.2. Tributanoylated GlcNAc, GalNAc, and ManNAc: The Hexosamine Analogs Derived from Short-Chain Fatty Acid. Despite the widely reported anti-inflammatory and chondrogenic differentiation properties of GlcN, its direct influence on the regeneration of cartilage had remained inconclusive for a long time [113, 114]. From a perspective of tissue engineering, the effective treatment of osteoarthritis requires a strategy that can both reduce inflammation and increase tissue production.

Among the efforts in reducing inflammation, NF-κB signaling is an intriguing therapeutic target in OA disease since it regulates the expression of many inflammatory mediators and matrix-degrading enzymes [115].

As it is mentioned in Section 4.5, the NF-κB signaling pathway can be modulated via MGE products [105], and the inhibition of NF-κB in cancer cells was previously observed with the short-chain fatty acid- (SCFA-) modified hexosamine analogs (Figure 2(b)Bd), such as tributanoylated 3,4,6-O-Bu3ManNAc and 3,4,6-O-Bu3ManNLev [101, 116]. After cellular uptake, those tributanoylated sugars can be naturally metabolized to their downstream byproducts, for example, from 3,4,6-O-Bu3ManNAc to ManNAc with three butyrate groups. The hexosamine part acted as a "core" for the biosynthesis of glycosaminoglycans (GAG), while the butyrate moieties modulated inflammation signaling pathways.

In addition to 3,4,6-O-Bu3ManNAc, the tributanoylated GlcNAc analog (3,4,6-O-Bu3GlcNAc) downregulated the NF-κB activity in those cancer studies too [116], which was reminiscent of the therapeutic effect of GlcNAc analogs in OA. And not surprisingly, the same effect of the tributanoylated GlcNAc (3,4,6-O-Bu3GlcNAc) was observed in OA chondrocytes, which promoted their ECM accumulation and inhibited inflammation [117], indicating that 3,4,6-O-Bu3GlcNAc might have the potential to reproduce cartilage tissue.

To get a comprehensive grasp of the characteristic of tributanoylated hexosamine analogs and to optimize their therapeutic effect in OA, Coburn et al. synthesized three analogs named as 3,4,6-O-Bu3GalNAc, 3,4,6-O-Bu3ManNAc, and 3,4,6-O-Bu3GlcNAc, for evaluating their effect on chondrocytes and mesenchymal stem cells, [118]. All the analogs inhibited the expression of NF-κB and increased the cartilage-like ECM accumulation in OA chondrocytes, while the GalNAc-Bu3 induced the strongest responses at a concentration with negligible cytotoxicity.

The *in vivo* investigation of GalNAc-Bu3 in rat OA models by Kim et al. showed that GalNAc-Bu3 induced cartilage tissue production both in MSCs and human OA chondrocytes by regulating the Wnt/β-catenin signaling, the negative pathway engaged in OA same as NF-κB [119]. Furthermore, GalNAc-Bu3 extended the survival of MSCs despite the rapid clearance rate of the synovial fluid.

Notably, the therapeutic effects of these tributanoylated analogs are not simply due to their catabolized metabolites, since their isomers are incapable to suppress the NF-κB activity (such as 1,3,4-O-Bu3GlcNAc), suggesting that the specific location of butyrate influences those effects [117].

Taken together, the potential of hexosamine analogs derived from SCFA could be translated as suitable drug candidates for OA disease, and the prospect of MGE-based carbohydrates in inducing chondrogenic differentiation of MSCs is calling for further investigations.

5.2. Prospect of MGE in Intervertebral Disc Regeneration. The intervertebral disc (IVD) is comprised of nucleus pulposus (NP), annulus fibrosus (AF), and endplates (EPs), while the NP is the highly hydrated region that is located at the inner central part of IVD [120]. It is well-known that the aging and dysfunction of NP cells lead to the degeneration of NP, which is an important initial process in the pathology of intervertebral disc degeneration (IVDD) [121].

5.2.1. Values and Obstacles of Stem Cells in IVDD Regeneration. Exogenous supplementation of mesenchymal stem cells (MSCs) has been proved to enhance the height and water content of the intervertebral disc [122, 123]. However, the harsh microenvironment in the degenerated NP (hypoxia, lack of blood supply, acidity, and hyperosmolality) severely hinders the survival and function of any transplanted cells [124–126].

As has been discussed earlier, the effective adhesion between stem cells and ECM can optimize cell behavior and tissue regeneration [127]. In the context of discs, it has been reported that the high affinity between MSCs and type

II collagen promoted the differentiation towards NP cells and helped MSCs maintain NP-like phenotypes [128, 129]. But a proper method to ensure the adhesion between transplanted cells and ECM components *in vivo* remains unclear in IVDD therapy. Herein, the MGE technique might help.

5.2.2. Enhance Nucleus Pulposus Regeneration by MGE. Based on previous studies demonstrating that modification of cell-surface glycan can modulate the adhesion property of cells [89], MGE might stimulate inner bounding to ECM that facilitates the differentiation of MSCs and then regenerate the disc.

An example from our laboratory validated that ManNProp, introducing extra -CH3 on sialic acid, greatly enhanced the adhesion ability of ADSCs, especially the selective adhesion with type II collagen [130]. Moreover, this cellular response promoted the efficiency of differentiation towards NP-like cells in a β1-integrin-dependent manner, by stimulating FAK/ERK pathway.

In rat IVDD models, better mechanical performance, increased water content, and the reconstruction of NP structure were observed after the transplantation of engineered cells with collagen scaffolds. It manifested that the MGE strategy not only promoted stem cells to overcome the harsh microenvironment in degenerated NP tissues but also benefits the regeneration of NP.

Designing biomaterials to improve cell adhesion is not a novel topic. However, the current efforts are mainly focusing on ECM engineering to "lure" passive adhesion by adding purified proteins and molecules [131, 132]. But the complexity of the synthetic procedure and the instability of proteins have confined the translational research of ECM modification. To address these limitations, we have highlighted the feasibility of cell membrane modification to produce "active adhesion" via glycoengineering and thus benefit disc regeneration.

Meanwhile, cartilage is an analogous connective tissue of IVD with a similar biofunction. And the type II collagen scaffolds carrying stem cells have also been exploited for cartilage defects [133]. Consequently, we assume that the MGE based on ManNProp is also potential in cartilage repair.

6. Outlook and Conclusion

Metabolic glycoengineering (MGE), a technique that prospered for three decades, is now shedding new light on extensive fields. In this review, we highlighted the determinable role of glycan in regulating cell microenvironment and analyzed how MGE modifies these glycans to regulate cell fate.

Beyond those biological activities discussed in this manuscript, the MGE strategy has also been applied in a variety of biomedical fields, such as visualizing glycoconjugates for *in vivo* tracking [134], targeting agents to diagnose or kill cancer cells [135], homing of therapeutic cells [136, 137], drug delivery to promote disease recovering [138], and cell vaccine-based immunotherapies [139]. Notably, to realize the full potential of MGE, it will be necessary to explore its unveiled opportunities in regenerative medicine and bridge the gap in the current research.

In the future, we can assume the direction of MGE as (1) extend MGE application to other glycosylation pathways beyond sialic acid and discover novel metabolic precursors; (2) determine the effect of MGE in more disease models and cell types that require better regenerative distribute, such as myocardial infarction (cardiomyocytes), diabetes (pancreatic beta cells), leukemia (hematopoietic stem cells), or any other disease suitable for stem cell transplantation; (3) bioorthogonal modification of stem cells holds great prospect since the click chemistry allows more complex combination, which may lead to broader methods in altering the stem cell niche; (4) the sustainability of modified groups due to natural catabolism of glycans ought to be ameliorated; and (5) translating the current development of MGE into clinical practice has to be on its way.

In conclusion, this review summarized the MGE's application in tissue engineering and regenerative medicine, and the booming diversity of MGE ensures a broad prospect for this technique in the future.

Data Availability

Data sharing is not applicable to this article as no new data was created or analyzed in this study.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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