

Review

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Review

Glycosphingolipids in Osteoarthritis and Cartilage Regeneration Therapy: Mechanisms and Therapeutic Prospects

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Abstract: Glycosphingolipids (GSLs), a subtype of glycolipids containing sphingosine, are critical components of vertebrate plasma membranes, playing a pivotal role in cellular signaling and interactions. In human articular cartilage in osteoarthritis (OA), GSL expression is known notably to decrease. This review focuses on the roles of gangliosides, a specific type of GSL, in cartilage degeneration and regeneration, emphasizing their regulatory function in signal transduction. The expression of gangliosides, whether endogenous or augmented exogenously, is regulated at the enzymatic level, targeting specific glycosyltransferases. This regulation has significant implications for the composition of cell surface gangliosides and their impact on signal transduction in chondrocytes and progenitor cells. Different levels of ganglioside expression can influence signaling pathways in various ways, potentially affecting cell properties, including malignancy. Moreover, gene manipulations against gangliosides have been shown to regulate cartilage metabolisms and chondrocyte differentiation in vivo and in vitro. This review highlights the potential of targeting gangliosides in the development of therapeutic strategies for osteoarthritis and cartilage injury and addresses promising directions for future research and treatment.

Keywords: glycosphingolipids (GSLs); osteoarthritis; articular cartilage; gangliosides; chondrocyte differentiation; cartilage regeneration

1. Introduction

Articular cartilage is a specialized connective tissue that resides at the interface between bones and joint space [1]. This highly specialized tissue comprises chondrocytes and a specific extracellular matrix (ECM) containing types II, IX, and XI collagen and proteoglycans, but notably lacks type I collagen. Referred to as hyaline cartilage, it is characterized by its elasticity, which plays a crucial role in absorbing and distributing loads during weight-bearing. Another function is to provide a smooth, lubricated surface facilitating a range of motion and enabling load transfer with minimal friction. Articular cartilage is avascular, aneural, lymphatic, and hypocellular, limiting its capacity for standard tissue repair mechanisms [2]. Most important, articular cartilage has a limited capacity for intrinsic healing and repair.

Osteoarthritis (OA), the most common joint disease, affects over 300 million people worldwide and contributes to an economic burden on both patients and society [3,4]. The disease costs the United States economy more than \$80 billion per year [5]. OA is characterized by progressive degradation of articular cartilage and ECM, while its pathogenesis remains largely unknown despite extensive gene- and protein-based research [6]. Articular cartilage does not possess access to the nutrients or circulating chondrogenic progenitor cells and cartilage lacks the natural potential to overcome a sufficient healing response by possessing a nearly acellular nature [7]. Consequently, articular cartilage has limited healing potential; therefore, it can lead to cartilage degeneration and ultimately result in OA. In particular, the relationship between OA and glycolipids began to receive attention after the finding that the composition of glycosphingolipids (GSLs) is markedly altered in the

articular cartilage of OA patients [8–10]. Following this report, the possibility that biomembrane glycolipids, the subject of post-genomic studies, are involved in the pathogenesis of OA has come to be considered.

GSLs are key components of cell membranes, comprising a hydrophobic ceramide and a hydrophilic oligosaccharide residue. Ceramides are embedded in the outer leaflet of the plasma membrane, while oligosaccharides project into the extracellular space [11,12]. GSLs cluster on the cell membrane surface, modulating transmembrane signaling and mediating intercellular and cell-matrix interactions [11–14]. An enzyme called glucosylceramide synthase encoded by the UDP-glucose ceramide glucosyltransferase (Ugcg) gene is responsible for directing the first committed step in GSL synthesis [15–18]. Glucosylceramide is formed when a glucose moiety is transferred from UDP-glucose to ceramide, which is the precursor of most cellular GSLs. Mice with a global disruption in UGCG are embryonic lethal (E7.5), suggesting that GSLs are essential for embryonic development and differentiation [15–17,19]. It is now well established that some sphingolipids can regulate key biological functions, and these include cell growth and survival, cell differentiation, angiogenesis, autophagy, cell migration, or organogenesis [20]. Furthermore, specific bioactive sphingolipids have been linked to various pathologies, including inflammation-related diseases like atherosclerosis, rheumatoid arthritis, type II diabetes, obesity, cancer, and osteoarthritis.

Here, we mainly discuss the usefulness of GSLs expressed on cell membranes as biomarkers for quality control in cartilage regenerative medicine and as therapeutic target molecules for OA.

2. Impact of GSLs on the Cartilage Homeostasis

After it was shown that a major component of glycolipids (ceramide) stimulates the mRNA expression of collagenase-1/MMP-1 and stromelysin-1/MMP-3 in human fibroblasts through the activation of three different mitogen-activated protein kinases (MAPKs), ERK1/2, SAPK/JNK and p38 in cartilage, ceramide was also found to be involved in cartilage degeneration and apoptosis [21,22]. The ceramide pathway activator suppressed the production of inflammatory cytokines and activation of the MAPK pathways [23]. As mentioned above, systemic knockout mice of the Ugcg gene are embryonic lethal because UGCG is the first committed step in the synthesis of the majority of GSLs [15–18]. GSLs form clusters on the plasma membrane and play diverse roles in regulating membrane-mediated signal transduction and mediating cell-cell and cell-extracellular matrix interactions [24–27]. Therefore, an attempt was made to sort out even the most characteristic downstream glycolipid molecules by sequential knockout of upstream glycosyltransferase genes involved in the impairment of cartilage homeostasis in chondrocytes. The contents of such research studies are summarized in Figure 1 and Table 1.

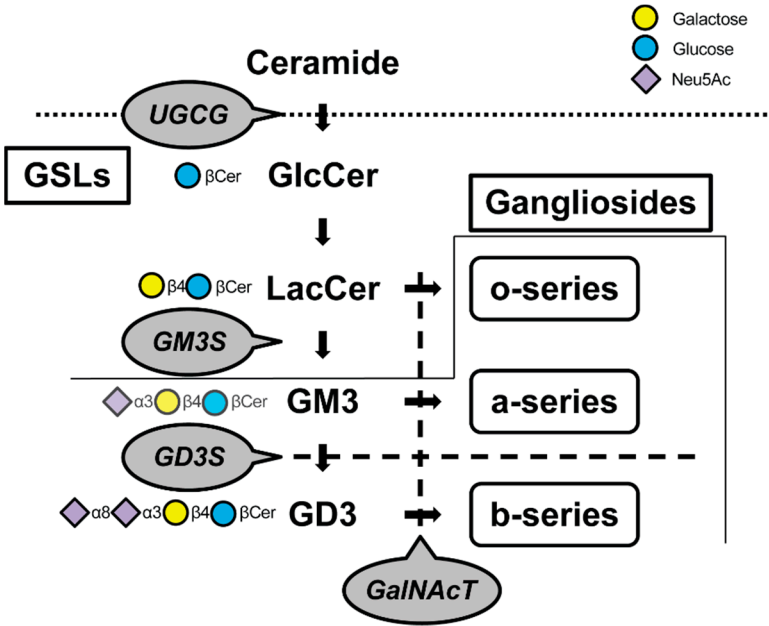


Figure 1. Schematic of the biosynthetic pathway for gangliosides. Glucosylceramide (GlcCer) synthase, encoded by the *Ugcg* gene, synthesizes GlcCer from ceramide. Gangliosides are classified as o-, a-, and b-series according to the number of sialic acids attached to galactose. GM3 synthase (GM3S) is required for GSL synthesis downstream of LacCer, including the a-series and b-series. b-series gangliosides are synthesized from the common precursor molecule GD3, which is the product of GD3 synthase (GD3S, encoded by the *Gd3s* gene). β 1, 4-N-acetylgalactosaminyltransferase (GalNAcT) activity is required for the elaboration of the o-, a-, and b-series precursors LacCer, GM3, and GD3, respectively. Cer, ceramide; GSLs, glycosphingolipids; GlcCer, glucosylceramide; LacCer, lactosylceramide.

A decrease in all major gangliosides, contrasting with a marked increase in the GM3, has been demonstrated in osteoarthritic fibrillated cartilage. Some previous studies have shown that gangliosides have tissue-protective effects against oxidative stress or apoptosis in neuronal, cardiac, and hepatic cells [28–32]. The results of the series of studies indicate that loss of gangliosides results in greater cartilage vulnerability to interleukin (IL)-1 stimulation in the cartilage degradation process by increasing MMP-13 secretion and chondrocyte apoptosis. On the other hand, There have been indications that replenishing cells with the missing gangliosides can restore normal activation [33,34]. Based on the previous studies, it is reasonable to consider a treatment that supplements the o-, a-, and b-series gangliosides below GalNAcT. Future therapeutic trials are pending.

Glycosidase inhibitors are also considered to be an important target for cartilage regeneration. They are directly linked to osteoarthritis because N-acetyl-beta-hexosaminidase is the predominant glycosidase released by chondrocytes to degrade glycosaminoglycan [35,36]. Stimulation of chondrocytes with IL-1 β selectively increases extracellular hexosaminidase activity among many enzymes, suggesting that hexosaminidase is the cartilage matrix-degrading enzyme activated by inflammatory stimuli. The inhibitor of this hexosaminidase was shown to modulate intracellular levels of glycolipids, including GM2 and GA2 (o- and a-series gangliosides).

Table 1. Genetic defects in mouse glycan formation and physiologic consequence.

Glycosyltransferase	Lost glycolipids	Consequences of depletion of its glycolipid	References
UGCG (Glucosylceramide synthase)	GSLs	Embryonic death. Reduced insulative capacity of the myelin sheath. Col2- <i>Ugcg</i> ^{-/-} mice enhance the development of OA	[16,17,19,37–39]
ST3GalIV (GM3S)	Gangliosides other than the o-series	GM3 plays an immunologic role. Heightened sensitivity to insulin. Severely reduced CD4 ⁺ T cell proliferative response and cytokine production. Promote OA and RA but cartilage regeneration	[34,40–43]
ST8Sia I (GD3S)	b-series ganglioside	Tumor-associated carbohydrate antigens (TACA) in neuro-ectoderm-derived cancers. Suppression of age-related bone loss. Deteriorates OA with aging	[33,44–47]
GalNAcT (GM2/GD2S)	Almost all gangliosides except GM3, GD3, and GT3	Age-dependent neurodegeneration, movement disorders associated with it. Defects in spermatogenesis and learning. Exacerbating OA progression	[33,48–50]

3. Role of GSLs in Cartilage Repair and Differentiation Processes

GSLs play a crucial role in the repair and differentiation processes of articular cartilage. These processes are essential for addressing cartilage defects, commonly observed in osteoarthritis. The unique properties of GSLs facilitate the regeneration of cartilage tissue, which is key to restoring joint function. Articular cartilage repair models have demonstrated the potential of specific cell types and biological factors in facilitating cartilage repair. These models highlight the importance of understanding the underlying mechanisms of cartilage regeneration, particularly focusing on the role of GSLs in this process.

In this chapter, various aspects of cartilage repair will be explored, with a focus on the role of glycolipids in promoting the regeneration and repair process through chondrogenic differentiation.

3.1. Endogenous Potential to Heal in Articular Cartilage

To enhance endogenous cell recruitment to the injury site, the biological process within a living organism after articular cartilage injury needs to be clarified. Considering species differences, appropriate animal models to replicate human articular cartilage repair processes are reported in mice [51–53], rats [54], rabbits [55–57], horses [58], and canines [59], thus molecular reaction after articular cartilage injuries is continuously disclosed [60–64]. Even though the species are different, the major ganglioside in articular cartilage appears to be GM3 [65,66]. GSLs consist of several types of glycolipids and are classified into several groups depending on their structural features, which include neo-lacto-series, globo-series, isoglobo-series and ganglio-series (gangliosides) [24,40]. GM3 serves as a precursor molecule for most of the more complex ganglioside species [41] and ganglioside expression pattern in cells during differentiation changes in response to cytokine and growth factor stimulation [67–69]. During the articular cartilage healing process, GM3 was first expressed in injured bone marrow on day 1 and gradually decreased at 2 weeks after articular cartilage injury GM3 next transiently expressed cartilage in the vicinity of remnant cartilage which co-expressed with type X collagen with a peak 6 weeks postoperative [42]. Notably, the depletion of gangliosides in mice suppressed hypertrophic differentiation in the vicinity of remnant cartilage, resulting in enhanced articular cartilage regeneration. These results suggested that gangliosides have dual roles in the recruitment of chondrogenic precursor cells to the injury site and induction of hypertrophic differentiation in chondrocytes. Manipulation of ganglioside expression may future direction in articular cartilage regeneration, however, the site- and time-specific intervention to manipulate glycosphingolipids in articular cartilage is needed.

3.2. Changes in the Glycan Structure during Chondrogenic Differentiation

Chondrogenic differentiation is the well-organized process by which cartilage is formed from condensed mesenchyme tissue, which differentiates into chondrocytes and begins secreting the molecules that form the extracellular matrix [70]. Extracellular enzymes, which include the matrix metallopeptidases, lead to the activation of cell signaling pathways and gene expression in a temporal-spatial-specific manner during the development process. The recruited mesenchymal stem cells may attempt to differentiate into chondrocytes after articular cartilage injury [71,72]. However, the response after injury is not a fully recapitulated process of development, resulting in regenerated cartilage-like tissue that does not possess typical biomolecules of hyaline cartilage such as type II collagen and aggrecan, and the proportion of the chemical constituents of them differ from those in original cartilage [73,74]. Molecules promoting the selective differentiation of multipotent mesenchymal stem cells into chondrocytes have been reported to stimulate the repair of injured articular cartilage [75]. Therefore, the regulation system for chondrogenic differentiation is attracting attention.

Glycosylation is one of the posttranslational modifications in cell surface proteins and extracellular matrix proteins, which regulate a variety of biological functions, including enhancement

of protein stability, controlling cell-to-cell communication, and adhesion [76]. In addition, this process is known to contribute to the pathogenesis of various kinds of diseases [77,78]. We previously performed quantitative and qualitative analyses of N-linked glycans and glycoproteins during chondrogenic differentiation using the glycoblotting method [79] and subsequent glycoform-focused reverse proteomics and genomics using mouse pre-chondrogenic cell line, ATDC5 cell line [80]. The levels of high-mannose type N-glycans increase during chondrogenic differentiation, suggesting that N-glycans may have key roles in differentiation and/or homeostatic maintenance of chondrocytes [81]. As for hypertrophic differentiation in chondrocytes, Yan et al. reported that resting chondrocytes exposed to concanavalin A, which binds specifically to high mannose-type structures, selectively differentiated to the hypertrophic stage [82,83]. We previously performed comprehensive glycomics, including N-glycans, O-glycans, free oligosaccharides (fOSs), glycosaminoglycans (GAGs), and GSLs and showed dynamic alterations in all classes of glycoconjugates following the differentiation process [84]. Hierarchical clustering analysis based on quantitative glycomic profiles showed that the levels of various N-glycans dramatically increased with hypertrophic progression, in contrast, those of GSL-glycans and fOSs significantly decreased. Changes in the levels of O-glycans and GAGs were transient. These results suggested that glycan markers can be used as differentiation biomarkers for chondrogenic differentiation and may help to evaluate the regenerative product after articular cartilage injury.

4. Cell Sources

As we explained in the Introduction section, articular cartilage is a type of hyaline cartilage that enables smooth movements between bones in articulating joints, which requires both weight-bearing and low-friction capability [85]. Therefore, the regenerated cartilage requires these high qualities of properties. The ultimate goal for ideal cartilage regeneration is to restore these key properties of the original hyaline cartilage in terms of histological structure and biomechanical functions, which seems to be only achieved by replacing it with healthy cartilage tissue [86]. As for now, several types of cell-based approaches have been introduced [87]. Representative strategy includes autologous cartilage implantation, mesenchymal stem cells, and induced pluripotent stem cells. Table 2 summarizes three representative cell types and reports on cartilage regenerative medicine. Here, mainly based on glycobiology, we overview the recent cell-based regenerative medicine in articular cartilage.

Table 2. Cell sources and cartilage regenerative medicine.

Clinical practice	Cell source	Lesion size (cm ²) / OA grade	Performances	References
Microfracture	Mesenchymal stem cell (MSC)	2.0-4.0	Microfracture is most likely to be successful for small femoral condylar defects	[88–92]
Autologous matrix-induced chondrogenesis (AMIC)	MSC	1.3-5.3	Effective procedure for the treatment of mid-sized cartilage defects. Low failure rate with satisfactory clinical outcome	[88,89,93–99]
Autologous chondrocyte implantation	Chondrocyte	2.0-10.0	Superior structural integration with native cartilage tissue compared to microfracture and AMIC, but a two-stage treatment burden exists	[89,100–103]

Osteochondral autograft transplantation	Chondrocyte	0.1-20.0 / OA grade I -III	Osteochondral autograft transfer system and mosaicplasty appear to be an alternative for the treatment of medium-sized focal chondral and osteochondral defects of the weight-bearing surfaces of the knee. Chondrocyte sheet and auricular cartilage micrograft for treatment of early-stage OA has been tried	[104–107]
Allogenic transplantation	Chondrocyte, iPSC	2.2-4.4 / OA grade II -IV	Osteoarticular allograft transplantation was used to treat high-grade cartilage defects or arthritis. iPSC-derived cartilages are used in preclinical studies that are in the middle to late stages when clinical trials are within range	[108–115]
Intra-articular injection with stem cell	adipose-derived stem cell, MSC	OA grade II -IV	Lower degenerative grades improve outcomes but are less effective for end-stage OA. The results of intra-articular administration of stem cells are better with BMSC.	[116–122]

4.1. Autologous Cartilage/Chondrocyte Implantation

In 1993, R Langer and J P Vacanti advocated the concept of tissue engineering, which applied the principles of biology and engineering to the development of functional substitutes for injured tissue by manipulating cells, scaffolds, and stimuli such as cytokine [123]. The first target organ by tissue engineering was thought to be skin or cartilage due to the less variation of cell types, and avascular and two-dimensional nature. However, in terms of articular cartilage, it was proved to be incorrect as far as known. Articular cartilage does not possess access to the nutrients or circulating chondrogenic progenitor cells and cartilage lacks the natural potential to overcome a sufficient healing response by possessing a nearly acellular nature [124]. Consequently, articular cartilage has limited healing potential; therefore, it can lead to cartilage degeneration and ultimately result in OA.

In 1994, Brittberg et al. first introduced a cell-based therapy consisting of two staged procedures for full-thickness defects of articular cartilage in the knee [125]. This procedure requires the first harvest of chondrocytes from a non-weight-bearing area of articular cartilage. After a culture of 4 to 6 weeks, a second-stage procedure is undertaken to implant amplified chondrocytes into the defect. Considering the limited healing potential for articular cartilage, these procedures seem to be ideal. Since the first clinical report was published, several authors have demonstrated successful clinical outcomes of this procedure for cartilaginous lesions [126–128]. However, there remain concerns about the dedifferentiation of chondrocytes during the culture period due to the limited proliferative capacity [129–131]. The cartilage extracellular matrix possesses various glycosylated proteins, which contribute to the maintenance of its specific functions [132]. Sialic acids are negatively charged sugars expressed at the terminal positions of N- and O-linked oligosaccharides, which are attached to cell surfaces or secreted glycoproteins. As a result of their non-reducing terminal position, sialic acids are involved in highly specific recognition phenomena [133,134]. Primary human chondrocytes

predominantly express α -2-6-specific sialyltransferases and α -2-6-linked sialic acid residues in glycoprotein N-glycans [135]. Interestingly, inflammation stimuli induced a shift from α -2-6-linked towards α -2-3-linked sialic acid, suggesting that α -2-6-linked sialic acid can be used as a biomarker for quality control in amplified human cartilage.

4.2. Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells are multipotent stem cells and can be obtained from various organs including bone marrow, synovium, periosteum, adipose tissue, and skeletal muscle [136]. MSCs are attractive cell sources in regenerative medicine based on their abilities to self-renew and differentiate into mesenchymal tissue lineage [137]. During the last few years, the use of MSCs and their cell-free derivatives has seen an increasing number of applications in disparate medical fields, including chronic musculoskeletal conditions [138–142]. All of these approaches would require cell harvesting and transplantation. One of the concerns is that MSCs are heterogeneous populations, whose capability to differentiate varies depending on the tissues harvested and donor age. Although MSCs are grouped together by the common characteristics of CD44, 73, 90, 105 positivity and CD31, 45 negativity, these do not necessarily define "stem cells." These cells are then expanded in vitro until a required cell number is reached, therefore, isolation methods, culture conditions, and passages should be given consideration.

Glycosylation features associated with bone marrow-derived MSCs included high-mannose type N-glycans, linear poly-N-acetylglucosamine chains, and α 2-3-sialylation [143]. Their cellular differentiation stage can be determined using these glycomics. Tateno et al. carried out glycome analysis on different passages of adipose-derived human MSCs (hMSCs) using high-density lectin microarray to identify glycan markers that distinguish MSCs to have enough capability to differentiate [144]. This report indicated that α 2-6-linked sialic acid-specific lectins showed stronger binding to early passage of adipose-derived hMSCs with differentiation ability to adipocytes and osteoblasts than did late passage cells without the ability. They also reported quantitative glycome analysis targeting both N- and O-glycans from early and late passages of adipose tissue-derived hMSCs and showed the expression of α 2-6-sialylated N-glycans varies depending on the differentiation potential of stem cells but not O-glycans, suggesting that α 2-6-sialylated N-glycans can be used biomarker for quality control of hMSCs [145]. The presence of α 2-6-linked sialic acid structure is a characteristic of pluripotent stem cells that possess higher differentiation potential. This may serve as an indicator of their differentiation potential. Ryu et al. showed that the gangliosides GM3 and GD3, which contain α 2-3- and α 2-8-linked sialic acids, were expressed after the chondrogenic differentiation of synovium-derived hMSC aggregates [146]. In the same way, GM3 expression increased temporarily following the chondrogenic differentiation of hMSCs derived from bone marrow [147]. Considering OA cartilage is characterized by a decrease in most gangliosides, these gangliosides may be useful in developing therapeutic agents for MSC-based articular cartilage regeneration in articular cartilage disease.

4.3. Induced Pluripotent Stem Cells (iPSCs)

In 2006, Takahashi and Yamanaka reported pluripotential stem cells from mouse embryonic or adult fibroblasts by introducing four factors Oct3/4, Sox2, c-Myc, and Klf4 [148]. The method to establish human iPSCs dramatically evolved and simplified [149]. This progress provides us with opportunities to understand the disease mechanisms and promote regenerative medicine [150,151]. To avoid the rejection of differentiated cells originating from iPSC transplantation, autogenous transplantation with iPSCs originating from donors is ideal. However, these procedures require time to establish iPSCs with high enough quality for transplantation as well as the cost. In contrast, iPSCs induced from individuals with a homozygous human leukocyte antigen haplotype (HLA-homo) is a significant candidate for allogeneic transplantation on the basis that HLA-homo iPSCs might not be rejected by HLA haplotype-matched patients [152–154]. iPSC banking recruited from healthy, consenting HLA-type homozygous donors and is made with peripheral blood-derived mononuclear

cells or umbilical cord blood [155]. Many research groups have been trying to apply iPSCs-based therapy to patients, and some of them are already being administered in clinical trials [156].

As for cartilage metabolisms, the main techniques to confirm chondrogenic differentiation from iPSCs are based on the detection of upregulated chondrogenic genes or histological analysis of the extracellular matrix. We previously performed a quantitative GSL-glycan analysis to compare human iPSCs, iPSC-derived MSC-like cells, iPS-MSC-derived chondrocytes (iPS-MSC-CDs), and bone marrow-derived MSCs [147,157]. GSL-glycan profiles differed among cell types, and the GSL-glycome underwent a characteristic alteration during the process of chondrogenic differentiation. Undifferentiated human iPSCs mainly expressed globo- and lacto-series GSLs, which shifted to ganglio-series GSLs, such as GM3 when iPSCs differentiated into iPS-MSCs. After chondrogenic differentiation of both bone marrow-derived MSCs and iPS-MSC-like cells, the expression of GM3 increased temporarily. Furthermore, the GSL-glycome of normal human cartilage was closely similar to that of iPS-MSC-CDs.

In terms of tumorigenicity for iPSCs themselves, while clinical applications are going forward, the concerns that transplantation of differentiated iPSC might lead to teratoma formation in the recipient should be clarified [158,159]. Matsumoto et al. reported that R-17F antibody detects undifferentiated iPSCs harboring the Lacto-N-fucopentaose I (LNFP I) of GSLs, and as a result, exerts cytotoxic activity [160]. Recently, we developed the aminolysis-sialic acid linkage-specific alkylamidation (SALSA) method [161], which enables discrimination of sialic acid linkage isomers on GSL-glycans by mass spectrometric analysis and analyzed the GSL-glycome of co-cultured undifferentiated iPSCs and chondrocytes by glycoblotting-SALSA method. Most GSL-glycans from ~100 cells of iPSCs could also be detected. Utilizing this technique, we showed that R-17 antibody detects undifferentiated iPSCs harboring the Lacto-N-fucopentaose I (LNFP I) of GSLs have selective removal of residual iPSCs even by chondrocytes co-cultured with iPSCs [162]. Furthermore, the experiment was successfully performed to determine whether R-17 antibodies could prevent teratoma formation induced by residual iPSCs [163]. The GSLs-Glycome analysis is useful to determine the optimal condition for the removal of undifferentiated iPSCs to a level safe for transplantation.

5. Conclusions

Glycomics of mesenchymal stem/progenitor cells can be utilized to evaluate their stage of cellular differentiation. Furthermore, this review highlighted the potential that supplementing missing GSLs could play significant roles in tissue regeneration and disease modification. To guide the regeneration of degenerated or injured cartilage into articular cartilage, a multifactorial methodology that incorporates GSLs, which are closely related to cartilage homeostasis, should be developed in the future.

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