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Metabolic-Glycoengineering-Enabled Molecularly Specific Acoustic Tweezing Cytometry for Targeted Mechanical Stimulation of Cell Surface Sialoglycans

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Abstract: This study developed a novel type of dibenzocyclooctyne (DBCO)-functionalized microbubbles (MBs) and validated their attachment to azide-labelled sialoglycans on human pluripotent stem cells (hPSCs) generated *via* metabolic glycoengineering (MGE). This enabled the application of mechanical forces to sialoglycans on hPSCs *via* molecularly specific acoustic tweezing cytometry (mATC), *i.e.*, displacing sialoglycan-anchored MBs using ultrasound (US). It was shown that subjected to forces of US pulses, sialoglycan-anchored MBs exhibited significantly larger displacements and faster, more complete recovery after each pulse than integrin-anchored MBs, indicating that sialoglycans are more stretchable and elastic than integrins on hPSCs in response to mechanical force. Furthermore, stimulating sialoglycans on hPSCs using mATC reduced stage-specific embryonic antigen-3 (SSEA-3) and GD3 expression but not OCT4 and SOX2 nuclear localization. Conversely, stimulating integrins decreased OCT4 nuclear localization but not SSEA-3 and GD3 expression, suggesting that mechanically stimulating sialoglycans and integrins initiated distinctive mechanoresponses during the early stages of hPSC differentiation. Taken together, the results demonstrated that MGE-enabled mATC uncovered not only different mechanical properties of sialoglycans on hPSCs than integrins but also their different mechanoregulatory impacts on hPSC differentiation, validating MGE-based mATC as a new, powerful tool for investigating the roles of glycans and other cell surface biomolecules in mechanotransduction.

Introduction

Cells in the body are exposed to a constantly changing extracellular environment. While the impacts of soluble factors (*e.g.*, morphogens, growth factors, cytokines, etc.) on cellular functions have been well established, the influences of insoluble "solid-state" signals of the cell microenvironment, particularly extracellular matrix (ECM) rigidity and external mechanical forces, on cellular behaviors, human development, and diseases have been less investigated.[1] Existing studies support that cells sense

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biophysical cues from the cellular microenvironment and convert them into intracellular biochemical signals to influence cellular function, a process termed "mechanotransduction".[2] For example, substrate rigidity has been exhibited to regulate self-renewal and differentiation of adult stem cells.[2-3]

It is well understood that during cellular mechano-sensing and mechanotransduction, cell surface adhesion molecules (such as integrins) engage ECM proteins to transmit external forces to the actin cytoskeleton (CSK) and other molecules within the cell. However, all cells are covered by a thick layer of glycans or carbohydrates, called the cell glycocalyx, which serves as the first point of contact for most cells to interact with each other or the extracellular environment.[4] Therefore, the cell glycocalyx can experience forces from the cellular microenvironment to mediate mechanotransduction directly or *via* interaction with ECM proteins and other cell surface adhesion molecules.[5] Sialoglycans, *i.e.*, glycans containing one to several *N*-acetylneuraminic acid (Neu5Ac, commonly known as the "sialic acid") residues, are ubiquitous and abundant in the cell glycocalyx. Neu5Ac, usually present at the non-reducing end of glycans, can directly detect various biophysical signals including mechano-stimuli to regulate recognition and binding events.[6] As such, sialoglycans play a critical role in cellular functions, including stem cell pluripotency and differentiation[7] as well as cell recognition.[8]

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), possess the properties of unlimited self-renewal and pluripotency to differentiate into all human germ layers both *in vitro* and *in vivo*.[9] Controlled and directed differentiation of hPSCs is important to realize their potential for studying human development, drug screening, disease modeling, and applications in regenerative medicine. While the intrinsic mechanosensitivity of hPSCs has been exploited to direct their differentiation and fate specification,[10] previous studies have primarily focused on mechano-regulated hPSC differentiation mediated by integrin-ECM interactions.[11] The mechanotransductive roles of the cell glycocalyx in hPSC differentiation remain largely underexplored, despite the omnipresence of glycans in hPSCs and their important involvement in development.[12]

Mechanoglycobiology, a new interdisciplinary field focusing on mechanotransduction and mechanobiology of cell surface glycans, investigates how cells sense and respond to mechanical signals *via* glycans. It extends beyond the scope of traditional glycobiology and is gaining recognition in many fields.[5, 13] However, the lack of appropriate technologies that can selectively apply controlled mechanical forces to specific glycans on live cells makes the study of mechanoglycobiology challenging.

To tackle these difficulties and facilitate mechanoglycobiological investigations, we have developed a new technology that combines acoustic tweezing cytometry (ATC) and cell metabolic glycoengineering (MGE). ATC is a technique that uses ultrasound (US) pulses to displace lipid-encapsulated gaseous microbubbles (MBs) (radius ~ 2 μm) designed to attach to specific molecules on the cell surface, thereby capable of applying defined mechanical strains or forces to cells *via* the MB-molecule-cell axis.[14] We have shown the versatility of ATC to exert mechanical forces to selected cell adhesion molecules (*e.g.*, integrins) in a variety of cell types for cell mechanical phenotyping and cellular behavior modulation, *e.g.*, hPSC differentiation.[14-15] MGE permits the functionalization of cell surface glycans *via* supplementing cells with a chemically modified sugar precursor for its metabolic incorporation in glycans. As a result, cells can be engineered to express glycans carrying a unique molecular handle for the attachment of various molecular labels for biological studies and applications.[16] In this study, we used MGE to functionalize sialoglycans on the surface of hPSCs with azide.[16b] In the meantime, we developed a new type of MBs that are labeled with alkyne. Then, we were able to attach alkyne-functionalized MBs to azide-labeled sialoglycans on hPSCs by click chemistry[17] to enable a new technology, *i.e.*, molecularly specific ATC (mATC), to apply molecular-targeted forces to cells. Moreover, we employed the new, MGE-based mATC technology to disclose the unique mechanical properties of sialoglycans and the impacts of targeted mechanical stimulation of cell surface sialoglycans on hPSC differentiation, showcasing the feasibility and potential of this technology.

Results and Discussion

MGE-based mATC for mechanoglycobiological studies

We designed a novel, MGE-based mATC technology for mechanoglycobiological studies (**Fig. 1**). Cells are treated with an azide-modified mannosamine derivative, 1,3,4,6-tera-*O*-acetyl-2-*N*-azidoacetylmannosamine (AcManNAz) that is widely used for MGE of cell surface sialoglycans,[16] to express azido-sialoglycans. Thereafter, MBs functionalized with alkyne can selectively attach to the azido-sialoglycans on cells *via* a click reaction under mild and bioorthogonal conditions,[17] enabling targeted mechanical stimulation of sialoglycans by mATC.

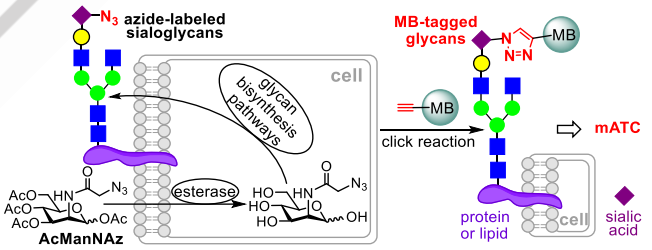

Figure 1. Working principle and protocol for MGE-based mATC. Upon treatment with AcManNAz, cells are metabolically engineered to express azide-labeled sialoglycans, enabling attachment of alkyne-functionalized MBs to the azido-sialoglycans on cells by a click reaction for mATC.

Alkyne-functionalized MBs to bind specific molecules on cells via click chemistry

The large contrast of acoustic impedance between the gas core (*e.g.*, perfluorobutane or PFB) of MBs and the surrounding liquid medium results in efficient interaction of US with MBs. This leads to robust and concentrated effects, including pronounced volume pulsation and translational movement by the acoustic radiation force (ARF) of an US field.[18] Given their strong interactions with US, MBs have been used as contrast agents for US imaging.[19] In mATC, MBs are modified with a specific ligand to facilitate their attachment to targets on cells such that defined mechanical forces can be applied by displacing the cell-bound MBs using the ARF of US pulses.[14b, 15c, 20] Usually, MBs are made of phospholipids, such as distearoylphosphatidylcholine (DSPC). Previously, we

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decorated MBs with biotin via incorporating polyoxyethylene 40-stearate-biotin in their lipid shells to allow MB attachment to avidinated ligands, *e.g.*, streptavidinated Arg-Gly-Asp (RGD) that recognizes and specifically binds integrins on cells.[14-15]

To use mATC to mechanically stimulate sialoglycans on hPSCs that are metabolically engineered to express azido-sialoglycans, alkyne-functionalized MBs (**Fig. 1**) are required. To this end, we explored a new type of MBs that are functionalized with the dibenzocyclooctyne (DBCO) group (**Fig. 2**). DBCO contains a strained and highly reactive C/C triple bond that facilitates strain-promoted alkyne-azide cycloaddition (SPAAC) easily achievable under biocompatible copper-free conditions.[17, 21] Accordingly, a mixture of phosphatidylethanolamine (DSPE)-PEG2000-DBCO and DSPC in 1:19 molar ratio was utilized to prepare MBs with a gaseous PFB core (**Fig. 2**). The resulting DBCO-MBs can be used to bind azide-modified molecules (*e.g.*, azido-sialoglycans) on live cells *via* SPAAC. Although a DBCO-containing MB was described previously,[22] it had a different composition from our MBs and was designed for different purpose and application.

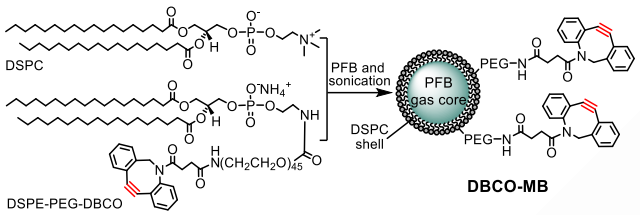

Figure 2. DBCO-functionalized MBs to target cell surface molecules for mATC. DSPE-PEG-DBCO and DSPC (1:19) were used to prepare DBCO-MBs with a gaseous PFB core for attachment to azido-molecules on cells *via* SPAAC.

Validation of hPSC MGE and DBCO-MB attachment to azido-sialoglycans on hPSCs

To validate the coupling of our newly formulated DBCO-MBs with azido-molecules, we incubated DBCO-MBs with Alexa Fluor 488-azide. Our results show the efficient labeling of these MBs by the green fluorophore (**Fig. 3a-c**), confirming the successful linkage of DBCO-MBs to azide-modified molecules *via* SPAAC. Our results also show that the DMCO-MBs are uniform in size and fluorescent intensity (**Fig. S1**, **SI**), indicating an even distribution of DBCO on MB shells. We then examined DBCO-MB addition to sialoglycans on hPSCs glycoengineered with AcManNAz. After incubation with DBCO-Alexa Fluor 594, hPSCs were efficiently labeled with this red fluorophore (**Fig. 3d-f**), verifying hPSC MGE to express azido-sialoglycans and attachment of DBCO-modified molecules to cells *via* SPAAC. The intensity of red fluorescence in hPSCs stabilized after a day of incubation with AcManNAz (**Fig. S2**, **SI**), suggesting that the efficiency of MGE was not affected by hPSC proliferation into colonies. As anticipated, increasing AcManNAz concentration enhanced the fluorescent intensity in hPSCs (**Fig. S3a** and **b**, **SI**). Finally, we also validated the successful attachment of DBCO-MBs to azido-sialoglycans on hPSCs after their treatments with AcManNAz (**Fig. 3g** and **3h**).

Comparative studies on the mechanical properties of the MB-sialoglycan and MB-integrin axes in hPSCs

Next, we tested the feasibility of utilizing mATC to mechanically stimulate cell surface sialoglycans. Here, we applied mATC to DBCO-MBs attached to azido-sialoglycans on the hPSC surface (**Fig. 4a**) using US pulses of 1.25 MHz center frequency, 0.035 MPa acoustic pressure, and 1 Hz pulse repletion frequency (PRF). For comparisons, we also performed mATC experiments under the same US conditions using hPSCs with integrin-bond DBCO-MBs *via* azido-RGD and SPAAC (**Fig. 4b**) and with integrin-bound biotin-MBs *via* streptavidin-RGD (**Fig. 4c**), similar to our previous studies.[14-15] Thus, sialoglycans and integrins are the “anchors” of MBs on hPSCs, and azide/DBCO, RGD-azide/DBCO, and RGD-avidin/biotin are the “linkers” bridging MBs and cells *via* a glycan or integrin anchor (**Fig. 4a-c**). Our results show that subjected to US pulses, the cell-bound MBs exhibited cyclic displacements corresponding to the “on” and “off” periods of the US pulses (**Fig. 4d-f**, **Fig. S4**, **SI**).

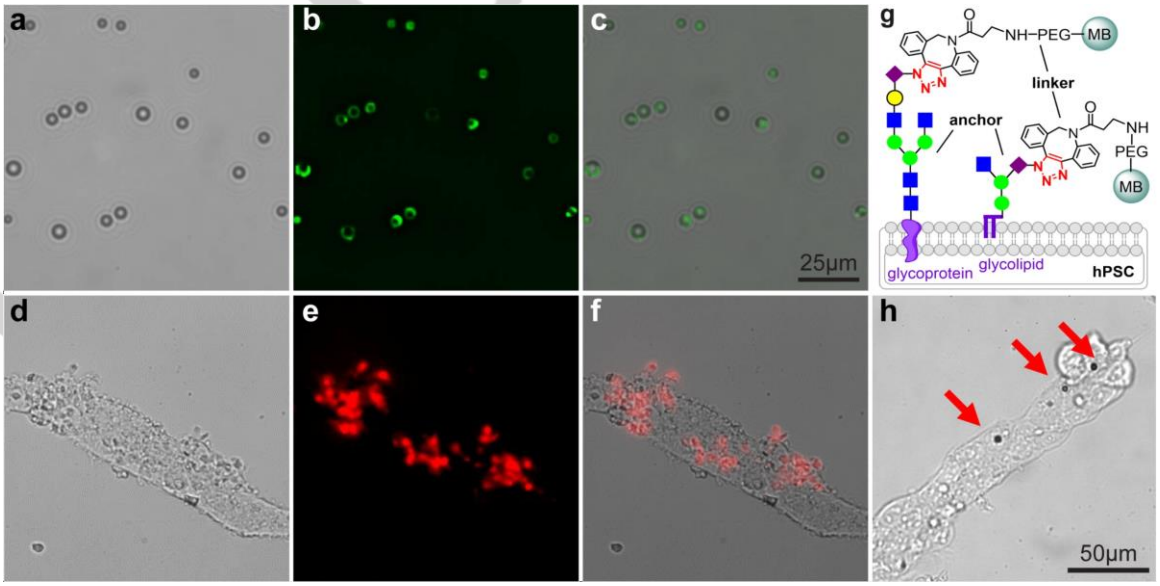

Figure 3. Validation of hPSC MGE and DBCO-MB attachment to azide-labeled sialoglycans on hPSCs. **a-c**). Examples of bright field (BF), fluorescent, and overlay images showing attachment of azide-Alexa Fluor 488 (green) to DBCO-MBs *via* SPAAC. **d-f**). Examples of BF, fluorescent, and overlay images of a colony of AcManNAz-engineered hPSCs after treatment with Alexa Fluor 594-DBCO, confirming hPSC expression of azido-sialoglycans *via* MGE and then their labeling with the red fluorophore *via* SPAAC. **g**). Schematics of DBCO-MB attachment to azide-labeled glycans on cells *via* SPAAC. **h**). BF image of a colony of engineered hPSCs with AcManNAz having DBCO-MBs (red arrows) bound to cell surface azido-sialoglycans.

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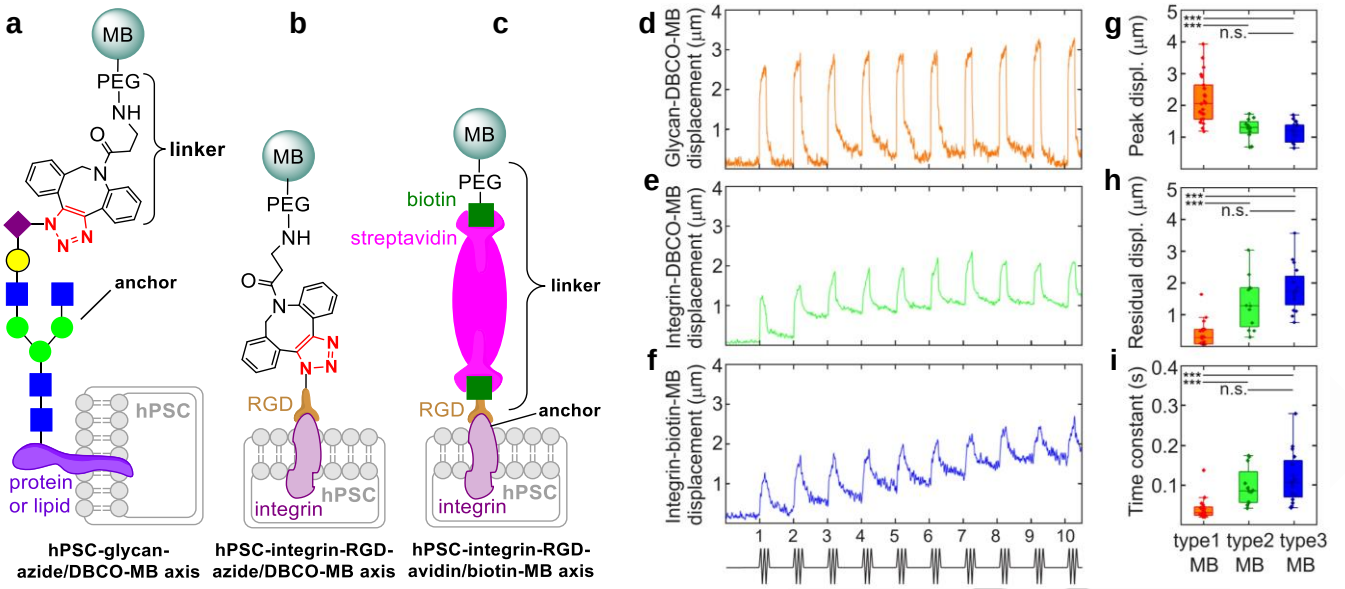

Figure 4: mATC-induced displacements of MBs anchored to hPSCs. Schematics of DBCO-MB attachment to **a**) azide-labeled sialoglycans (*via* azide/DBCO linker) and **b**) azide-RGD-labeled integrins (*via* RGD-azide/DBCO linker), as well as **c**) biotin-MB attachment to streptavidin-biotin-RGD-labeled integrins (*via* RGD-biotin/streptavidin/biotin linker), on hPSCs. **d-f**). Examples of displacement of MBs attached to hPSCs *via* the glycan-azide/DBCO, integrin-RGD-azide/DBCO, and integrin-RGD-avidin/biotin linkages, respectively, during US pulses. **g-i**). Peak and residual displacements and time constants of sialoglycan-anchored DBCO-MBs (type 1, orange; N = 22), integrin-anchored DBCO-MBs (type 2, green; N = 13), and integrin-anchored biotin-MBs (type 3, blue; N = 16), respectively, after each US pulse. n.s.: no statistical significance ($p \geq 0.05$); ***: $p < 0.001$.

The underlying mechanism of MB displacements is the impact of ARF generated by US pulses on gaseous MBs.[14, 18] The primary ARF of an US pulse in mATC exerts concentrated effects on MBs and effectively displaces molecule-bound MBs on the cell surface, while having negligible effects on the cell culture medium or cells themselves.[23] The ARF acting on an MB is dependent upon US parameters, e.g., acoustic pressure P_A and angular frequency ω_0 of the US pulse, $F \cong \frac{2\pi P_A^2 R_0}{\delta_{tot}\rho_0 c\omega_0}$,[14, 18] where R_0 is MB radius, δ_{tot} is damping constant (~ 0.16), ρ_0 is medium density ($1000 kg \cdot m^{-3}$), and c is the speed of sound in aqueous liquid medium ($1500\, m \cdot s^{-1}$). In our mATC experiments with US pulses at 1.25 MHz and 0.035 MPa, the ARF exerted on an MB with 1.5-2.0 μm radius is ca. 17.0-25.0 nN.[14, 18] During each US pulse (with a duration of 0.2 s), this force displaced MBs without detachment, thus inducing MB-linker/anchor stretch and cell deformation. Given the constant ARF during each US pulse, the characteristics of time-dependent MB displacement essentially reflect the stiffness or compliance of the MB-integrin-cell and MB-sialoglycan-cell axes during a creep test. As expected, our measurements of MB displacements during mATC show that all MBs bound to cells *via* different linkages (**Fig. 4a-c**) exhibited characteristic viscoelastic behaviors, *i.e.*, reaching a peak displacement at the end of each US pulse, followed by a recovery after each pulse (**Fig. 4d-f**). Multiple US pulses induced cyclic displacements of the cell-bound MBs, indicating cyclic mechanical strains to hPSCs *via* extension or stretch along the MB-sialoglycan-cell and MB-integrin-cell axes.

Interestingly, sialoglycan-anchored DBCO-MBs showed different characteristics from that of integrin-anchored MBs with either the RGD-DBCO/azide or RGD-streptavidin/biotin linker (videos **SV1-3**, **SI**). Sialoglycan-anchored DBCO-MBs sustained significantly greater peak displacements than integrin-anchored MBs using an RGD-DBCO/azide or RGD-streptavidin/biotin linker (**Fig. 4d** *vs* **4e/f**, and **Fig. 4g**), suggesting that the MB-sialoglycan-hPSC axis is more stretchable than the MB-integrin-hPSC axis. Meanwhile, no statistically significant differences were seen between integrin-anchored MBs by RGD-DBCO/azide and RGD-biotin/streptavidin linkers. These results indicate that the observed stretchability of the MB-hPSC axis depends on the molecular anchors (*i.e.*, RGD-integrins *vs* sialoglycan) directly associated with hPSC membrane, rather than the linkers (azide/DBCO *vs* avidin/biotin) between the anchors and the MBs. Furthermore, our measurements show that compared to integrin-anchored MBs, displaced sialoglycan-bound MBs recovered more quickly and completely after each US pulse, revealed by their smaller residual displacements (**Fig. 4d** *vs* **4e/f**, and **Fig. 4h**) and time constant of recovery (**Fig. 4i**, 0.03 *vs* 0.12 s), suggesting distinctly different viscoelasticity of the sialoglycan-hPSC and integrin-hPSC axes. Again, no significant difference was observed in integrin-anchored MBs by different linkers (RGD-azide/DBCO *vs.* RGD-avidin/biotin). The different viscoelastic characteristics of sialoglycan-hPSC and integrin-hPSC axes may result in different cellular impact of mATC and forces transmitted to hPSCs through different molecular entities.

Influences of mechanical stimulation of integrins on hPSC differentiation

Because integrin-anchored MBs *via* RGD-avidin/biotin and RGD-azide/DBCO linkers exhibited similar mechanical responses or characteristics, next, we tested if DBCO-MBs and avidin/biotin-MBs, both targeting integrins, had similar effects on differentiation of hPSCs when subjected to mATC. We have previously shown that ATC-mediated cyclic forces applied via biotin-MBs targeting integrins had a significant impact on hPSC differentiation.[15a, 20, 24] Similarly, mATC treatment of hPSCs with integrin-bound DBCO-MBs also show a significant impact on hPSC differentiation in this study (**Fig. 5**). In specific, mATC-mediated stimulation of integrins

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via DBCO-MBs or biotin-MBs decreased nuclear localization of octamer-binding transcription factor 4 (OCT4) but caused no significant changes in SRY-Box transcription factor 2 (SOX2) expression (**Fig. 5**). OCT4 is a core component of the regulatory circuitry underlying the pluripotency of hPSCs, and it is crucial for maintaining hPSC pluripotency. SOX2 is a transcription factor that is vital in maintaining the pluripotency of hPSCs as well. However, an increase in SOX2 expression was observed previously using biotin-MBs.[20] This discrepancy may be attributed to the higher duty cycle (50%) and longer pulse (0.5 s) used in previous studies than those in this study (20% and 0.2 s). It is expected and has been proven that higher duty cycles and longer durations lead to greater phenotypic changes in hPSCs[24b] due to the longer time of forces applied to cells. Our previous studies using biotin-MBs examined only OCT4 and SOX2 expression.[20, 24] In this study, we also analyzed the impacts of mATC on stage-specific embryonic antigen-3 (SSEA-3) and GD3 (also called TRA-1-60) in hPSCs, which are glycolipid antigens indicative of undifferentiated state of hPSCs. The reduction in their expression levels signifies changes in the glycan composition during hPSC transition from pluripotent state to lineage-committed state.[25] Again, no significant difference in SSEA-3 and GD3 were seen in hPSCs using the two types of MBs (**Fig. 5**). The results demonstrate that the anchor (integrins), rather than the linker (RGD-azide/DBCO *vs* RGD-avidin/biotin) bridging MB and integrin, is responsible for the mechanobiological effects on hPSC differentiation.

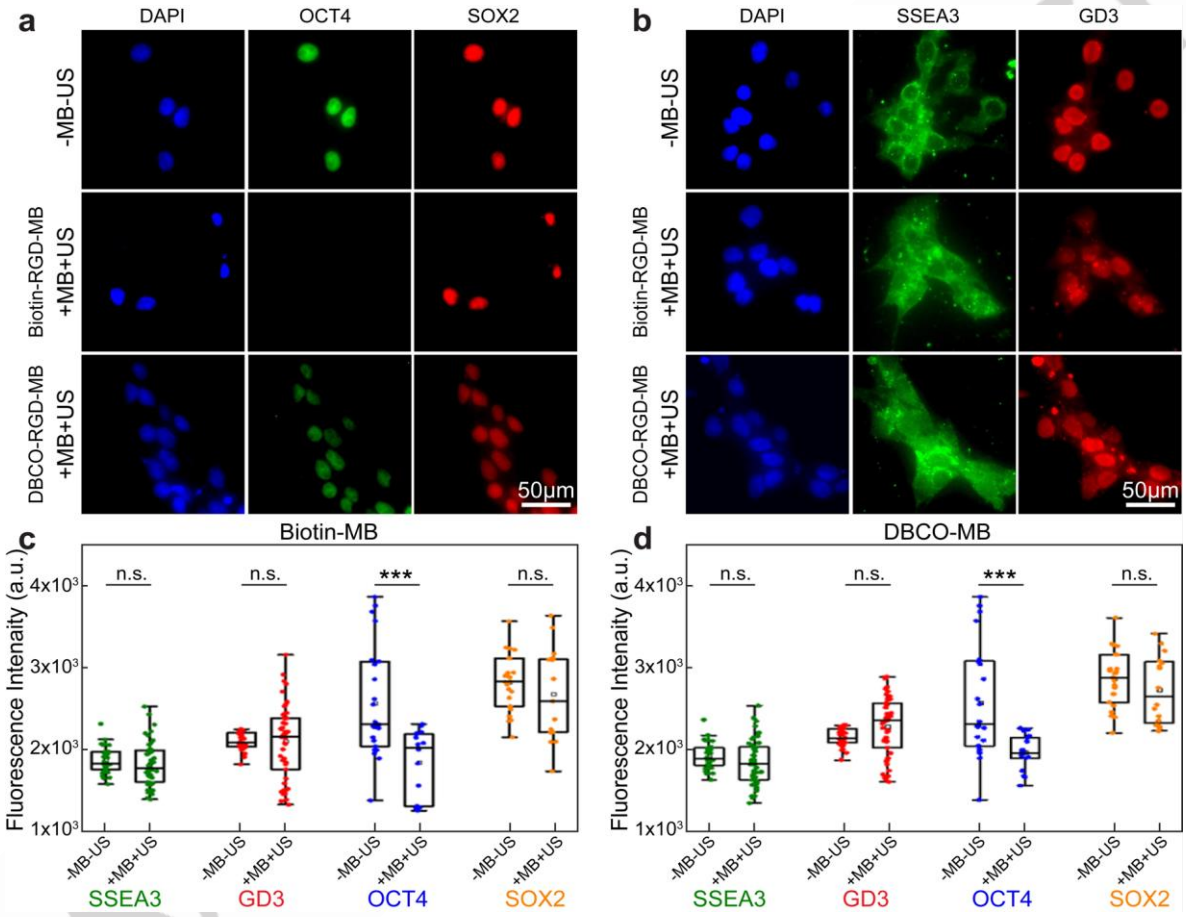

Figure 5: Effects of integrin-targeted mATC on hPSCs using biotin-MBs and DBCO-MBs. a,b). Examples of fluorescent images of DAPI, OCT4, SOX2, SSEA-3, and GD3 in hPSCs subjected to mATC using biotin-MBs and DBCO-MBs (+MB, +US), and the control without mATC (-MB, -US). **c,d**). Fluorescent intensities of OCT4 and SOX2 in hPSCs subjected to mATC using biotin-MBs (N = 17) and DBCO-MBs (N = 15), compared to the control (N = 23), as well as SSEA-3 and GD3 using biotin-MBs (N = 46) and DBCO-MBs (N = 39), compared to the control (N =30). n.s.: no statistical significance; ***: $p < 0.001$.

Influences of mechanical stimulation of sialoglycans on hPSC differentiation

Lastly, we used mATC to examine how mechanical stimulation of cell surface sialoglycans impact hPSC differentiation. To this end, we treated hPSCs with AcManNAz to install azide in sialoglycans, and then attached DBCO-MBs to azido-sialoglycans on hPSCs (**Fig. 1**). Next, the hPSCs were subjected to mATC studies. Our measurements of the expression levels of OCT4, SOX2, SSEA-3, and GD3 in these cells disclose decreased SSEA-3 and GD3 expression but not OCT4 or SOX2 nuclear localization (**Fig. 6**). These observations are in contrast to those from mechanical stimulation of integrins, where no changes in SSEA-3 or GD3 expression but a significant reduction of OCT4 nuclear localization were detected, as shown in **Fig. 5** and our previous studies.[20, 24] These findings reveal the intriguing possibility that mATC stimulation of sialoglycans and integrins may have initiated different mechanotransduction mechanisms in the early stages of hPSC differentiation. This also echoes our observation of different mechanical properties and responses of the MB-sialoglycan-cell and MB-integrin-cell axes in hPSCs (**Fig. 4**).

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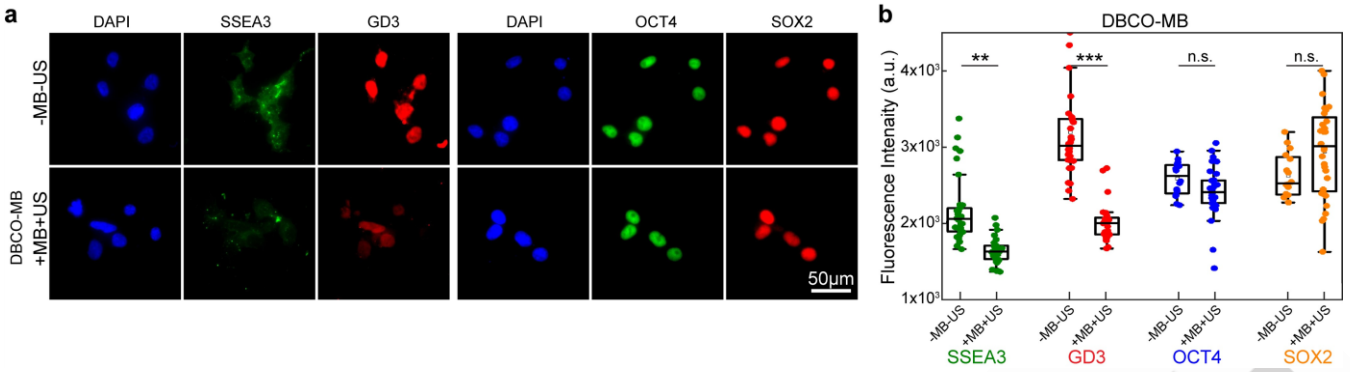

Figure 6. **Effects of sialoglycan-targeted mATC on hPSCs using DBCO-MBs**. **a**). Examples of fluorescent images of DAPI, SSEA-3, GD3, OCT4, and SOX2 in hPSCs subjected to sialoglycan-targeted mATC using DBCO-MBs (+MB, +US), and the control without mATC (-MB, -US). **b**). Florescent intensities of SSEA-3 and GD3 (N = 28) as well as OCT4 and SOX2 (N = 32) in hPSCs subjected to sialoglycan-targeted mATC using DBCO-MBs, compared to the controls without mATC (N = 34 and 16, respectively). n.s.: no statistical significance; **: $p < 0.01$; ***: $p < 0.001$.

Discussion of the results and future directions

The investigation of cell mechanotransduction and biophysics has benefited greatly from techniques like atomic force microscopy (AFM)[26] and optical tweezers[27] that can apply controlled subcellular forces to individual cells. However, these techniques are not suitable for high-throughput assays and for generation of forces to induce sustained cellular responses and, hence, have limited utility for large scale studies or for harnessing the intrinsic mechanosensitivity of hPSCs in translational application. Magnetic twisting cytometry (MTC)[28] applies forces by pulling or twisting magnetic beads attached to cells, which is useful for the study of force-mediated regulation of gene expression and stem cell differentiation.[29] However, solid magnetic beads can elicit cellular internalization and are difficult to remove, limiting continuous use of cells for further investigation. Flow shear stress and substrate stretching techniques, although capable of mechanical stimulation of many cells at once, lack the versatility and precision to target specific receptors on the cell surface, whereas this is important in mechanotransduction.[30]

In contrast, mATC applies molecule-specific forces to many cells simultaneously[14-15] *via* biocompatible MBs attached to targeted molecules on cells. MBs with a lipid or protein shell have been used as safe contrast agents for clinical US imaging, and they exhibit no cell internalization.[19] The new DBCO-MBs contain a reactive alkynyl group for convenient and efficient coupling with molecules containing an azido group *via* click chemistry. When combined with MEG to install azido-sialoglycans on cells, these MBs enable a new and practical mATC technology to target and mechanically stimulate sialoglycans on live cells for the first time.

The distinct viscoelastic properties of MB-sialoglycan-hPSC and MB-integrin-hPSC axes in response to mATC, independent of the linker (*e.g.*, MB-DBCO-azide-RGD or MB-alkyne-azide) between MBs and the cell surface molecules/anchors, may be attributed to the different chemical structures and microenvironments of cell surface glycans and integrins and potentially reflect their distinct interactions with other molecules on the cell surface and/or within the cells. While it is important to further elucidate the underlying mechanisms on hPSC differentiation, the focus of this study is to demonstrate the feasibility of the new ATC approach enabled by MGE and a new type of MBs (DBCO-MBs), in terms of influencing cellular behaviors such as hPSC differentiation. Nevertheless, our results of the different mechanical properties of sialoglycans and integrins on cells and their different impacts on cellular behaviors reveal intriguing and different functional roles of these molecules in cell mechanotransduction. For example, compared to proteins such as integrins, glycans are structurally flexible, lacking well-defined 3D structure/conformation. Glycans in the cell glycocalyx are more exposed to the external space than integrins that are buried underneath the cell glycocalyx. Furthermore, integrins may form stronger connections with intracellular structures, such as the CKS. Consequently, displacing surface glycans should face less hindrance than displacing integrins, leading to greater MB displacements and cellular deformation during mATC under the same US condition. The smaller residual displacement and faster restoration time of sialoglycan-anchored MBs after each US pulse, compared to integrin-anchored MBs, indicate less plasticity and permanent deformation of sialoglycan-associated molecules than the integrin-CSK linkage in hPSCs. These findings disclose new insights into the mechanical behaviors of cell surface sialoglycans and support that sialoglycans, or the cell glycocalyx overall, are uniquely positioned and suitable as sensors and transmitters of mechanical signals in mechanotransduction.[5d, 13j]

Our results show the superior sensitivity of hPSCs to mechanical forces applied to integrins and sialoglycans, as evidenced by the rapid differentiation and changes in their expression of SSEA-3, GD3, and OCT4 after only 30 min of mATC stimulation, whereas such changes typically take 24-48 h to occur when soluble factors are used.[11b] Moreover, hPSCs exhibited different responses to mechanical stimulation of sialoglycans or integrins in SSEA-3 and GD3 expression as well as in OCT4 nuclear localization. Taken together, these results suggest that sialoglycans may represent a distinctive mechanotransduction axis orthogonal to the integrin axis in regulating hPSC differentiation at least during the early stages.[31] It is worth noting that this study shows the feasibility of MGE-enabled mATC for mechanostimulation of sialoglycans on hPSCs and the immediate impact on hPSC differentiation. The results underscore the importance of further in-depth investigation of molecular mechanisms involved in initiation of cellular signaling pathways and alteration of gene expression.

While this study focused on hPSCs and their surface sialoglycans, MGE-enabled mATC technology could be widely applicable. First, MGE with other sugar precursors can be used to target different

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glycans and attach MBs for mATC. Second, MGE-based mATC can be applied to different cell types. Moreover, the new DBCO-MBs can be employed to conveniently and effectively target other azide-modified molecules on cells for mATC, as demonstrated in this work by targeting integrins on hPSCs using DBCO-MBs. As a result, the new DBCO-MBs and MGE-based mATC technology offer great molecular specificity and versatility for mechanoglycobiological studies.

Furthermore, AcManNAz-based MGE was utilized in this study to target a select group of glycans, *i.e.*, sialoglycans. However, due to the heterogeneity of natural glycans, the observed influence of mATC on hPSCs is the combined result of different sialoglycans. In the meantime, glycans on cells usually exist as protein and lipid conjugates, in which glycans and proteins/lipids are inseparable and function jointly. Therefore, forces applied to glycans can also influence the conjugated proteins and lipids. To gain insights into the functional roles of any specific glycans or glycoconjugates in mechanotransduction, we can combine mATC with other glycan engineering methods, e.g., using enzymes or structurally defined probes, which is one of our future directions. Finally, glycans on cells can sense biophysical cues and transduce signals by varied mechanisms. For example, the structures of glycoproteins and glycolipids and their interaction with other biomolecules on cells can be directly influenced by mATC forces to regulate transmembrane signals. Applied forces may also cause changes in the membrane organization and fluidity of cells to modulate the functions of transmembrane proteins.[32] As such, integrins and sialoglycans may influence each other during mechanotransduction[33] as the activities of integrins are affected by their glycosylation patterns and interaction with other glycans.[5i] This posts another interesting problem that is beyond the scope of this study but important to pursue in future investigations.

Conclusion

We have developed a new type of MBs, DBCO-MBs, and verified their capability to couple with azide-labeled molecules on cells *via* click chemistry. Metabolic labeling of sialoglycans on hPSCs with an azide facilitated DBCO-MB attachment to them, thus allowing targeted mechanostimulation of surface sialoglycans in hPSCs using mATC. When subjected to the cyclic forces of US pulses, sialoglycans and integrins on hPSCs showed remarkably different viscoelastic properties. Sialoglycans on hPSCs appeared more flexible and elastic than integrins on the cell surface, suggesting that sialoglycans can be excellent sensors and transducers of mechanical signals. Mechanostimulation of sialoglycans and integrins on hPSCs initiated rapid but different changes in pluripotency markers and transcription factors indicative of early stages of hPSC differentiation, providing new insights into the role of sialoglycans as an important mechanotransduction element affecting hPSC fate. Our results validate the feasibility of MGE-based mATC as a versatile and useful platform for probing the functional roles of glycans and other cell surface targets in cellular mechanotransduction and underscore the importance of further investigation on the mechanisms of mechanoglycobiology in hPSC differentiation as well as other applications.

Supporting Information

Supporting Information includes materials, methods, experimental procedures, and other biological results. The data underlying this study are available in the published article and its Supporting Information.

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Keywords: Acoustic tweezing cytometry • Glycoengineering • Sialoglycans • Mechanobiology • Human pluripotent stem cells

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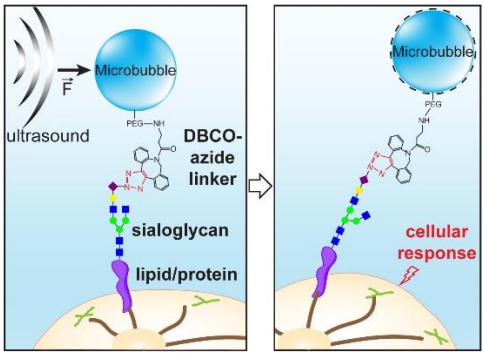

DBCO-functionalized microbubbles were attached to azide-tagged sialoglycans on the cell surface generated via cell metabolic engineering to enable molecularly specific acoustic tweezing cytometry. This novel technology was utilized to study the roles of sialoglycans in mechanotransduction, revealing different mechanical properties and mechanoregulatory activities of sialoglycans from protein sensors like integrins.

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