Supporting Information

for

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

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I. Experimental Section

Materials and general methods

Chemicals and materials were purchased from commercial sources and used as received without further purification unless otherwise noted. AcManNAz was synthesized in our labs according to previously reported methods and was fully characterized with 1D and 2D ^{1}H and ^{13}C NMR and high-resolution MS data. Biotin-MBs were purchased from Advanced Microbubbles Laboratories, LLC, and DBCO-MBs were designed by our labs but were custom-produced by Advanced Microbubbles Laboratories. Cyclo Arg-Gly-Asp-D-Phe-Lys-PEG-Biotin (biotin-PEG-RGD) was purchased from Peptides International. Streptavidin was purchased from Thermo Fisher. DSPE-PEG2000-DBCO and DSPC were purchased from Avanti Polar Lipids. Azide-RGD was purchased from Biosynth, UK. Alexa Fluor 594-DBCO and Alexa Fluor 488-azide were purchased from Fisher Scientific. Anti-SOX2, -OCT4, -SSEA-3, and -GD3 antibodies were purchased from Santa Cruz Biotechnology. Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 555-conjugated goat anti-human IgM antibodies were purchased from Invitrogen.

Cell culture

H9 human embryonic stem cells (WA09, WiCell; NIH registration number: 0062) were maintained in a standard feeder-free culture system using mTeSR1 medium (STEMCELL Technologies) and lactate dehydrogenase-elevating virus (LDEV)-free hESC-qualified reduced growth factor basement membrane matrix Geltrex (Thermo Fisher Scientific; derived from Engelbreth-Holm–Swarm tumours similarly as Matrigel) in accordance with the manufacturer's guidelines. Each passage involved a visual inspection to confirm the absence of spontaneously differentiated, mesenchymal-like cells in the culture. Only hPSCs before P70 were used in this work. For ATC experiments, cells are seeded at a density of 15,000 cells cm^{-2} in mTeSR in a glass bottom dish (MatTek Corporation) coated with 1% Geltrex on day 0. ROCK inhibitor Y-27632 (10 μM; Tocris) was supplemented in the culture medium to prevent dissociation-induced apoptosis. On day 1, mTeSR1 was replenished without Y-27362. SB-431542 (10 μM, Stem cell Technologies) and mTeSR1 medium were added to inhibit TGF-β signaling and disrupt the pluripotency-maintaining gene circuit in hPSCs. mATC experiments were performed on day 2.

For hPSC MGE to install sialoglycans onto the cell surface, AcManNAz (100 μM) was added to the culture of hPSCs in mTeSR1, which was incubated at 37 ^{o}C and 5% CO_2 for 24 h. To verify

azide-labeled sialogylcan presentation on hPSCs, the cells were washed with phosphate buffered saline (PBS; Thermo Fisher Scientific, pH 7.4, sterile-filtered) and incubated with Alexa Fluor 594-DBCO (20 μM) for 1 h in the incubator rt to allow for click reaction to take place. After the staining, cells were washed with PBS 3 times and imaged with a Nikon Ti Eclipse Microscope to detect the fluorescent signals.

Attachment of biotin-MBs to integrins in hPSCs

Biotin-MBs (diameter of 4 μm) were incubated streptavidin (10 mg/mL) (20:1 ratio, v/v) at room temperature (rt) for 1 h. After washing with PBS to remove excess streptavidin, the streptavidin-biotin-MBs were mixed with biotin-PEG-RGD (2 mg/mL) at a ratio of 10:1 (v/v) and incubated at rt for another h, followed by washing with PBS to remove excess biotin-PEG-RGD. The solution of resultant RGD-streptavidin/biotin-MBs was diluted 10 times with mTESR. The MBs in MTeSR solution (final concentration $2 \times 10^8/ml$) were attached to hPSCs following a procedure described previously. Briefly, the medium was removed from the cell culture dish to leave a minimal amount of mTeSR on the adherent hPSCs, followed immediately by addition of the RGD-streptavidin/biotin-MB solution (100 μL). The dish was then flipped upside down and placed in an incubator (37 ^{o}C) for 30 min to allow the MBs to float up against the hPSCs, thereby enabling their attachment to integrins of hPSCs. The dish was flipped back and unbound MBs were removed by washing with PBS three times before ATC experiments.

Formulation of DBCO-MBs

DBCO-MBs were prepared by the same protocols used for the formulation of biotin-MBs. Briefly, PFB was bubbled into the suspension of DSPE-PEG2000-DBCO and DSPC (in 1:19 molar ratio) in a buffer with sonication under well-controlled conditions to form the desired DBCO-MB solution, which was directly used in this study or used after dilution.

To verify their functionalization with DBCO, these MBs were incubated with Alexa Fluor 488-azide (20 μM) at rt for 1 h, followed by washing with PBS 3 times using gravitation. The stained DBCO-MBs were placed on a glass slice and imaged using a Nikon Ti Eclipse Microscope with excitation/emission peaks of 495/519 nm.

Attachment of DBCO-MBs to integrins in hPSCs

DBCO-MBs obtained above were mixed with azide-RGD (2 mg/mL) at a ratio of 10:1 (v/v), and the mixture was incubated at rt for 1 h, followed by washing with PBS to remove unattached azide-

RGD. The resultant RGD-azide/DBCO-MB solution was diluted 10 times in mTESR medium and then attached to hPSCs by the same protocols employed to attach RGD-streptavidin/biotin-MBs as described above.

Attachment of DBCO-MBs to sialoglycans on metabolically engineered hPSCs

DBCO-MBs in mTeSR (100 µL) were added to the culture of AcManNAz (100 µM)-treated hPSCs obtained above. The mixture was incubated at 37 ^{o}C for 30 min and then rinsed with PBS 3 times to remove unbounded MBs, following the same MB attachment procedure as described above. The resultant DBCO-MB-labeled cells were subjected to mATC studies.

Experimental setup for mATC studies

A single-element planar transducer (1.25 MHz center frequency, 3.5 mm radius, 9 mm Rayleigh distance; Advanced Devices, MA) was used for mATC. The transducer was fixed at an angle of 45^{o} relative to the vertical axis facing downward and placed at the Rayleigh distance from the hPSCs seeded at the bottom of glass bottom dish, with its active surface submerged in mTeSR for acoustic coupling. Two waveform generators (Agilent Technologies) and a 75 W power amplifier (Amplifier Research) were utilized to drive the transducer and generate US pulses with desired acoustic pressure, pulse duration, and PRF. The US transducer was calibrated in free field in water using a needle hydrophone (HNR-0500, Onda). In this study, mATC was applied to displace MBs on hPSCs for a total duration of 30 min using US pulses with an acoustic pressure of 0.035 MPa, PRF of 1 Hz, and duty cycle of 20% (duration of each pulse 0.2 s).

Monitoring and characterization of MB displacement during ATC

Movements of MBs anchored to the surface of hPSCs during mATC were recorded with an inverted microscope (Nikon Eclipse Ti-U) and a high-speed camera (Photron FASTCAM SA1) operated at 1000 frames/s and a subpixel resolution of 330 nm. MB displacement was determined by tracking the location of MBs over time from the recorded video employing an automated, custom-develop MATLAB script. Peak displacement, residual displacement (the non-recovered displacement after each US pulse), and time constant of the displacement recovery after each pulse were determined from the measured displacement of each MB.

Immunofluorescence staining and quantification

hPSCs were fixed immediately after mATC treatment by applying 4% paraformaldehyde (Electron Microscopy Sciences) at rt for 30 min and permeabilized with 0.1% sodium dodecyl sulfate (SDS;

Sigma-Aldrich) for another 30 min. To ascertain changes in OCT4 and SOX2, after incubated with 4% Donkey serum (Sigma-Aldrich) at rt for 1 h to block non-specific binding, hPSCs were treated with anti-SOX2 or anti-OCT4 primary antibodies and then with secondary antibodies at 4 ^{o}C for 1 h, respectively. Cell nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; Thermo Fisher Scientific) by standard protocols. For the analysis of surface glycolipid antigens, the hPSCs were incubated first with anti-SSEA-3 (1:50 dilution) or -GD3 (1:50 dilution) primary antibodies at 4 ^{o}C overnight and then with secondary antibodies Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 555-conjugated goat anti-human IgM (1:1,000 dilutions) at 4 ^{o}C also overnight. Immunofluorescent images of the hPSCs were taken with a Nikon Ti Eclipse Confocal Microscope. Using ImageJ (NIH), the expression levels of OCT4, SOX2, SSEA3, and GD3 in each cell were determined by quantifying the immunofluorescent intensities. Specifically, the fluorescent intensity of nuclear marker was determined through the following process: first, DAPI channel images were converted to 8-bit and then, a threshold was automatically determined from the average background signal to demarcate the nucleus region and determine nuclear signal intensity relative to the average background signal. After applying binary masking, images of each marker are obtained, and fluorescent intensity was determined. To address variations in staining at different batches, the fluorescent intensity values are normalized to average DAPI intensity for each image.

Statistical Analysis

All experiments were repeated independently at least three times. For data complying with normal distribution, two-sample *t*-test is applied. Nonparametric Mann-Whitney test is performed for the data which does not follow the normal distribution. * $p < 0.05$, statistically significant; ** $p < 0.01$ and *** $p < 0.001$, statistically very significant.

II. Additional Biological Results, Images, and Videos

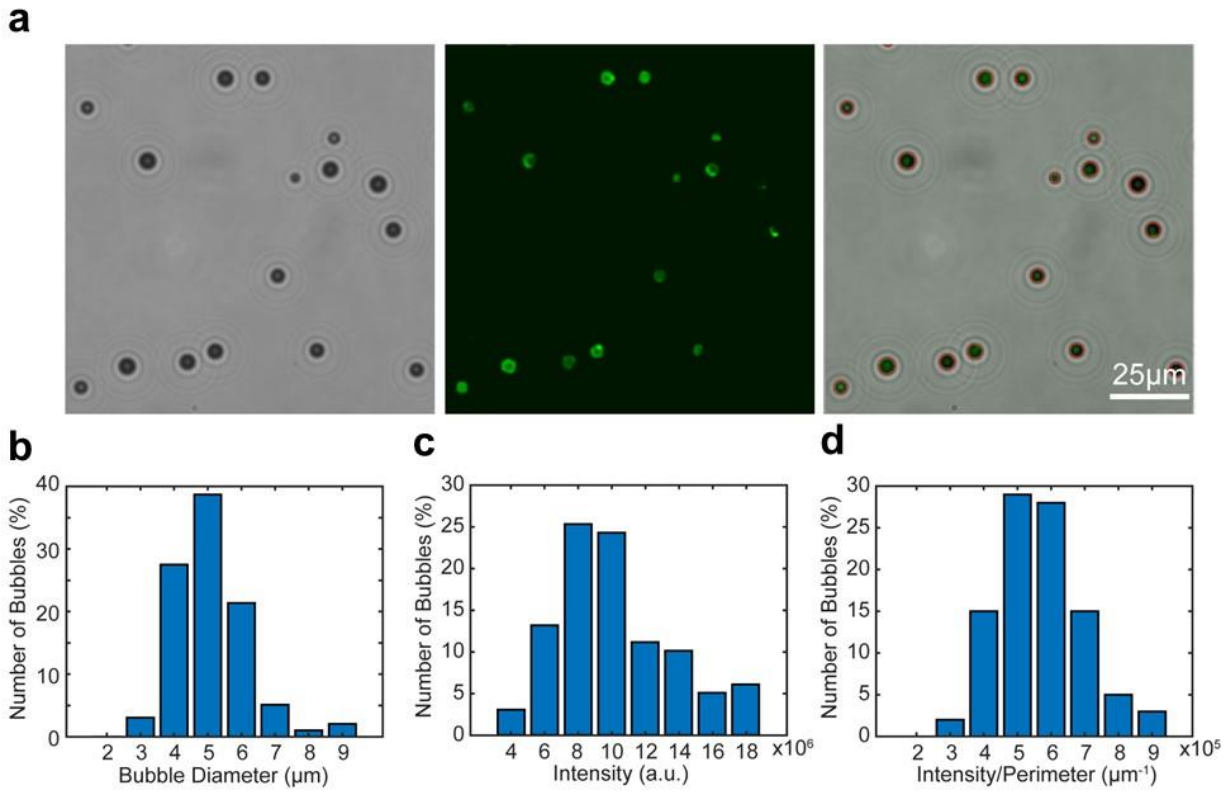

Figure S1: **a**). Bright field (BF), fluorescent, and overlay images of DBCO-MBs treated with azide-Alexa Fluor 488 (green fluorophore), with MB positions shown by the red circles; **b**) MB diameter distributions; **c**) MB fluorescence intensity distribution; **d**) MB fluorescence intensity distribution divided by MB perimeter.

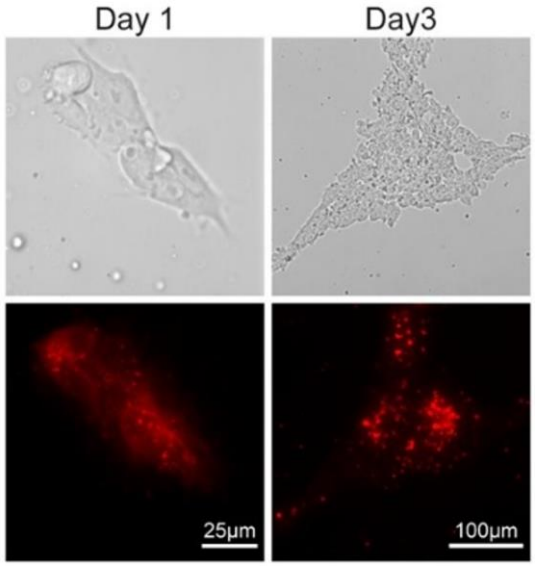

Figure S2: BF and fluorescent images of hPSCs treated with 100 μM of AcManNAz for 1 and 3 days, respectively, and then with Alexa Fluor 594 (red fluorophore)-DBCO.

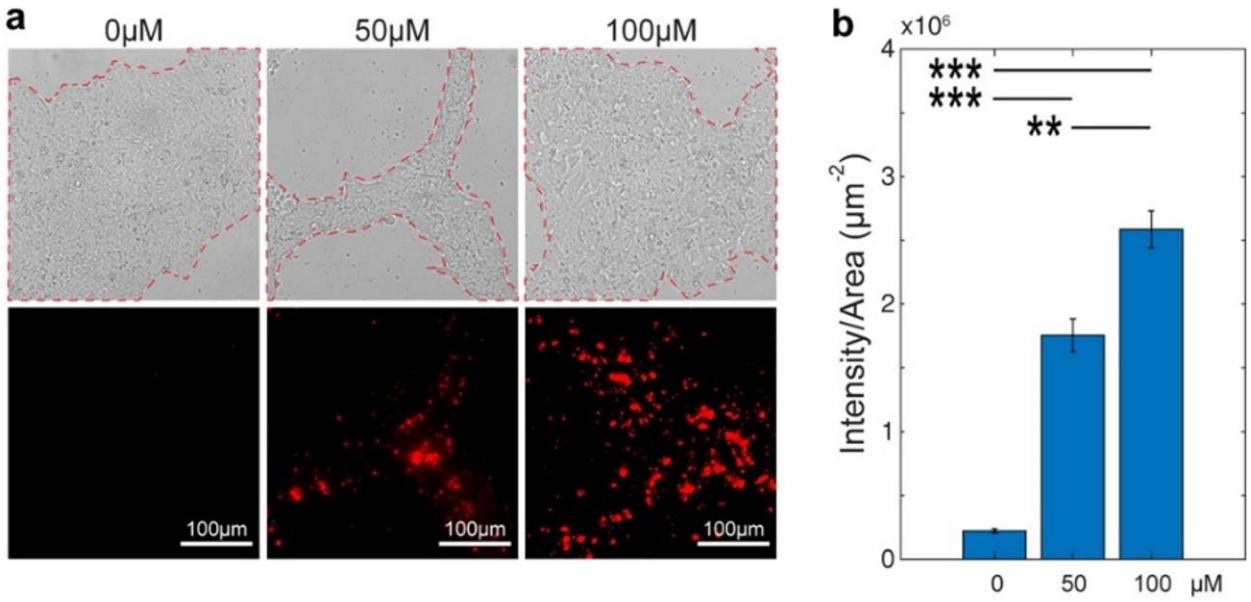

Figure S3: **a**) BF and fluorescent images and **b**) quantification of fluorescent intensities (divided by areas) of hPSCs treated with 0, 50, and 100 μM of AcManNAz, respectively, for 3 days and then with Alexa Fluor 594-DBCO. **: $p < 0.01$; ***: $p < 0.001$.

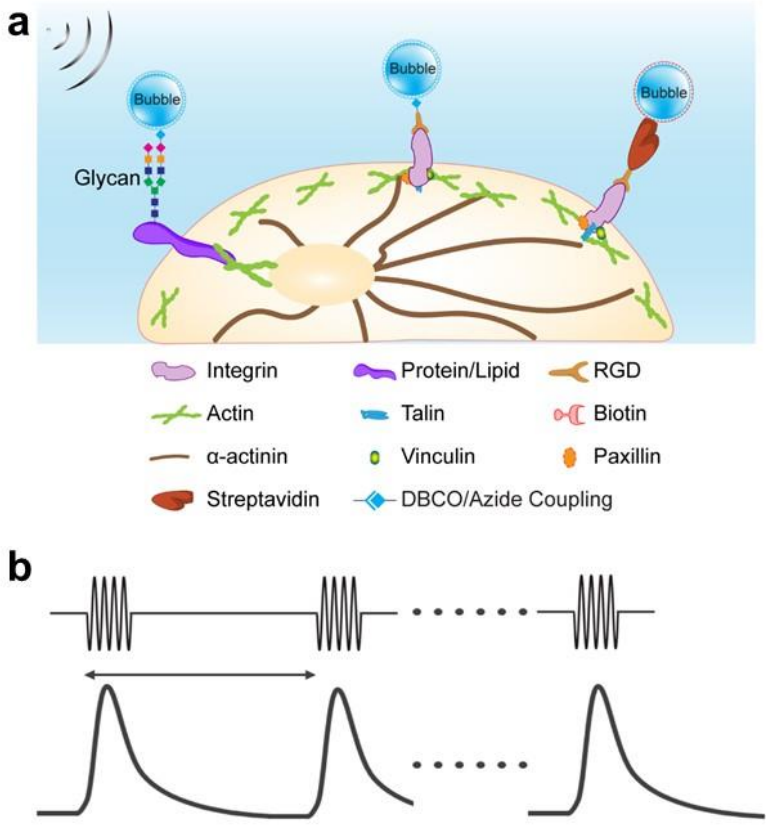

Figure S4: Diagrams showing the experimental settings for mATC. **a**). Schematics depicting the attachment of MBs to hPSCs via three different types of linkers and anchors; **b**). schematics of ultrasound pulses used to displace cell-bound MBs during mATC.

Supplemental video SV1: displacement of DBCO-MB attached to sialoglycans of hPSCs

Supplemental video SV2: displacement of DBCO-MB attached to integrins of hPSCs via RGD

Supplemental video SV3: displacement of Biotin-MB attached to integrins of hPSCs via RGD