Supporting Information

Sliding hydrogels with mobile molecular ligands and crosslinks as 3D stem cell niche

Xinming Tong, and Fan Yang

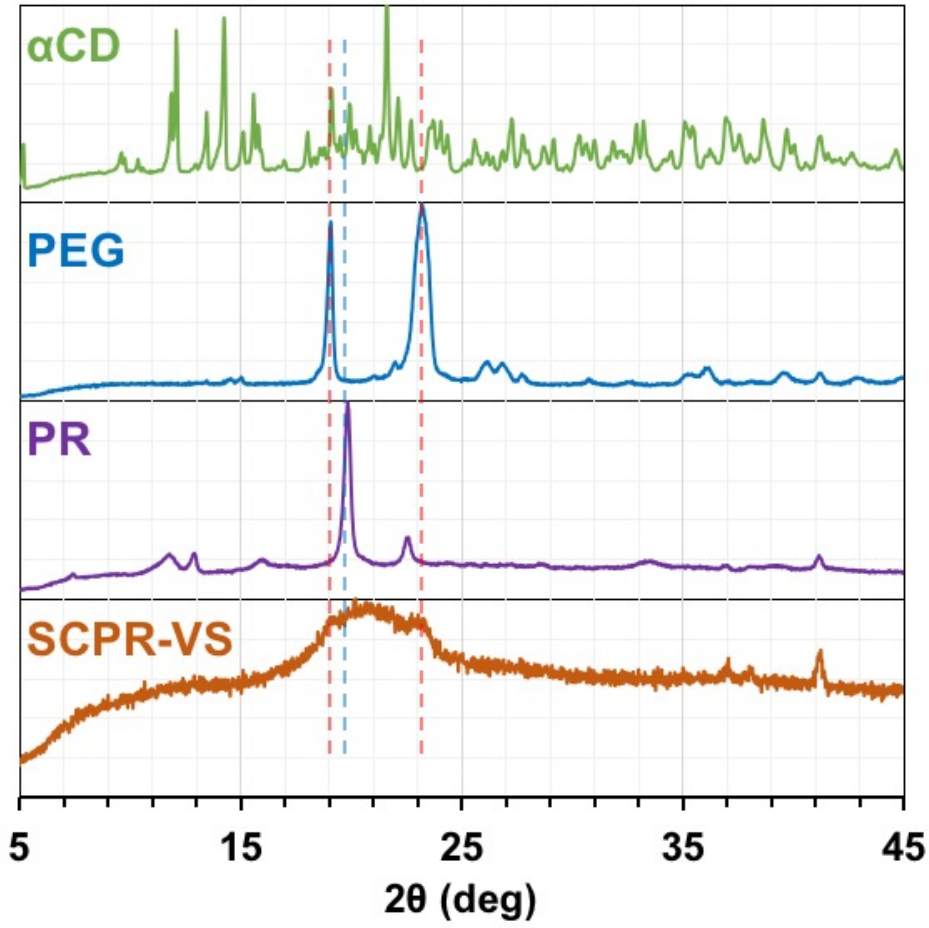

Figure S1. X-ray diffraction patterns of naïve free α-cyclodextrins (αCDs), polyethylene glycol (PEG), polyroxatane (PR), and succinic-polyrotaxane with vinyl sulfone groups (SCPR-VS).

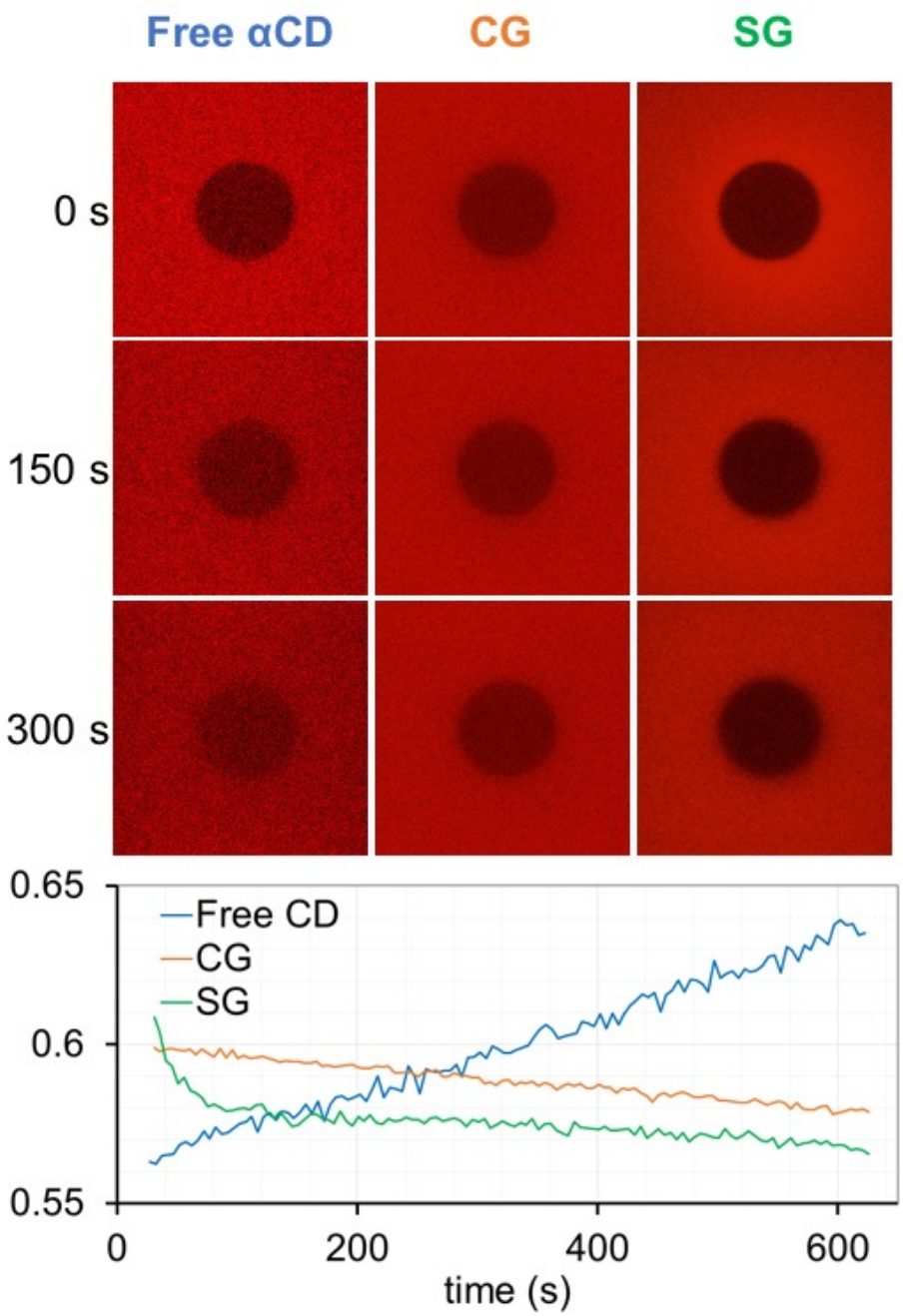

Figure S2. Confocal images (top) and normalized fluorescent intensity (bottom) of fluorescence recovery after photobleaching reveal diffusivity characteristics. Free αCD, hydrogel with free α-cyclodextrins labeled with tetramethylrhodamine. CG, chemical hydrogel with chemically immobilized tetramethylrhodamine-labeled ligands. SG, sliding hydrogel with sliding tetramethylrhodamine-labeled ligands. Only the data from free αCD indicated fluorescence recovery after photobleaching and thus intra-construct mobility.

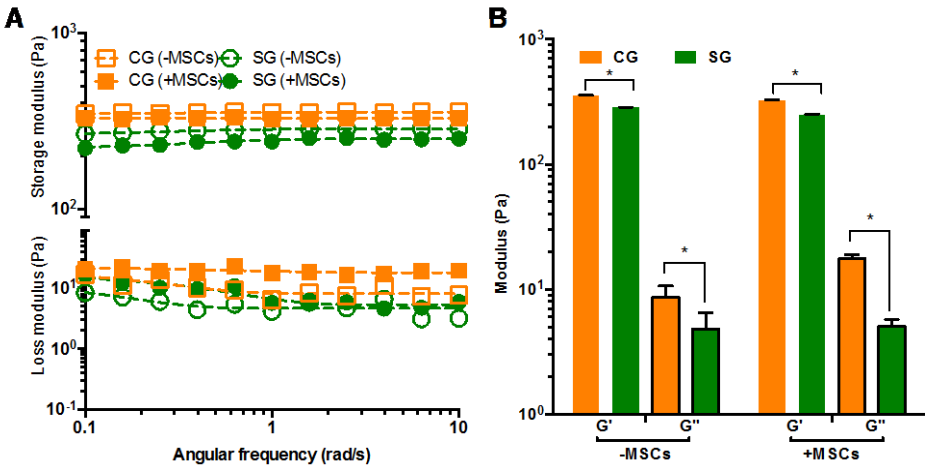

Figure S3. (A) Storage moduli and loss moduli of chemical hydrogel (CG) and sliding hydrogel (SG), with and without MSCs encapsulated; (B) modulus obtained under 1 rad/s with strain of 5 %.

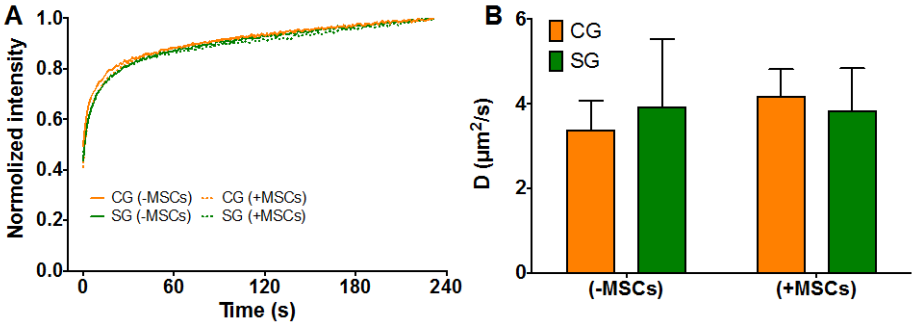

Figure S4. (A) Representative fluorescent intensity recovery curve for FITC-BSA in chemical hydrogel (CG) and sliding hydrogel (SG), with and without MSCs encapsulated. **(B)** Calculated diffusion coefficient of FITC-BSA in CG or SG hydrogels. No significant difference was observed among all groups tested.

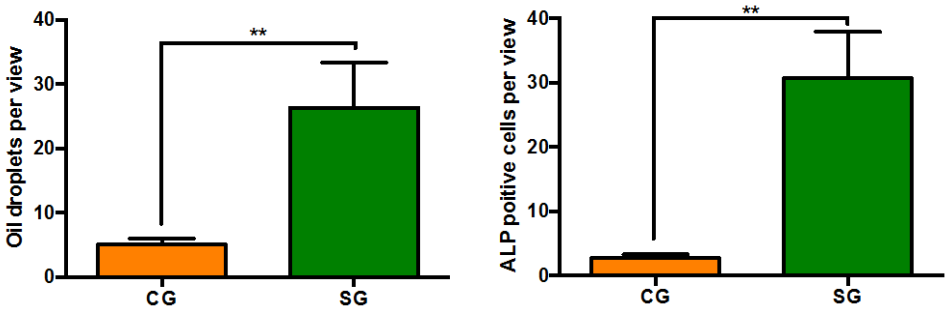

Figure S5. Quantification of differentiation towards adipogenesis or osteogenesis by counting oil droplets or ALP positive cells. SG significantly increased both adipogenesis and osteogenesis in 3D than CG. **: $p < 0.01$.

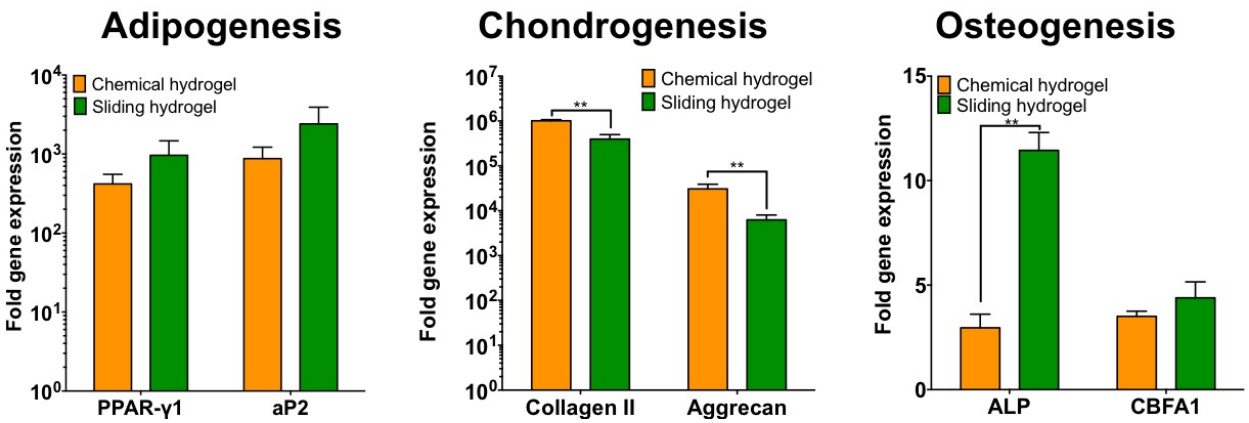

Figure S6. Quantitative expression of genes encoding biomarkers of cell fate reflects stem-cell differentiation. hMSCs were cultured in chemical hydrogels (orange) or sliding hydrogels (green) for 14 days. Adipogenesis markers, peroxisome proliferator-activated receptor gamma 1 (PPAR-γ1) and adipocyte protein 2 (aP2); chondrogenesis markers, collagen II and aggrecan; osteogenesis markers, alkaline phosphatase (ALP) and core-binding factor alpha 1(CBFA1). Data were presented as average ± standard deviations with 3 samples per group. **: $p < 0.01$.

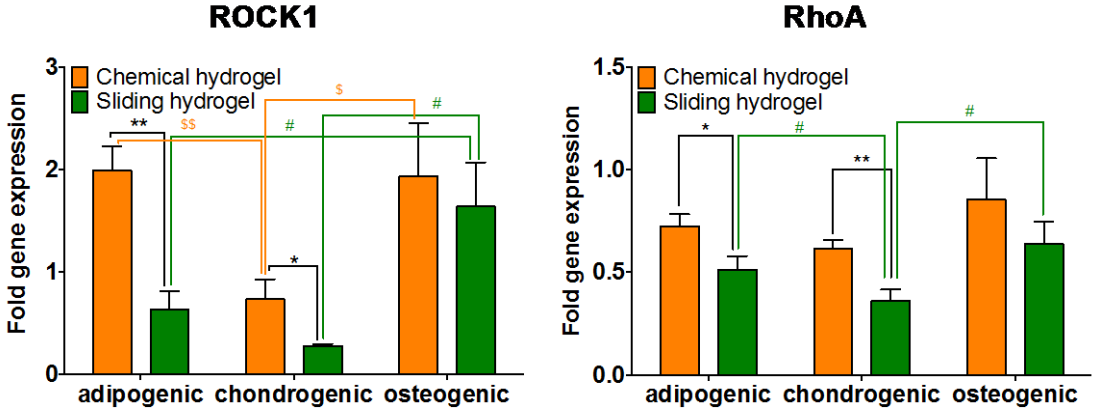

Figure S7. Quantitative expression of mechanosensing genes (rho-associated, coiled-coil-containing protein kinase 1, ROCK1 and ras homolog gene family, member A, RhoA) by hMSCs in chemical hydrogels (orange) and sliding hydrogels (green) reflects stem-cell differentiation. Culture was carried out in adipogenic, chondrogenic, or osteogenic medium for 14 days. Data were presented as average ± standard deviations with 3 samples per group. (* and **: statistically different gene expressions between chemical hydrogel and sliding hydrogel, with *: $p < 0.05$, **: $p< 0.01$; $ and $$: statistically different gene expressions in chemical hydrogel in different induction medium, with $: $p < 0.05$ and $$: p value < 0.01; #: statistically different gene expression in sliding hydrogel in different induction medium with #: $p < 0.05$).

Synthesis of succinic-polyrotaxane with vinyl sulfone groups (SCPR-VS)

α-cyclodextrin (αCD; 2.4 g; Biosynth) and 1.3 g polyethylene glycol with thiol groups (PEG-thiol; MW 20 kDa; NOF America) were mixed in 50 mL dionized water and stirred overnight. The resulting turbid suspension was freeze-dried and the powder was mixed with 1 g mono-βCD-vinyl sulfone in 0.1 M Tris-HCl buffer (pH 8.0) and stirred overnight. The resulting suspension was mixed with dimethyl sulfoxide until a clear solution was obtained; this solution was dialyzed against deionized water (MWCO 13 kDa) for 2 days. The dialysate was centrifuged; the precipitated solid was washed with deionized water three times and freeze-dried as a powder. One gram of this polyrotaxane was reacted with 1 g succinic anhydride (Sigma) in 10 mL pyridine (Fisher) overnight, precipitated in diethyl ether, and vacuum dried into a solid powder. This succinic polyrotaxane was reacted with 1 mL divinyl sulfone (Sigma) in 50 mL Na_2CO_3 buffer (pH 10.5, 0.1 M) for 2 h. The solution was neutralized with concentrated HCl and dialyzed against deionized water (MWCO 13 kDa) for 2 days. The dialysate was freeze-dried into a powder (succinic-polyrotaxane with vinyl sulfone groups).

Synthesis of PEG-thiol and 4arm-PEG-thiol

PEG (MW 6 kDa, Sigma) and 4arm-PEG (MW 10 kDa, JenKem) were converted into PEG-diamine and 4arm-PEG-amine, respectively, by reacting them with phthalimide (Sigma) catalyzed by diisopropyl azodicarboxylate/triphenylphosphine (Sigma) and deprotected using hydrazine (Sigma) in dichloromethane. The resulting PEG-diamine and 4arm-PEG-amine were reacted with thioglycolic acid in toluene catalyzed by toluenesulfonic acid (Sigma) at 160 °C overnight. The resulting PEG-dithiol and 4arm-PEG-thiol products, respectively, were precipitated in cold ethyl ether.

Hydrogel formation

To formulate a chemical hydrogel, 4arm-PEG-thiol and 4arm with vinyl sulfones (MW 10 kDa, JenKem) were dissolved in phosphate-buffered saline PBS at concentration of 4 w/v% and mixed in a volume ratio of 1:1. The mixed solution was left to crosslink at 37 °C for 30 min.

To obtain sliding hydrogel, PEG-dithiol and succinic-polyrotaxane with vinyl sulfone groups were dissolved in PBS at a concentration of 6 w/v% and mixed in a volume ratio of 1:1. The mixed solution was left to crosslink at 37 °C for 30 min.

Swelling ratio

Fifty-microliter hydrogels were formed and transfered to a 24-well plate with 1.5 mL PBS per well and incubated at 37 °C. The wet weight of each hydrogel was measured at each predetermined time point. The swelling ratio (Q) of the hydrogels was calculated using the expression $Q = W_{wet}/W_{dry}$, where W_{wet} is the wet weight of the hydrogel at a given time point and W_{dry} is the dry weight of the hydrogel at the beginning. To measure the dry weight of hydrogels in each group, separate hydrogels were formed under the same conditions and then washed in deionized water overnight and freeze-dried.

Mechanical testing

The hydrogels were formed into cylinders with a diameter of 6 mm and a thickness of 3 mm. The hydrogels were incubated in PBS overnight to reach equilibrium swelling prior to mechanical testing. Unconfined compression testing was used to measure the stiffness of all hydrogels using the Instron 5944 testing system equipped with a 10 N load cell (Interface Inc.). All tests were conducted in PBS at room temperature. The compressive modulus was calculated using the linear curve fits of the stress versus strain curve for strain ranges of 10-20%. To obtain the storage modulus and loss modulus as function of frequency, rheology test was performed using ARES-G2 (TA Instruments). Hydrogels were made into disk shape with diameter of 8 mm and thickness of 1 mm. The measurements were done at 37° C. Frequency sweeps were performed at 5% strain with frequency changed from 0.1 to 10 rad/s.

X-ray diffraction (XRD)

To prepare the hydrogel samples for XRD, the precursor samples were ground into powder and mounted on a glass slide. The hydrogels were first casted on a glass slide with 1 mm thickness and then air dried before testing. XRD measurement performed on a PANalytical X'Pert PRO XRD system. The radiation source was Ni-filtered, Cu Ka radiation. The voltage was set to 45 kV and the current was set to 40 mA. The scan was performed from $2\theta = 5°$ to 45° at a speed of 0.1°/s.

Fluorescence correlation spectroscopy (FCS)

FCS was performed on a Zeiss LSM 780 multiphoton laser scanning confocal microscope with the FCS function. Free αCD groups were labeled with tetramethylrhodamine (TRITC, Sigma) and mixed with chemical hydrogel precursor solution, with a concentration of αCD at 0.1 μM before crosslinking. Chemical hydrogels and sliding hydrogels were prepared by crosslinking the precursor solutions as described above plus an additional 0.1 μM CRGDSK-TRITC (Biomatik). Hydrogel samples were put on a glass coverslip and mounted onto the 40x lens, which was pre-immersed with a drop of water immersion liquid. Each sample was measured with 10 s measure time and 12 repetitions.

Fluorescence recovery after photobleaching (FRAP)

FRAP was performed on a Leica SP8 white light confocal microscope with the FRAP function. Free αCDs were labeled with TRITC as above and mixed with chemical hydrogel precursor solution, with a concentration of αCDs at 0.2 mM, before crosslinking. Chemical hydrogels and sliding hydrogels were prepared by crosslinking the precursor solutions as described above plus an additional 0.2 mM CRGDSK-TRITC. Hydrogels were sandwiched between two coverslides immersed in PBS before measurement. Photobleaching was achieved by focusing the laser to a spot (Φ = 10 μm) on the sample for 250 ms. Wide-field fluorescence images of the samples were acquired before and after photobleaching every 10 s. The fluorescence intensity was normalized to that before photobleaching.

To measure the diffusivity of FITC labeled bovine serum album (FITC-BSA) in hydrogels, both hydrogels made with and without cells and then soaked in PBS with FITC-BSA (1 mg/mL) overnight. The hydrogels were sandwiched between two coverslides immersed in PBS before measurement. Photobleaching was achieved by focusing the laser to a spot (Φ = 25 μm) on the sample for 10 s. Wide-field fluorescence images of the samples were acquired before and after photobleaching every 1.3 s. The fluorescence intensity was normalized to that before photobleaching.

Cell culture

Human mesenchymal stem cells (hMSCs) were obtained from Lonza and expanded until passage 6 in growth medium comprised of high-glucose Dulbecco's minimal essential medium (DMEM; Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/mL penicillin/streptomycin (Gibco), and 10 ng/mL basic fibroblast growth factor (PeproTech).

Cell encapsulation and viability confirmation

hMSCs were suspended in hydrogel precursor solutions as described above at a density of 10^7 cells per mL plus an additional 1.0 mM CRGDS. Cell-laden hydrogels were cast in a mold into volumes of 50 µL each. After culturing the cells in the hydrogels in growth medium for 24 h and 14 days, cells were stained with the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen; the kit contains calcein-AM and ethidium homodimer-1) in PBS for 20 min. Fluorescence images were taken using a Zeiss fluorescence microscope.

Fluorescence resonance energy transfer

hMSCs were suspended in hydrogel precursor solutions as described above at a density of 10^6 cells per mL with an additional 0.1 mM CRGDSK-TRITC and 0.1 mM CRGDS-fluorescein isothiocyanate and cast as above. Before imaging, the cell laden hydrogels were treated with Hoechst 34580 in medium for 1 hour. The hydrogels were sandwiched between two coverslides immersed in medium and mounted on a Leica SP8 white light confocal microscope. The hydrogels were excited at 488 nm and the fluorescence emitted between 570 and 620 nm (red emission) was collected.

Cell morphology (F-actin staining)

hMSCs were suspended in hydrogel precursor solutions as described above at a density of 5×10^6 cells per mL with an additional 1.0 mM CRGDS and cast as above. After culture in growth medium for 3 days, cell-laden hydrogels were fixed in 4% paraformaldehyde (Sigma) overnight and permeabilized in PBS with 3% bovine serum albumin (Fisher Scientific) and 0.1% Triton X-100 (Sigma) overnight. The hydrogels were then soaked in phalloidin-TRITC (Sigma, 1 µg/mL in PBS with 3% bovine serum albumin and 0.1% Triton X-100, supplemented with 1 µg/mL Hoechst 33342) overnight, followed by thoroughly washing in PBS for 2 days. Images were taken on a Leica SP8 white light confocal microscope.

Stem cell differentiation

hMSC-laden hydrogels (cell density 10^7 cells/mL) were cultured as follows to promote differentiation. Adipogenic medium was comprised of DMEM, FBS (10%), penicillin/streptomycin (100 U/mL),

dexamethasone (1 μM, Sigma), indomethacin (10 μM, Sigma), 3-isobutyl-1-methylxanthine (0.5 mM, Sigma), and insulin (10 μg/mL, Sigma). Chondrogenic medium consisted of DMEM, FBS (10%), penicillin/streptomycin (100 U/mL), dexamethasone (100 nM), ascorbic-2-phosphate (50 μg/mL, Sigma), proline (5 μg/mL, Sigma), ITS premix (5 μg/mL, Sigma), sodium pyruvate (1 mM, Gibco), and TGF-β3 (10 ng/mL, PeproTech). Osteogenic medium was comprised of DMEM, FBS (10%), penicillin/streptomycin (100 U/mL), dexamethasone (100 nM), ascorbic-2-phosphate (50 μg/mL, Sigma), and beta glycerol phosphate (10 mM, Sigma).

Quantitative analyses of gene expression

After culture in induction medium for 2 weeks, total cellular RNA was extracted and purified with TRIzol reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using SuperScript III reverse transcriptase (Invitrogen). Quantitative reverse transcription polymerase chain reaction was performed using a 7500 Fast Real-Time PCR System and SYBR Green master mix (Applied Biosystems). The relative expression of target markers was determined using the comparative C_T method, in which target gene expression was first normalized to that of the house-keeping gene encoding glyceraldehyde-3-phosphate dehydrogenase, followed by secondary normalization by the cells on day 1.

Histology

For histological analysis, cell-laden hydrogels (n = 2) were fixed in 4% paraformaldehyde (Sigma) for 30 min and washed with PBS thoroughly. To stain neutral lipid accumulation (a functional marker of adipogenesis), hydrogels were immersed in and stained with a Oil Red O (180 μg/mL in 60% isopropyl alcohol) for 1 h at room temperature. To stain alkaline phosphatase (an osteogenic biomarker), hydrogels were immersed in and stained with Fast Blue RR Salt (Sigma) and naphthol AS-MX phosphate alkaline solution (Sigma) in accordance with the manufacturer's instructions.

For safranin O staining (a functional marker of chondrogenesis), cell-laden hydrogels (n = 2) were fixed in 4% paraformaldehyde (Sigma) overnight and permeabilized in PBS with 3% bovine serum albumin and 0.1% Triton X-100 overnight. The hydrogels were then dehydrated in 30% sucrose and

embedded in OCT compound (Tissue-Tek®) before cryo-sectioning. Sectioned samples were stained with safranin O (Sigma) in accordance with the manufacturer's instructions.