Supporting Information

Shih et al. 10.1073/pnas.1321717111

SI Text

Preparation of Mineralized (Calcium Phosphate) Matrices. *N*-acryloyl 6-aminocaproic acid (A6ACA) and poly(ethylene glycol)-diacrylate (PEGDA6K) were synthesized as detailed previously (1, 2). To create PEG-A6ACA copolymer hydrogels, A6ACA (0.185 g) was dissolved in 1 N of sodium hydroxide (1 mL) to obtain a 1 M solution to which PEGDA6K (20 mg) was added to generate a 2% (wt/vol) solution of PEGDA6K. This solution was polymerized in BioRad 1-mm spacer glass plates for 20 min at room temperature by using 0.5% ammonium persulfate as an initiator and 0.15% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine as an accelerator. The hydrogels were immersed in PBS (pH 7.4) to equilibrate for 2 h and cut into 1-cm^2 circular discs. The hydrogels were mineralized (M) as described previously (3). Briefly, the circular hydrogel discs were equilibrated in deionized (DI) water for 6 h and dried for 24 h at 37 °C. The dried hydrogels were reswollen in modified simulated body fluid (m-SBF) containing 142 mM Na^+, 5 mM K^+, 1.5 mM Mg^{2+}, 2.5 mM Ca^{2+}, 103 mM Cl^-, 10 mM HCO^{3-}, 1 mM HPO_4^{2-}, and 0.5 mM SO_4^{2-}. The hydrogels were briefly rinsed in DI water for a few seconds and immersed in a solution of 40 mM Ca^{2+} and 24 mM HPO_4^{2-} (pH 5.2) for 60 min on an orbital shaker (VWR Mini Shaker, Radnor, PA) set at a speed of 200. The M discs were then briefly rinsed with DI water for a few seconds, immersed in fresh m-SBF for 48 h at 37 °C with daily exchange of m-SBF, and finally immersed in PBS for 5 h.

SEM Analysis of M Matrices. The morphology of nonmineralized (NM) and M hydrogels was examined by using SEM equipped with energy dispersive X-ray spectroscopy (EDS) (Philips XL30 ESEM). The samples were rinsed with water, flash-frozen in liquid N_2, and lyophilized for 24 h. The lyophilized samples were then sputter-coated with Ir for 10 s (Emitech K575X Sputter Coater) before the SEM imaging. Elemental analysis of the samples was performed using an EDS attachment and INCA software (Oxford Instruments).

X-Ray Diffraction. To examine the crystalline structure and the phase composition of M hydrogels through X-ray diffraction, samples were lyophilized and powdered. The powdered samples were analyzed by means of a Rigaku RU200Bh DMax-RB rotating anode diffractometer using Cu $K\alpha_1$ radiation ($\lambda = 0.154056$ nm). The diffractometer was operated at 40 kV of beam energy and 120 mA of beam current. A graphite monochromator with a [0002] orientation of crystal structure plane parallel to the hexagonal basal plane ($2d = 0.6708$ nm, d: interatomic spacing) was used, and the diffracted beam was collected into a horizontal goniometer covering an angular range of $5° \leq 20° \leq 80°$. The collected spectra were analyzed by means of MDI Jade X-Ray analysis software using the PDF-4+ (International Centre for Diffraction Data) database for peak identification.

Calcium and Phosphate Assays. To measure Ca^{2+} and PO_4^{3-} contents in NM and M hydrogels, samples were thoroughly rinsed with DI water, lyophilized, and homogenized in 0.5 N of HCl. The homogenate was vigorously vortexed for 24 h at 4 °C to achieve complete dissolution of calcium phosphate (CaP) minerals. The amount of Ca^{2+} in the homogenate was determined colorimetrically by using a calcium assay kit (Pointe Scientific, Inc.) as previously described (4). Briefly, 20 μL of the homogenate was mixed with 1 mL of assay solution, and absorbance (at 570 nm) of the resulting complex was measured using a Beckman Coulter DU 730 UV/Vis spectrophotometer. The amount of PO_4^{3-} in the homogenate was calculated using a molybdenum yellow assay according to previous reports (5). Briefly, 125 μL of sample solution was mixed with 1 mL of the assay solution, which contains two parts acetone, one part 5 N of H_2SO_4, and one part 10 mM ammonium molybdate. Absorbance of the resulting complex at 380 nm was then measured colorimetrically using a Beckman Coulter DU 730 UV/Vis spectrophotometer. The calculated Ca^{2+} and PO_4^{3-} contents were normalized to the dry weight of the sample. To determine the dissolution of CaP minerals of the M matrix into Ca and Pi ions, the M matrices were immersed in 1.5 mL of 0.050 M Tris·HCl [Tris = Tris (hydroxymethyl)aminomethane, pH = 7.4] at 37 °C. Every 24 h, 200 μL of the immersion solution was collected from each sample and replaced with 200 μL of fresh Tris buffer. Dissolved Ca and Pi ions from the solutions were measured as described above.

Cell Culture and Media. Human mesenchymal stem cells (p7071L; Institute for Regenerative Medicine, Texas A&M University) were cultured in growth medium containing DMEM (Invitrogen) and 10% (vol/vol) FBS (HyClone), supplemented with 100 units of penicillin, 1,000 units of streptomycin, and 2 mmol/L L-glutamine. Cells were trypsinized upon reaching 70% confluency, and passage 5 cells were seeded at an initial cell density of 4,000 cells per square centimeter for experiments. For cells cultured in osteogenic medium, the medium was prepared by supplementing growth medium with 10 nM dexamethasone (Sigma–Aldrich), 10 mM β-glycerophosphate (Sigma–Aldrich), and 0.2 mM ascorbic acid (Sigma–Aldrich). For cultures using growth medium with varying concentrations of PO_4^{3-}, NaH_2PO_4 and Na_2HPO_4 were mixed at a ratio of 1:4 to supplement the growth medium to final concentrations of 3, 4, and 5 mM PO_4^{3-} ions. For inhibition of ATP transport, *N*-ethyl maleimide was added at a concentration of 0.5 μM to the culture medium with a change of fresh media every 2 d. For pharmacological inhibition of adenosine receptors, the A1 receptor inhibitor DPCPX (Tocris Bioscience) and A2b receptor inhibitor PSB603 (Tocris Bioscience) were added to media at a concentration of 100 nM, with a change of media every 2 d. To inhibit purinergic receptor, suramin was added at a concentration of 30 μM and the media was changed every 2 d. For adenosine-mediated osteogenesis, adenosine (Sigma–Aldrich) was added at a concentration of 30 μg/mL to the growth medium, with fresh media changed each day.

Isolation of Cell Membrane Fraction and Western Blot Analysis. Cell membrane fractions were extracted with a Mem-PER membrane protein extraction kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, 2×10^6 cells per sample were initially pelleted in a 1.5-mL tube, and 150 μL of lysis buffer [(reagent A) containing aprotinin (1 μg/mL), leupeptin (1 μg/mL), PMSF (1 mM), Na_3VO_4 (1 mM), and NaF (1 mM)] was added. One part of reagent B was diluted with two parts of reagent C, and 450 μL of the mixture was added to each sample and incubated on ice for 30 min, with vortexing every 5 min. Samples were then centrifuged at 10,000 relative centrifugal force (rcf) for 3 min at 4 °C. The supernatant was transferred to new tubes and incubated for 20 min in a 37 °C water bath to separate the membrane protein fraction. Samples were then centrifuged at room temperature for 2 min at 10,000 rcf to isolate the hydrophobic fraction. The top layer was removed, and the bottom layer containing membrane proteins was kept for further sample

preparation. Samples were kept on ice during the experimental procedure. Each protein sample (100 μL) was then prepared with an SDS/PAGE Sample Prep Kit (Thermo Scientific) to eliminate interfering reagents for SDS/PAGE according to the manufacturer's guidelines. Briefly, the PAGE-prep protein binding resin was vortexed for 5 s, and 20 μL of resin slurry was transferred into the center of a spin cup. One hundred microliters of the sample was transferred into the resin and vortexed for 5 s. To this, 100 μL of DMSO was added and vortexed for 5 s. Samples were incubated for 5 min at room temperature with occasional mixing and centrifuged at 2,000 rcf for 2 min. The flow-through was discarded, and the resin-containing sample was washed with 300 μL of wash solution (one part DMSO and one part water) and vortexed until homogeneous. Samples were centrifuged at 2,000 rcf for 2 min, the flow-through was discarded, and the resin with the sample was washed for a total of two times. Samples were kept at −80 °C if not used immediately. For analysis of phosphorylated extracellular signal-regulated kinases 1/2 (phospho-ERK1/2; T202/Y204; Cell Signaling), cells were lysed with radioimmunoprecipitation assay buffer containing Tris·HCl (50 mM), 1% (wt/vol) Nonidet P-40, NaCl (150 mM), EDTA (1 mM), PMSF (1 mM), aprotinin (1 μg/mL), leupeptin (1 μg/mL), NaF (1 mM), and Na_3VO_4 (1 mM). The cell lysate was quantified with the Bradford assay. Briefly, protein assay solution (Bio-Rad) and protein samples were diluted fivefold and 100-fold, respectively, with Milli-Q (EMD Millipore Corporation) water. The BSA standards were diluted to achieve a range of 0–40 μg/mL per 100 μL of protein assay; 20 μL of sample or standard was then mixed in each well of a microtiter plate, and absorbance was detected at 595 nm with a spectrophotometer (Beckman Coulter DTX880). Gel electrophoresis was carried out on 8% (wt/vol) polyacrylamide-resolving gels at 120 V for 60 min and transferred to PVDF membranes at 100 V for 90 min. Membranes were blocked in 3% (wt/vol) BSA/TBS and Tween 20 and incubated with phospho-ERK1/2 (1:1,000; Cell Signaling), solute carrier family 20 (phosphate transporter), member 1 (PiT-1, 1:200; Santa Cruz Biotechnology), and tubulin (1:5,000; Abcam) primary antibodies overnight at 4 °C. Membranes were incubated with secondary HRP-conjugated antibodies (Cell Signaling) for 1 h at room temperature and then covered with chemiluminescent reagent (Thermo Scientific) for X-ray film exposure. Western blot images of phospho-ERK1/2 were quantified by ImageJ (National Institutes of Health).

RNA Isolation, RT, and Real-Time PCR. Total RNA was extracted with TRIzol (Invitrogen), phase-separated with chloroform, and precipitated using isopropanol. One microgram of RNA was reverse-transcribed using RTiScript (Bio-Rad) according to the manufacturer's instructions. SYBR green (Takara) was used to detect gene expression during amplification after initial denaturation at 95 °C for 30 s for one cycle, 95 °C for 5 s and 60 °C for 31 s for 40 cycles, and then 95 °C for 10 min on a PCR cycler (ABI Prism 7300). The primer sequences are as follows: Runx2 (forward: CCA CCC GGC CGA ACT GGT CC, reverse: CCT CGT CCG CTC CGG CCC ACA), OCN (forward: TGAGAGCCCTCACACTCCTC, reverse: ACCTTTGCTGGACTCTGCAC), type I collagen (forward: CAAGACAG TGATTGAATACAAAACCA, reverse: ACGTCGAAGCCGAATTCCT), OPN (forward: AATTGCAGTGATTTGCTTTTGC, reverse: CAGAACTTCCAGAATCAGCCTGTT), osterix (forward: CATCTGCCTGGCTCCTTG, reverse: CAGGGGACTGGAGCCATA), solute carrier family 20 (phosphate transporter), member 1 (SLC20a1) (forward: TCCCAT CAGTACAACACATTGTAAAA, reverse: CAGTCAACAGCCTTCTTGGA), and GAPDH (forward: AGCCACATCGCTCAGACAC, reverse: GCCCAATAC GACCAAATCC).

Isolation of Mitochondria. Mitochondria were isolated with a mitochondria isolation kit (Thermo Scientific) according to the manufacturer's instructions. Approximately 2×10^6 cells were obtained and transferred to a 1.5-mL tube, mixed with reagent A supplemented with aprotinin (1 μg/mL) and leupeptin (1 μg/mL), and incubated on ice for 5 min with vortexing at 1-min intervals. Reagent B was added and incubated on ice for 5 min. Reagent C was then added and centrifuged at 400 rcf for 10 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 12,000 rcf for 15 min at 4 °C. The supernatant was discarded, and reagent C was added to the pellet and centrifuged at 12,000 rcf for 5 min. The pellet of mitochondria was immediately measured for phosphate content.

Inorganic Phosphate Assay. Phosphate measurements were carried out using a phosphate assay solution prepared by combining two parts acetone, one part 5 N of H_2SO_4, and one part 0.01 M ammonium molybdate. All measurements were quantified against a standard made with KH_2PO_4 within a concentration range of 0–1 mM. To measure intracellular phosphate, 10^4 cells were suspended in 0.5 mL of DI H_2O, and 50 μL of the cell suspension mixed in 150 μL of phosphate assay solution was used. For intramitochondrial phosphate assay, the mitochondrial pellet isolated from 2×10^6 cells was suspended in 150 μL of phosphate assay solution. After a 5-min incubation of samples and standards with phosphate assay solution, mitochondrial and cellular phosphate contents were detected spectrophotometrically at an absorbance of 380 nm (Beckman Coulter DTX880).

Measurement of Mitochondrial Phospholipid. Mitochondrial membrane phospholipid was quantified as detailed previously to normalize intramitochondrial phosphate contents (6). Briefly, a thiocyanate reagent was prepared by dissolving 0.27 g of ferric chloride and 0.3 g of ammonium thiocyanate in 10 mL of Milli-Q water. Mitochondria isolated from 2×10^6 cells were first dissolved in 2 mL of chloroform in a glass tube, and 1 mL of thiocyanate reagent was added to this. After vortexing for 1 min, samples were spun at 300 rcf for 10 min to separate the mitochondrial lipid layer. After discarding the top layer, 100 μL of the red lower layer was harvested to measure absorbance at 488 nm (Beckman Coulter DTX880).

Immunofluorescent Staining. Samples were fixed in 4% (vol/vol) formaldehyde for 10 min, blocked/permeabilized in blocking solution (PBS, 3% BSA, 0.1% Triton-X) for 50 min, and then washed and incubated with primary osteocalcin (OCN) antibody (1:100; Abcam) in blocking solution overnight at 4 °C. After washing with PBS three times for 30 min, samples were incubated with secondary Alexa Fluor 488 antibody (1:250; Invitrogen) in blocking solution at room temperature for 1 h. Samples were then mounted with Vectashield-DAPI (Vector Laboratories) and imaged using a Zeiss Observer A1 microscope. To minimize the effects of scattering, the background was subtracted in ImageJ using a rolling ball algorithm (rolling ball radius: 750 pixels) for all images.

Luminescent ATP Analysis and Fluorescent ATP Stain. Intracellular ATP content was measured with an ATP bioluminescent somatic cell assay kit (Sigma–Aldrich). Briefly, 10^4 cells were suspended in 0.05 mL of medium and mixed with 0.1 mL of somatic cell ATP releasing reagent. The mixture was immediately mixed with 0.1 mL of ATP assay mix working solution and measured in a spectrophotometer (Beckman Coulter DTX880) for luminescence by using a black microtiter plate. Luminescent signal was compared with an ATP standard curve between 0 M and 2×10^{-7} M. For fluorescent staining of intravesicular ATP, cells were rinsed in PBS and incubated in 30 μM quinacrine dihydrochloride (Sigma–Aldrich) dissolved in Iscove's modified

Dulbecco's medium for 30 min before imaging with a Zeiss Observer A1 microscope.

HPLC. Media (0.5 mL) from the cell cultures were lyophilized for 24 h and frozen at −80 °C before experiments. The quantification of adenosine and ATP was analyzed by HPLC-UV, as previously described (7). The chromatic separations were performed on an Aeris Peptide 3.6u Xb-C18 phenomenex column (150 × 2.10 mm) using a Hitachi Elite LaChrom L-2130 pump equipped with a UV-Vis detector (Hitachi Elite LaChromL-2420). The flow rate was 0.23 $mL \cdot min^{-1}$, and the sample injection volume was 20 μL. Detection levels of adenosine and ATP were analyzed under isocratic conditions at 254 nm and 220 nm, respectively. The mobile phase for adenosine was composed of 0.5% acetonitrile, 5% (vol/vol) methanol, 94.5% (vol/vol) sodium acetate buffer (0.25 M, pH 6.3). The mobile phase for ATP was composed of 215 mM potassium dihydrogen phosphate, 2.3 mM tetra butyl ammonium hydrogen sulfate (TBHAS), 4% (vol/vol) acetonitrile, and 0.4% potassium hydroxide (1 M). The stock solutions of lyophilized cell samples were prepared in 1 mL of respective buffer solution. The sample injections were 20 μL, with a dilution factor of 10. Areas under the curve were used for quantification of adenosine concentration values. These data were given by the HPLC software (EZ Chrom Elite) integration tool. The limit of detection for ATP was determined by statistical analysis, where 3σ (threefold the SD) was determined to be a statistically significant signal for qualitative detection. Thus, the limit of detection was determined to be less than 100 ng/mL for ATP. High-resolution MS (HRMS) was used to confirm that the compound responsible for the integrated peaks was indeed adenosine. HRMS (electrospray ionization) m/e 290.0861 $[M^+Na^+]$ was calculated for $C_{10}H_{13}N_5O_4$ 290.0860.

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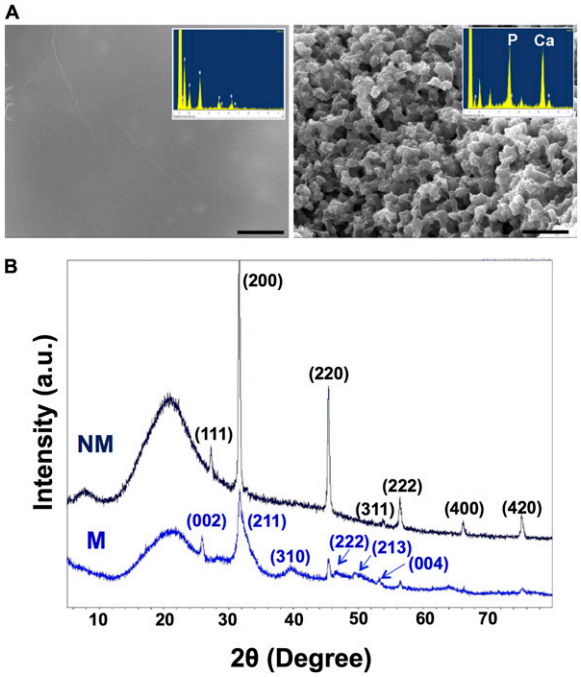

Fig. S1. (*A*) SEM and EDS (*Inset*) of M (*Right*) and NM (*Left*) hydrogels. (Scale bars: 2 μm.) (*B*) X-ray diffraction spectra of M and NM hydrogels. Arrows indicate hydroxyapatite peaks [PDF-4-010-6312, based on PDF4+ (International Centre for Diffraction Data) database].

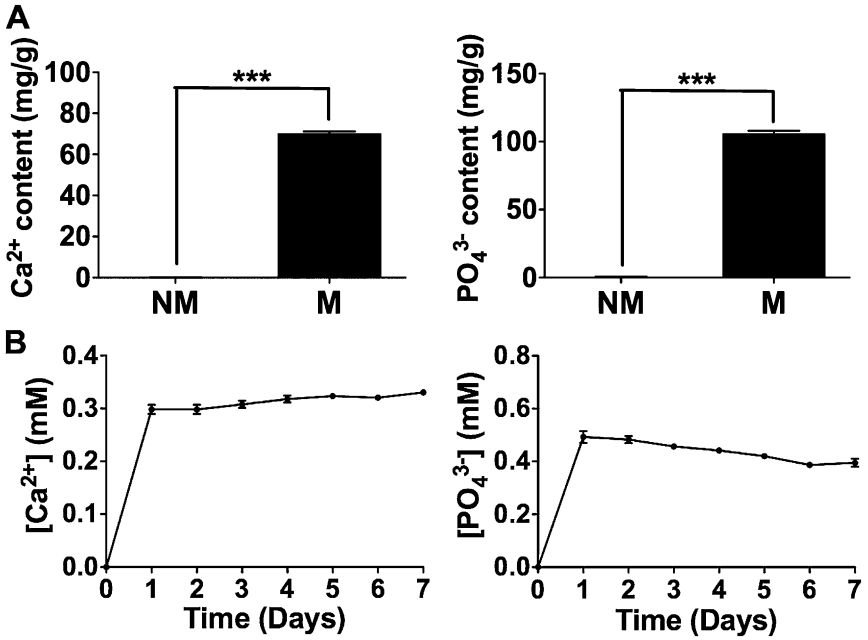

Fig. S2. (*A*) Calcium (Ca^{2+}) and phosphate (PO_4^{3-}) content of the M hydrogels. ***$P < 0.001$. (*B*) Dissolution of the CaP minerals into Ca^{2+} and PO_4^{3-} ions in Tris buffer at pH 7.4 as a function of time.

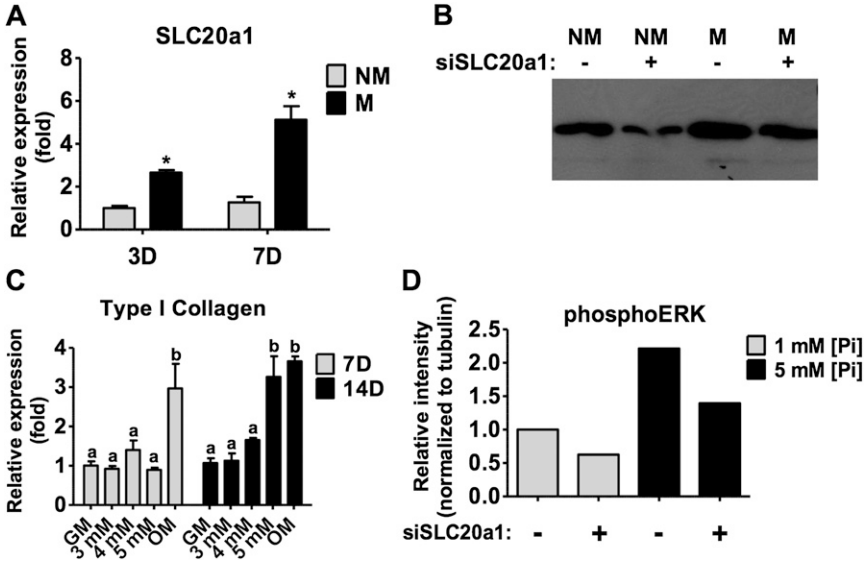

Fig. S3. (*A*) SLC20a1 gene expression of human mesenchymal stem cells (hMSCs) cultured on M and NM matrices after 3 d (3d) and 7 d (7d). Data are represented as the mean ± SD (two-tailed Student *t* test, *$P < 0.05$). (*B*) Western blot analysis of SLC20a1 protein of hMSCs on M and NM after 7 d of siRNA transfection. (*C*) Type 1 collagen gene expression of hMSCs after 7 d and 14 d of culture in growth medium containing varying amounts of PO_4^{3-} ions. (*D*) Semiquantitative analyses of phospho-ERK Western blot image in Fig. 2*D*. The plus (+) symbol denotes SLC20a1 siRNA, and the minus (–) symbol denotes scrambled siRNA. GM, growth medium (1 mM PO_4^{3-}); 3 mM, 3 mM PO_4^{3-}; 4 mM, 4 mM PO_4^{3-}; 5 mM, 5 mM PO_4^{3-}; OM, osteogenic medium; [Pi], concentration of PO_4^{3-}. Groups with different letters (a–c) are significant, $P < 0.05$; $n = 3$.

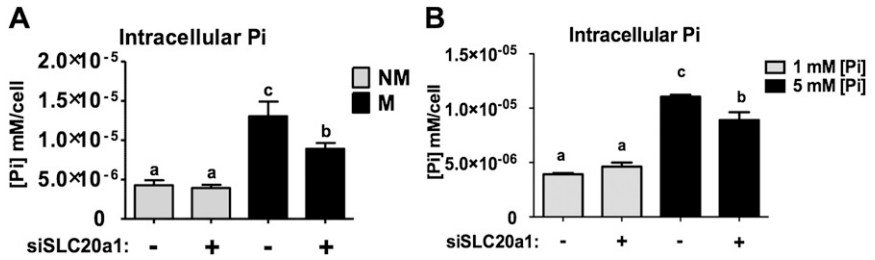

Fig. S4. (*A*) Intracellular PO_4^{3-} of hMSCs on M and NM matrices with and without SLC20a1 knockdown after 1 d of culture. (*B*) Intracellular PO_4^{3-} of hMSCs in 5 mM $PO4^{3-}$ medium with and without SLC20a1 knockdown after 1 d of culture. Pi, phosphate ion. Data are represented as the mean ± SD (one-way ANOVA, followed by Bonferroni post hoc test). Groups with different letters (a–c) are significant, $P < 0.05$; $n = 3$.

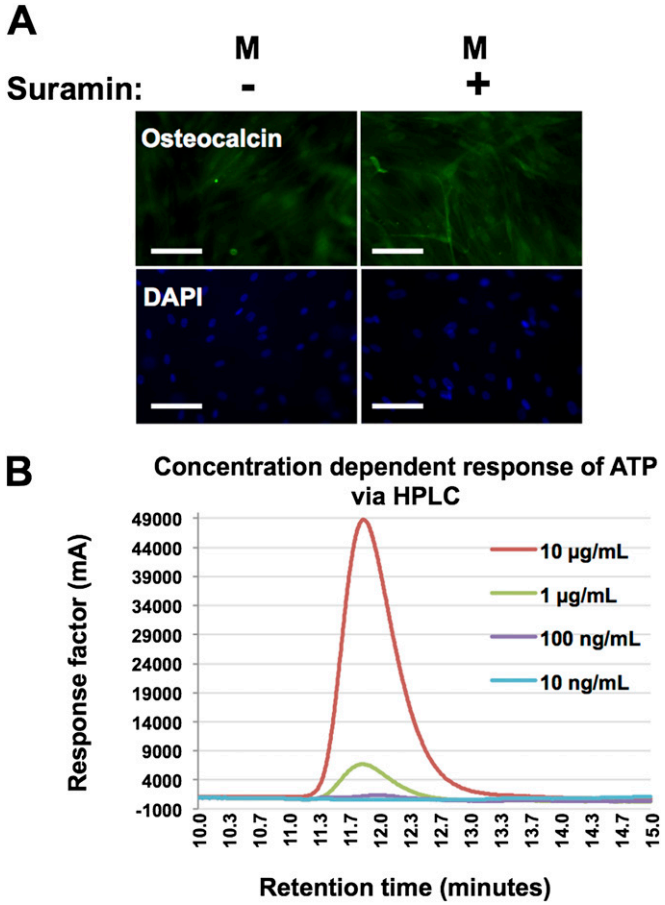

Fig. S5. (*A*) OCN immunofluorescent staining (green) and nucleus (blue) of hMSCs cultured on M and NM matrices after 21 d of culture in the presence of suramin, a pharmacological inhibitor for purinergic receptor. The plus (+) symbol denotes SLC20a1 siRNA, and the minus (–) symbol denotes scrambled siRNA. (*B*) Measurable threshold of ATP with HPLC. (Scale bars: 2 μm.)