Science Advances

AAAS

advances.sciencemag.org/cgi/content/full/2/8/e1600691/DC1

Supplementary Materials for

Small molecule–driven direct conversion of human pluripotent stem cells into functional osteoblasts

Heemin Kang, Yu-Ru V. Shih, Manando Nakasaki, Harsha Kabra, Shyni Varghese

Published 31 August 2016, *Sci. Adv.* **2**, e1600691 (2016)
DOI: 10.1126/sciadv.1600691

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at advances.sciencemag.org/cgi/content/full/2/8/e1600691/DC1)

Supplementary Figures

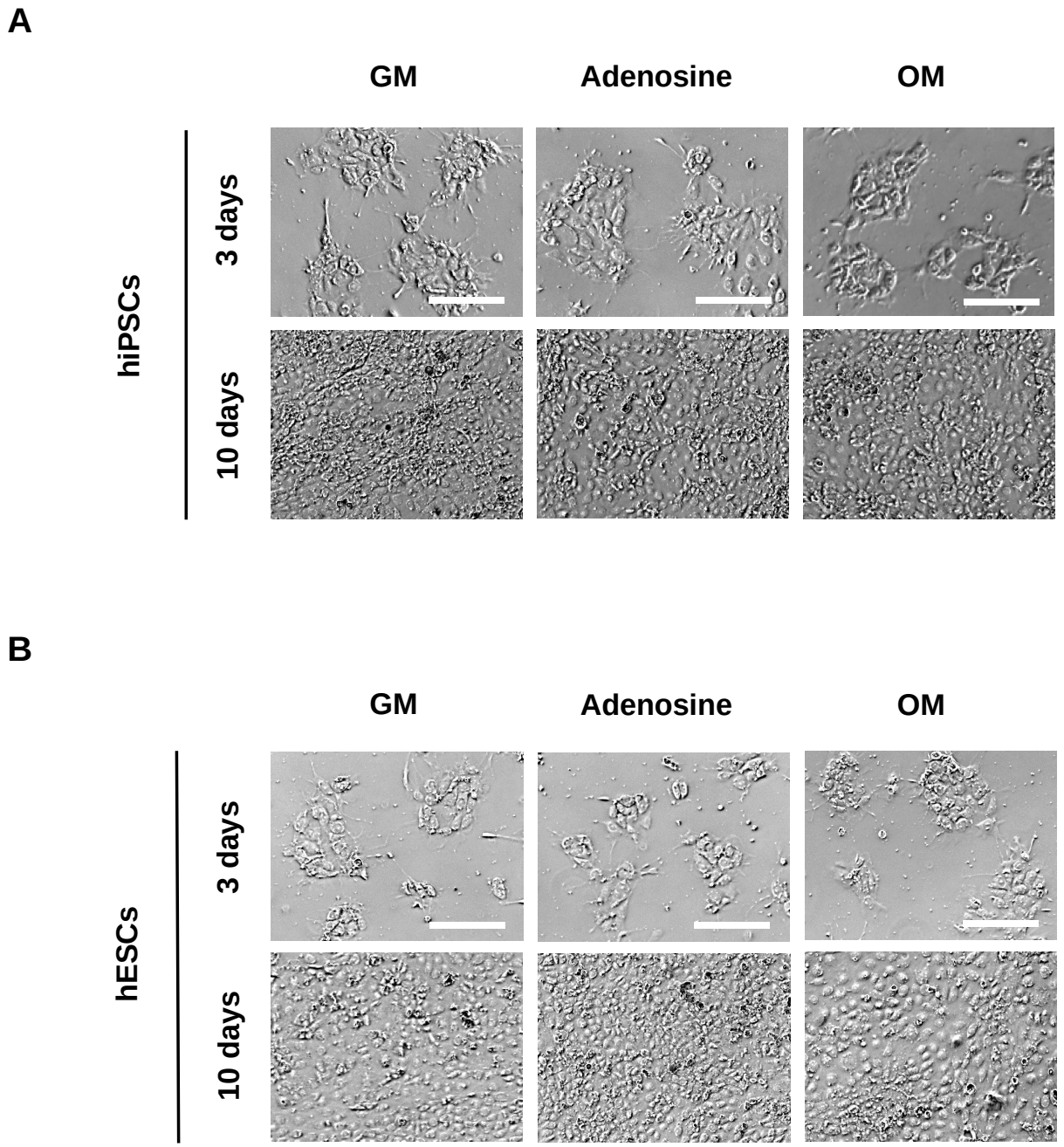


fig. S1. Attachment and growth of hPSCs cultured under various medium conditions. Bright-field images of (**A**) hiPSCs and (**B**) hESCs cultured for 3 and 10 days in growth medium (GM), growth medium supplemented with adenosine (Adenosine), and osteogenic-inducing medium (OM). Scale bars indicate 200 μm.

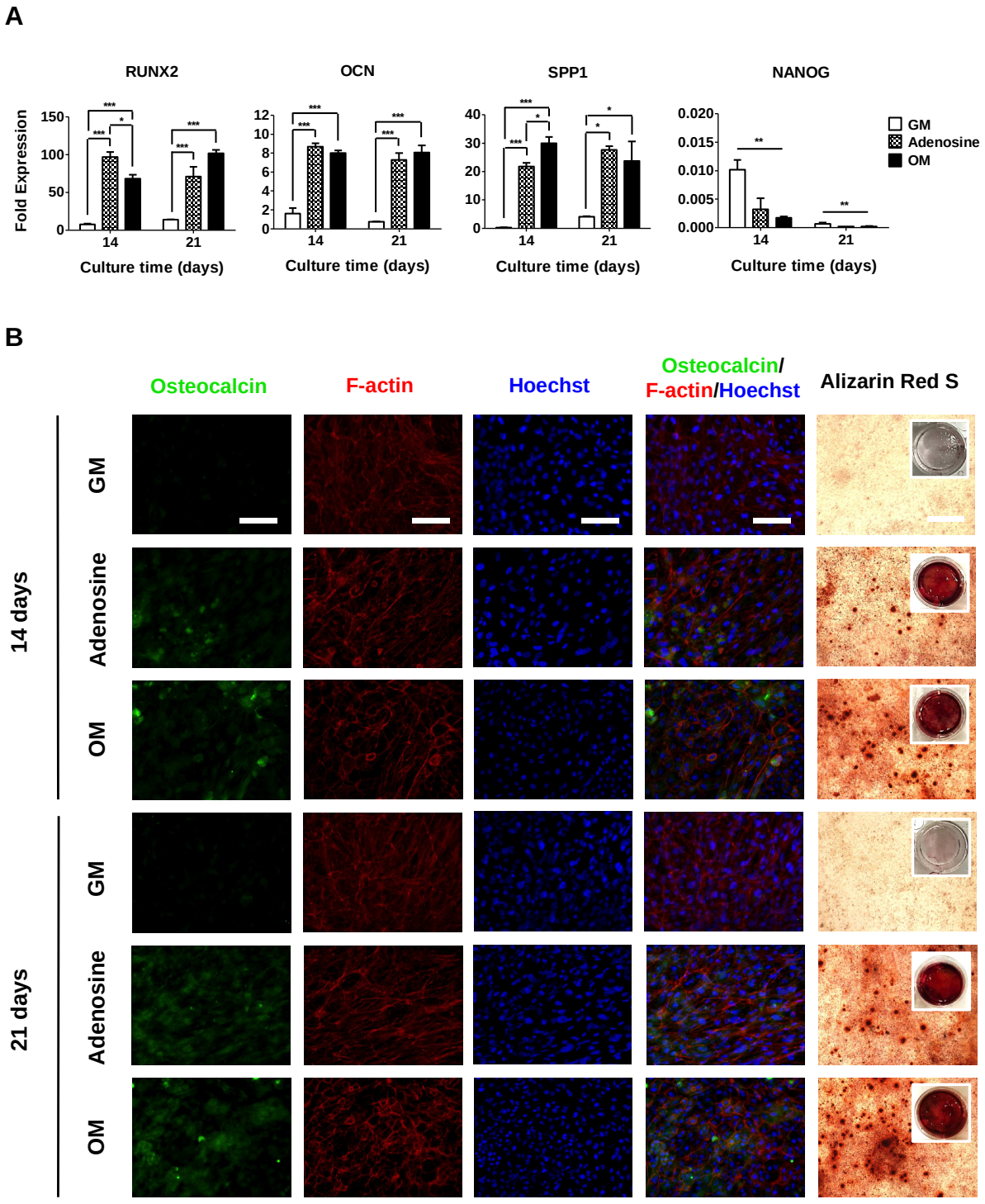


fig. S2. Exogenous adenosine induced osteogenic differentiation of hESCs. (**A**) Time-dependent quantitative analyses of gene expressions of hESCs for osteogenic markers (RUNX2, OCN, and SPP1) and pluripotent marker (NANOG) cultured in growth medium (GM), adenosine-supplemented growth medium (Adenosine), and osteogenic induction medium (OM).

(**B**) Staining of osteoblastic markers for hESCs cultured for 14 and 21 days in GM, Adenosine, and OM. Immunofluorescent staining for osteocalcin (green), F-actin (red), and nuclei (blue; Hoechst) as well as Alizarin Red S staining of hiPSCs cultured in various medium. Inset shows the stained images of whole wells. Scale bars indicate 100 μm. Data are shown as mean ± standard errors (n=3). Data are presented as fold expression of target genes after normalization to undifferentiated, pluripotent hESCs. For RUNX2, OCN, and SPP1, the groups with various medium conditions at the same culture time were compared by using one-way ANOVA with Tukey-Kramer post-hoc test. For NANOG, all the groups were compared to undifferentiated, pluripotent hESCs by two-way ANOVA with Bonferroni post-hoc test. Asterisks indicate statistical significances according to *p*-values (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

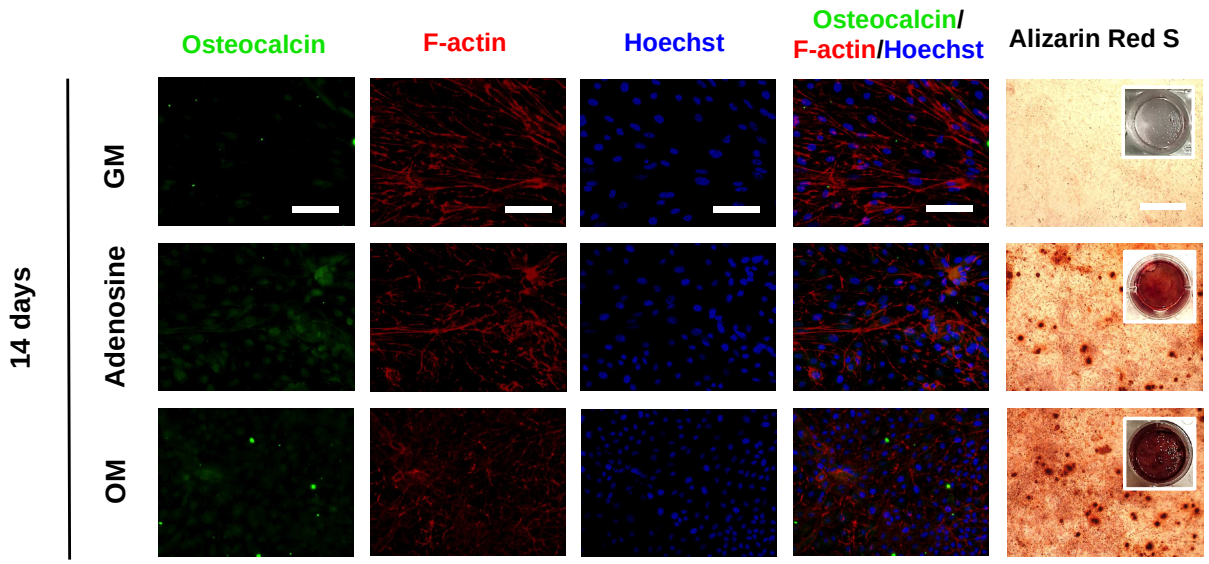


fig. S3. Exogenous adenosine induced expressions of osteoblastic markers for hiPSCs. Immunofluorescent staining for osteocalcin (green), F-actin (red), and nuclei (blue) as well as Alizarin Red S staining of hiPSCs cultured for 14 days in growth medium (GM), adenosine-supplemented growth medium (Adenosine), and osteogenic induction medium (OM). Inset shows the stained images of whole wells. Scale bars indicate 100 μm.

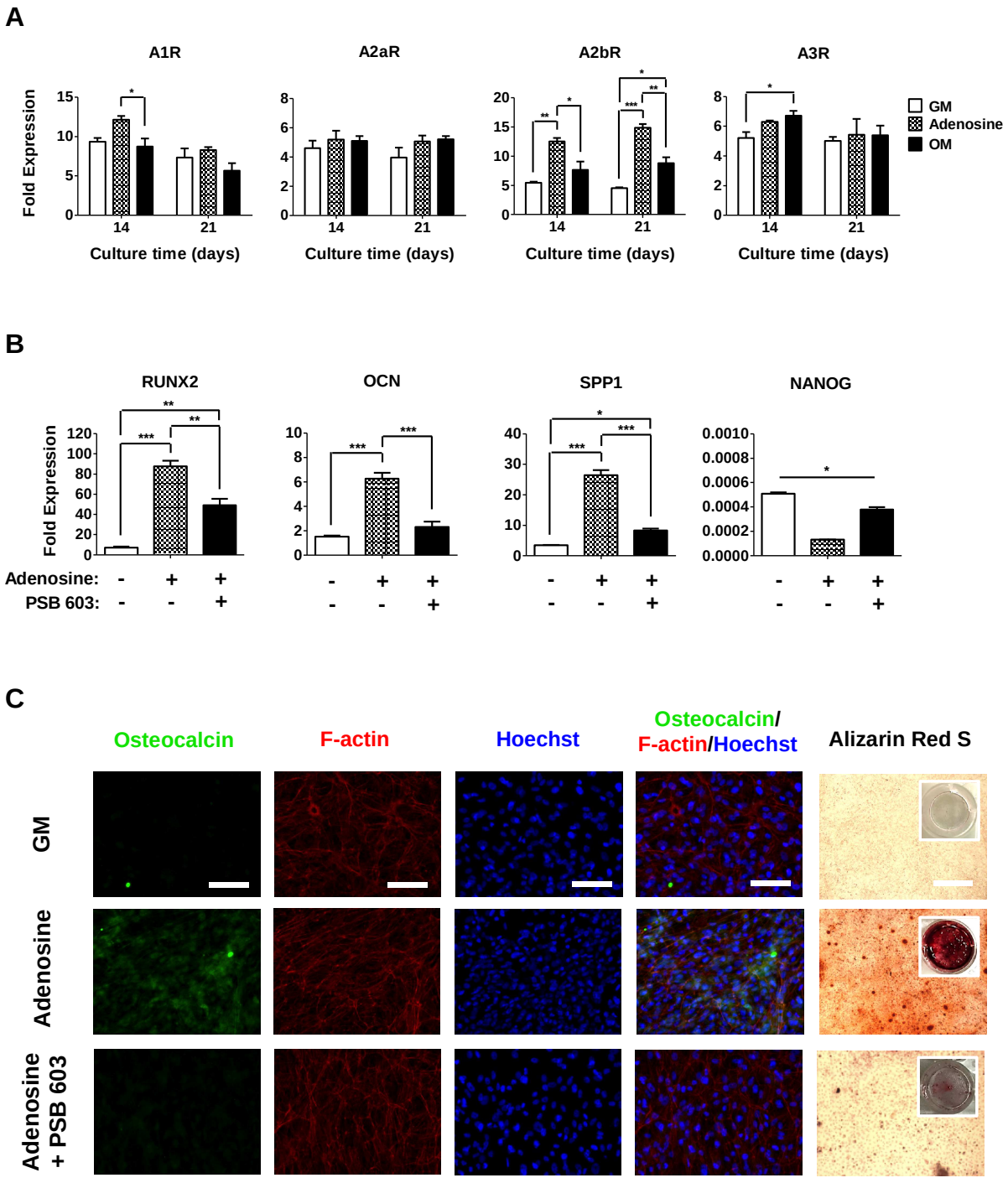


fig. S4. Adenosine-induced osteogenic differentiation of hESCs uses A2bR. (**A**) Gene expressions of adenosine receptor subtypes (A1R, A2aR, A2bR, and A3R) of hESCs cultured in growth medium (GM), adenosine-supplemented growth medium (Adenosine), and osteogenic induction medium (OM) as a function of time. (**B**) Gene expression levels of osteogenic markers (RUNX2, OCN, and SPP1) and pluripotent marker (NANOG) of hESCs at 21 days of culture in

presence or absence of adenosine and PSB 603 (an A2bR antagonist). The plus (+) and minus (-) denote the presence and absence of adenosine and PSB 603 in the culture medium. (**C**) Immunofluorescent staining for osteocalcin (green), F-actin (red), and nuclei (blue; Hoechst) as well as Alizarin Red S staining of hESCs cultured for 21 days in different medium conditions. The different medium conditions are growth medium (GM), growth medium containing adenosine (Adenosine), and growth medium containing adenosine and PSB 603 (Adenosine + PSB 603). Inset shows the stained images of whole wells. Scale bars represent 100 μm. Data are presented as mean ± standard errors (n=3). Data are shown as fold expression of target genes after normalization to undifferentiated, pluripotent hESCs. For A1R, A2aR, A2bR, and A3R as well as RUNX2, OCN, and SPP1, the groups with various medium conditions at the same culture time were compared by one-way ANOVA with Tukey-Kramer post-hoc test. For NANOG, all the groups were compared to undifferentiated, pluripotent hESCs by two-way ANOVA with Bonferroni post-hoc test. Asterisks were assigned to *p*-values with statistical significances (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

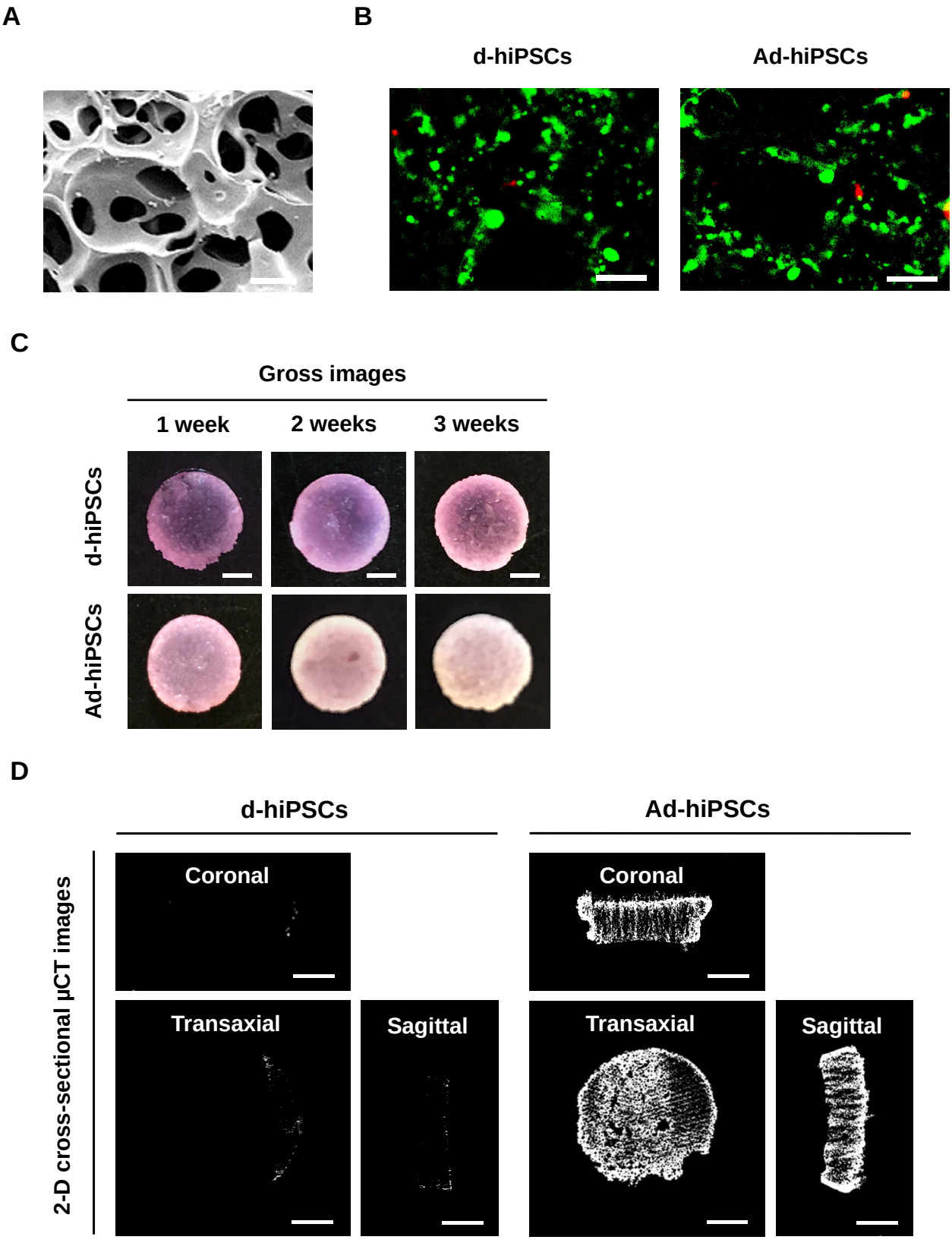

A
B
d-hiPSCs
Ad-hiPSCs
C
Gross images
1 week
2 weeks
3 weeks
d-hiPSCs
Ad-hiPSCs
D
d-hiPSCs
Ad-hiPSCs
2-D cross-sectional µCT images
Coronal
Transaxial
Sagittal
Coronal
Transaxial
Sagittal

fig. S5. In vitro hard tissue–forming ability of hiPSC-derived cells. (**A**) Scanning electron microscopy (SEM) image of macroporous matrices prepared by PMMA particle leaching. Scale bar indicates 50 μm. (**B**) Live-dead staining of the cell (Ad-hiPSC and d-hiPSC)-laden matrices after 3 days of *in vitro* culture. Scale bars represent 100 μm. (**C**) Gross images and (**D**) 2-D cross-sectional micro-computed tomography (μCT) images in coronal, transaxial, and sagittal planes of the d-hiPSCs and Ad-hiPSCs-laden matrices after 1, 2, and 3 weeks of *in vitro* culture. Scale bars represent 2 mm.

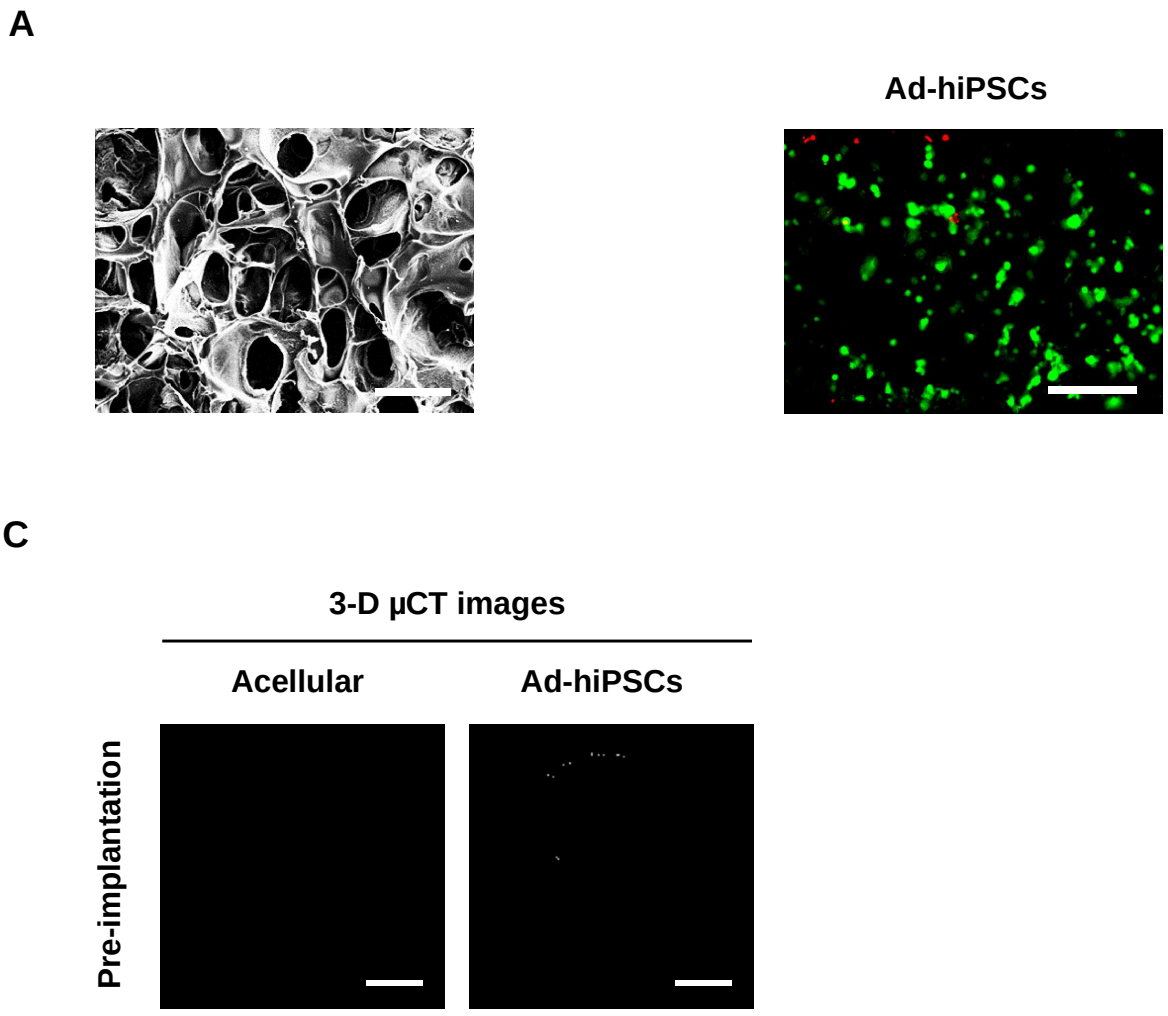


fig. S6. Minimal hard tissue formation for Ad-hiPSC–laden matrices before implantation. (**A**) Scanning electron microscopy (SEM) image of macroporous matrices prepared by cryogelation. Scale bar represents 50 µm. (**B**) Live-dead staining of Ad-hiPSCs-laden macroporous matrices after 3 days of in vitro culture. Scale bar indicate 100 µm. (**C**) 3-D micro-computed tomography (µCT) images of the acellular macroporous matrices alone (Acellular) or loaded with Ad-hiPSCs (Ad-hiPSCs) after 1 week of in vitro culture, prior to in vivo implantation. Scale bars represent 1 mm.

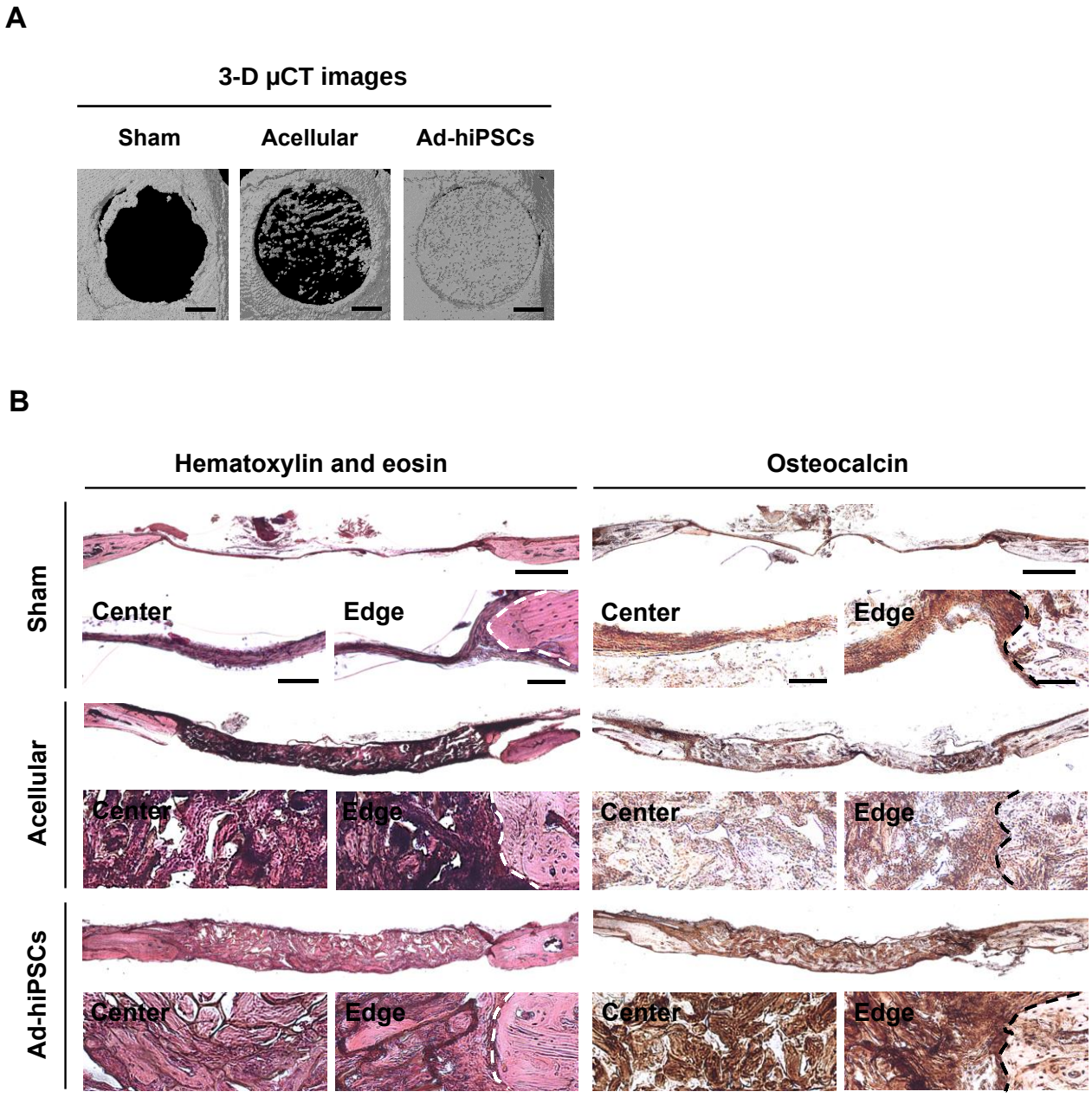
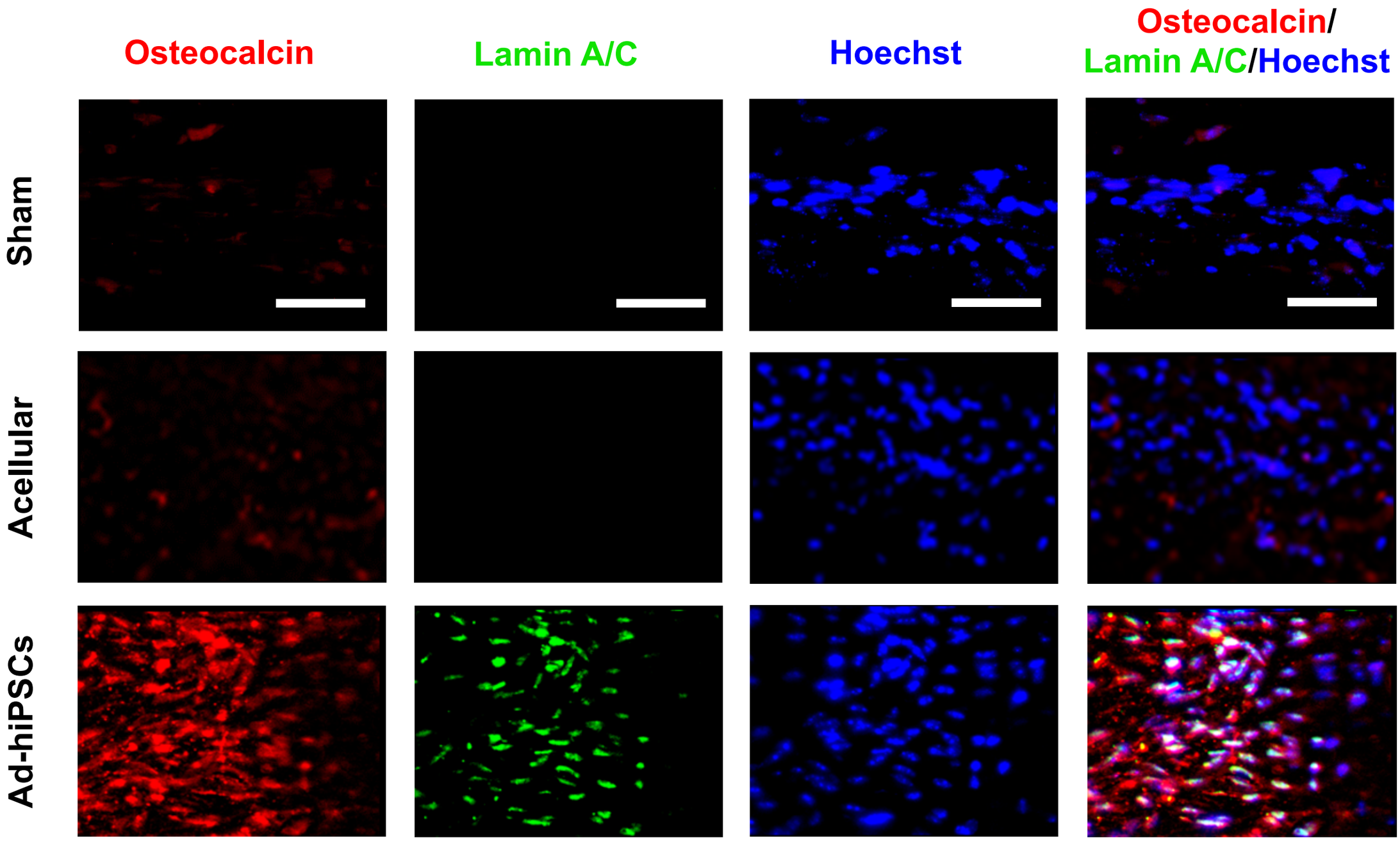


fig. S7. hiPSC-derived osteoblasts (Ad-hiPSCs) facilitate the repair of critical-sized bone defects. (**A**) 3-D micro-computed tomography (μCT) images and (**B**) hematoxylin and eosin (H&E) staining as well as immunohistochemical staining for osteocalcin of cranial bone defects treated with acellular and Ad-hiPSC-laden matrices, and sham group following 4 weeks of treatment. Scale bars for 3-D μCT images indicate 1 mm. Scale bars for staining images represent 500 μm. High magnification images show the center and edge of the treated bone

defects. White and black dotted lines mark the interface between engineered tissue and native bone tissue. Scale bars indicate 100 μm.

Fig. S8

A


Osteocalcin
Lamin A/C
Hoechst
Osteocalcin/
Lamin A/C/Hoechst
Sham
Acellular
Ad-hiPSCs

B

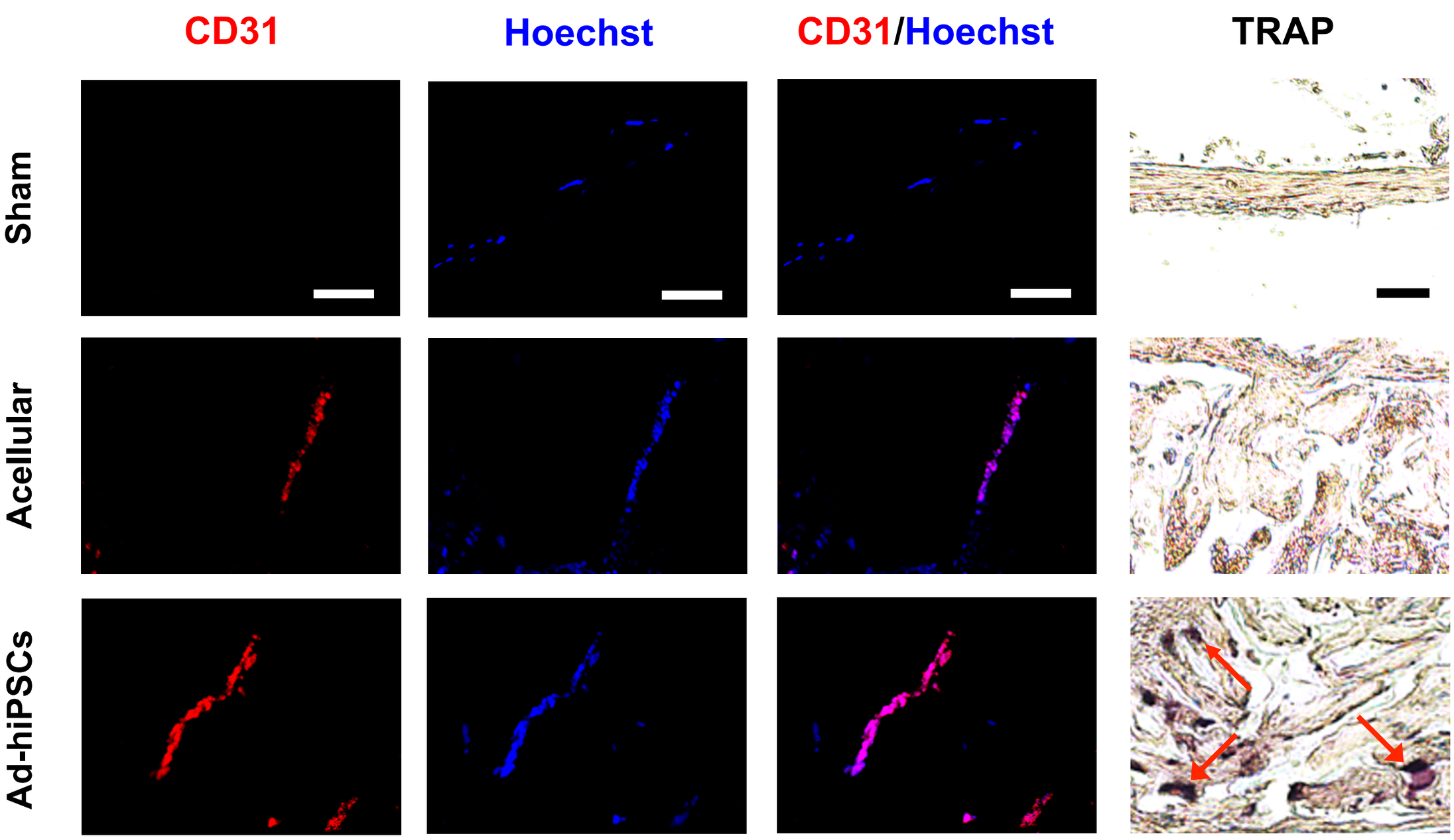

CD31
Hoechst
CD31/Hoechst
TRAP
Sham
Acellular
Ad-hiPSCs

fig. S8. Donor hiPSC–derived cells (Ad-hiPSCs) contribute to the regeneration of vascularized neobone tissue. Immunofluorescent staining for (**A**) osteocalcin (red) and human-specific lamin A/C (green) and (**B**) CD31 (red) along with nuclei (blue; Hoechst) as well as tartrate-resistant acid phosphatase (TRAP) staining of the bone defects treated with acellular, Ad-hiPSCs, and sham groups following 4 weeks of implantation. Red arrows indicate TRAP-positive stains. Scale bars indicate 50 μm.

table S1. List of primer sequences used for qPCR analysis.

Gene Name	Primer Sequence
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Forward: 5' CAT CAA GAA GGT GGT GAA GC 3'
	Reverse: 5' GTT GTC ATA CCA GGA AAT GAG C 3'
Runt-related transcription factor 2 (RUNX2)	Forward: 5' CCA CCC GGC CGA ACT GGT CC 3'
	Reverse: 5' CCT CGT CCG CTC CGG CCC ACA 3'
Osteocalcin (OCN)	Forward: 5' GAA GCC CAG CGG TGC A 3'
	Reverse: 5' CAC TAC CTC GCT GCC CTC C 3'
Secreted phosphoprotein 1 (SPP1)	Forward: 5' AAT TGC AGT GAT TTG CTT TTG C 3'
	Reverse: 5' CAG AAC TTC CAG AAT CAG CCT GTT 3'
NANOG	Forward: 5' GAT TTG TGG GCC TGA AGA AA 3'
	Reverse: 5' ATG GAG GAG GGA AGA GGA GA 3'
Adenosine A1 receptor (A1R)	Forward: 5' TGT TCC CTG GAA CTT TGG GC 3'
	Reverse: 5' CGA GGC AAG CAC CAT CCT G 3'
Adenosine A2a receptor (A2aR)	Forward: 5' CAC GCA GAG CTC CAT CTT CA 3'
	Reverse: 5' ACC AAG CCA TTG TAC CGG AG 3'
Adenosine A2b receptor (A2bR)	Forward: 5' TCT GTG TCC CGC TCA GGT AT 3'
	Reverse: 5' GTC AAT CCG ATG CCA AAG GC 3'
Adenosine A3 receptor (A3R)	Forward: 5' CCG TCA GAT ACA AGA GGG TCA C 3'
	Reverse: 5' CCC ACC AGG AAT GAC ACC AG 3'

movie S1. Minimal calcification from d-hiPSCs in vitro. 360° rotation views for 3-D micro-computed tomography (μCT) models of the in vitro engineered tissue from d-hiPSCs at 3 weeks.

movie S2. Dense and homogeneous calcified tissue formation from Ad-hiPSCs in vitro. 360° rotation views for 3-D micro-computed tomography (μCT) models of the in vitro engineered bone tissue from Ad-hiPSCs at 3 weeks.