Supplementary Information

Biomineralized matrix-assisted osteogenic differentiation of human embryonic stem cells

Heemin Kang [ab], Cai Wen [ac], Yongsung Hwang [a], Yu-Ru V. Shih [a], Mrityunjoy Kar [a], Sung Wook Seo [ad], and Shyni Varghese [*ab]

[a] *Department of Bioengineering and* [b] *Materials Science and Engineering Program, University of California, San Diego, La Jolla, CA 92093, USA.*

[c] *School of Chemistry and Chemical Engineering, Southeast University, Nanjing 210018, China.*

[d] *Department of Orthopaedic Surgery, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea.*

* Corresponding author: Associate Professor, Department of Bioengineering, University of California, San Diego, 9500 Gilman Drive, Mail Code 0412, La Jolla, CA 92093-0412, USA. Tel.: +1 858 822 7920; Fax: +1 858 534 5722. *E-mail address*: svarghese@ucsd.edu (S. Varghese).

Supplementary Figure Legends

Supplementary Figure S1. Characterization of mineralized hydrogels and the effect of different substrates on the pluripotency of hESCs. Release of (a) Ca^{2+} and (b) PO_4^{3-} from matrigel-coated or non-coated mineralized hydrogels incubated at 37 °C in Ca^{2+} and PO_4^{3-}-free Tris-HCl buffer as a function of time. Data are presented as mean ± standard deviations (n=3). (c) NANOG gene expression of HUES9 cells cultured in growth medium on non-mineralized (NM) and mineralized (M) hydrogels and coverslips (CS) as a function of culture time. Data are presented as mean ± standard errors (n=3). Two-way ANOVA with Bonferroni post-hoc test was used to compare multiple groups in different time points. Asterisks indicate statistical significances according to p-values (***: $p < 0.001$).

Supplementary Figure S2. Characterization of mineralized macroporous hydrogels and their effect on hESCs. (a) Bright field images of non-mineralized (NM) and mineralized (M) macroporous hydrogels in their swollen state. Scale bars represent 200 μm. (b) Ca^{2+} and (c) PO_4^{3-} release from M macroporous hydrogels in Tris-HCl buffer at 37 °C for 7 days. Data are presented as mean ± standard deviations (n=3). (d) Representative live-dead image of hESC-laden matrix (HUES9-laden macroporous hydrogels) after 3 days of culture. Scale bars indicate 200 μm. DNA contents of (e) HUES9-laden and (f) H9-laden macroporous hydrogels as a function of culture time. Data are presented as mean ± standard errors (n=3).

Supplementary Figure S3. Pluripotency of hESCs prior to their culture on various substrates. Representative immunofluorescent staining image of hESCs (HUES9 cells) for NANOG (green) and OCT4 (green) with corresponding nucleus (Hoechst; blue) confirming their pluripotency. Scale bars represent 200 μm.

Supplementary Figure S4. Adhesion and proliferation of hESCs on the matrices in 2-D culture. (a) Bright-field images of HUES9 after 1, 3, and 7 days of culture on non-mineralized (NM) and mineralized (M) matrices and coverslips (CS). Scale bars represent 500 μm. (b) Fluorescent staining for F-actin (green) and nucleus (Hoechst; blue) of

HUES9 cells after 3 days of culture on NM and M hydrogels and CS. Scale bars indicate 100 μm. Circularity index of the cells (bar graph) on various matrices was determined from F-actin images. Data are presented as mean ± standard errors (n=30 cells).

Supplementary Figure S5. Fluorescent staining for osteocalcin (green) and nucleus (Hoechst; blue; inset) of HUES9 cells after 14, 21, and 28 days of 2-D culture on non-mineralized (NM) and mineralized (M) hydrogels and coverslips (CS). Scale bars represent 100 μm.

Supplementary Figure S6. Fluorescent staining for F-actin (red) after 14, 21, and 28 days of 2-D culture for HUES9 cells on non-mineralized (NM) and mineralized (M) matrices and coverslips (CS). Scale bars represent 100 μm.

Supplementary Figure S7. μCT analyses of non-mineralized (NM) and mineralized (M) matrices prior to *in vivo* implantation confirming that the matrices themselves are not radio-opaque.

Supplementary Figure S8. μCT analyses of cell-laden matrices after *in vivo* implantation. 2-D cross-sectional μCT images in coronal, transverse, and sagittal planes of (a) HUES9 or (b) H9-laden non-mineralized (NM) and mineralized (M) matrices at 8 weeks post-implantation. Scale bars represent 2 mm.

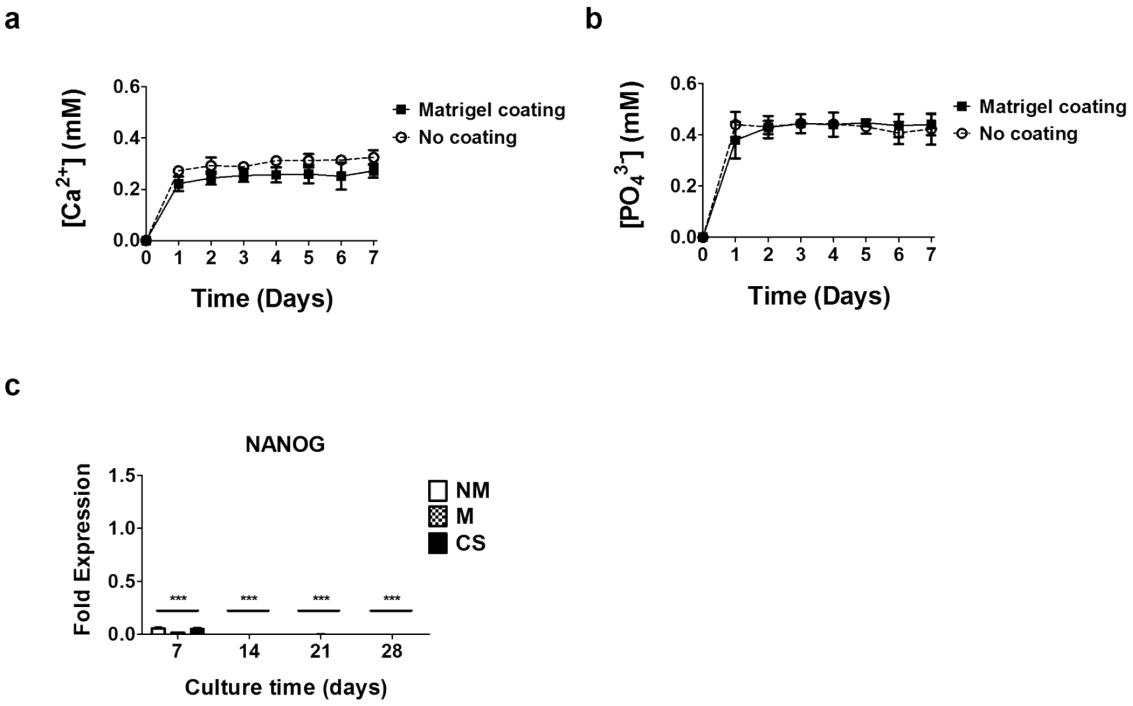
Supplementary Figure S9. Histochemical analyses of cell-laden matrices post *in vivo* implantation. (a) H&E staining of HUES9-laden and H9-laden non-mineralized (NM) macroporous hydrogels after 8 weeks of implantation. Scale bars indicate 500 μm. High magnification images of the peripheral mineralized region in the cell-laden matrices are also shown. Yellow arrows indicate bone-like matrix formation. Scale bars represent 100 μm. (b) Immunohistochemical staining of HUES9 and H9-laden NM and mineralized (M) macroporous hydrogels for lamin B following 8 weeks of implantation. Mouse skin tissue was used as a negative control. Scale bars indicate 100 μm.

Supplementary Table S1. List of primers used in qRT-PCR experiments.

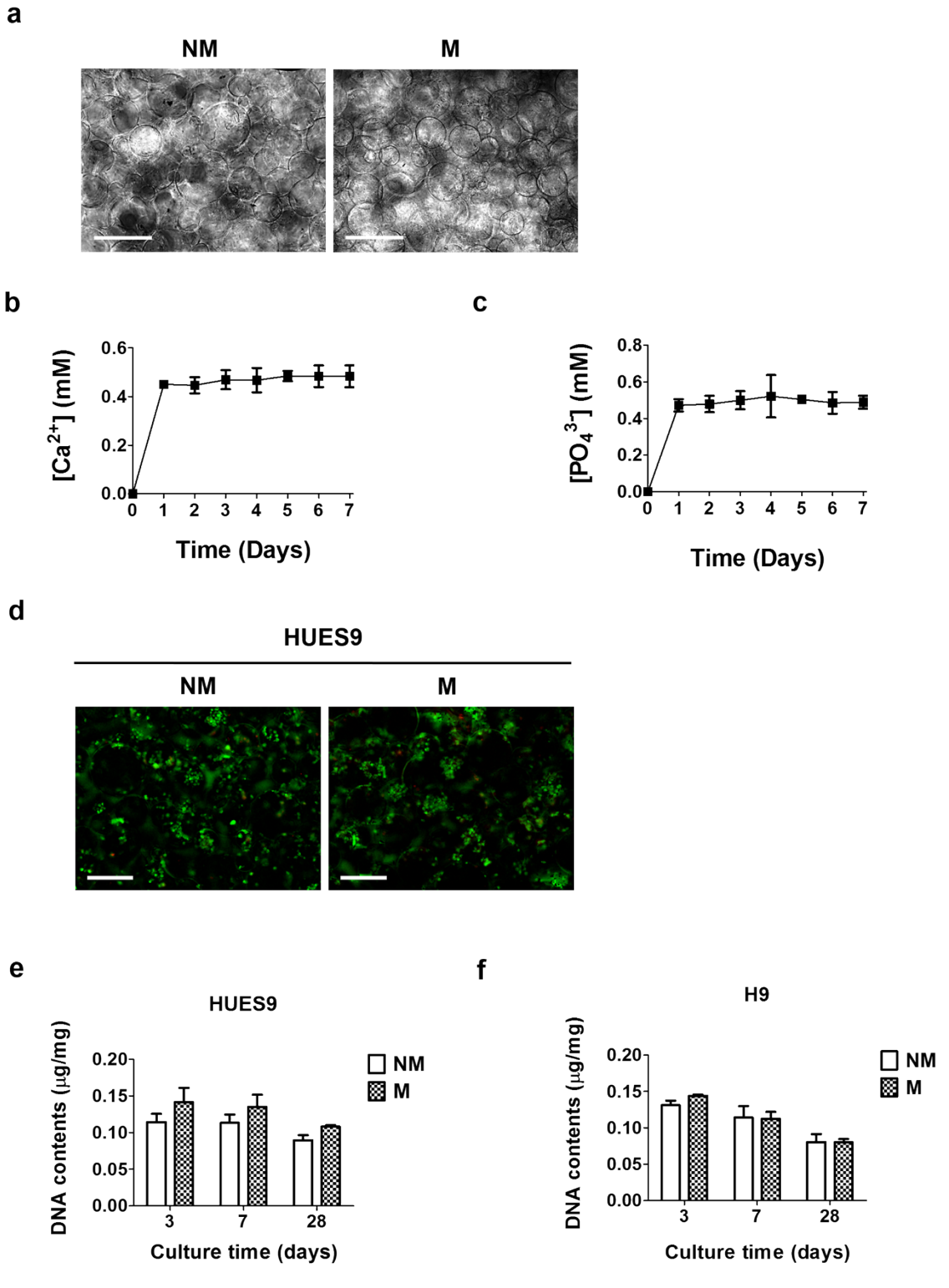
Supplementary Video S1. 3-D μCT views demonstrate uniform and pronounced mineralization in HUES9-laden mineralized matrices after 4 and 8 weeks of implantation.

Supplementary Video S2. 3-D μCT views demonstrate uniform and pronounced mineralization in H9-laden mineralized matrices after 4 and 8 weeks of implantation.

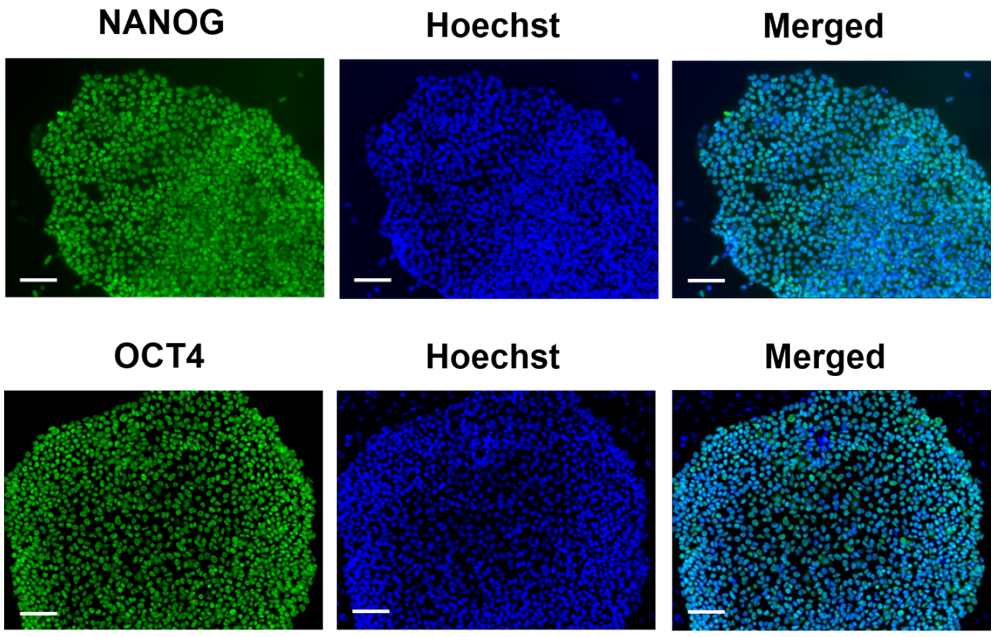
Supplementary Figure S1



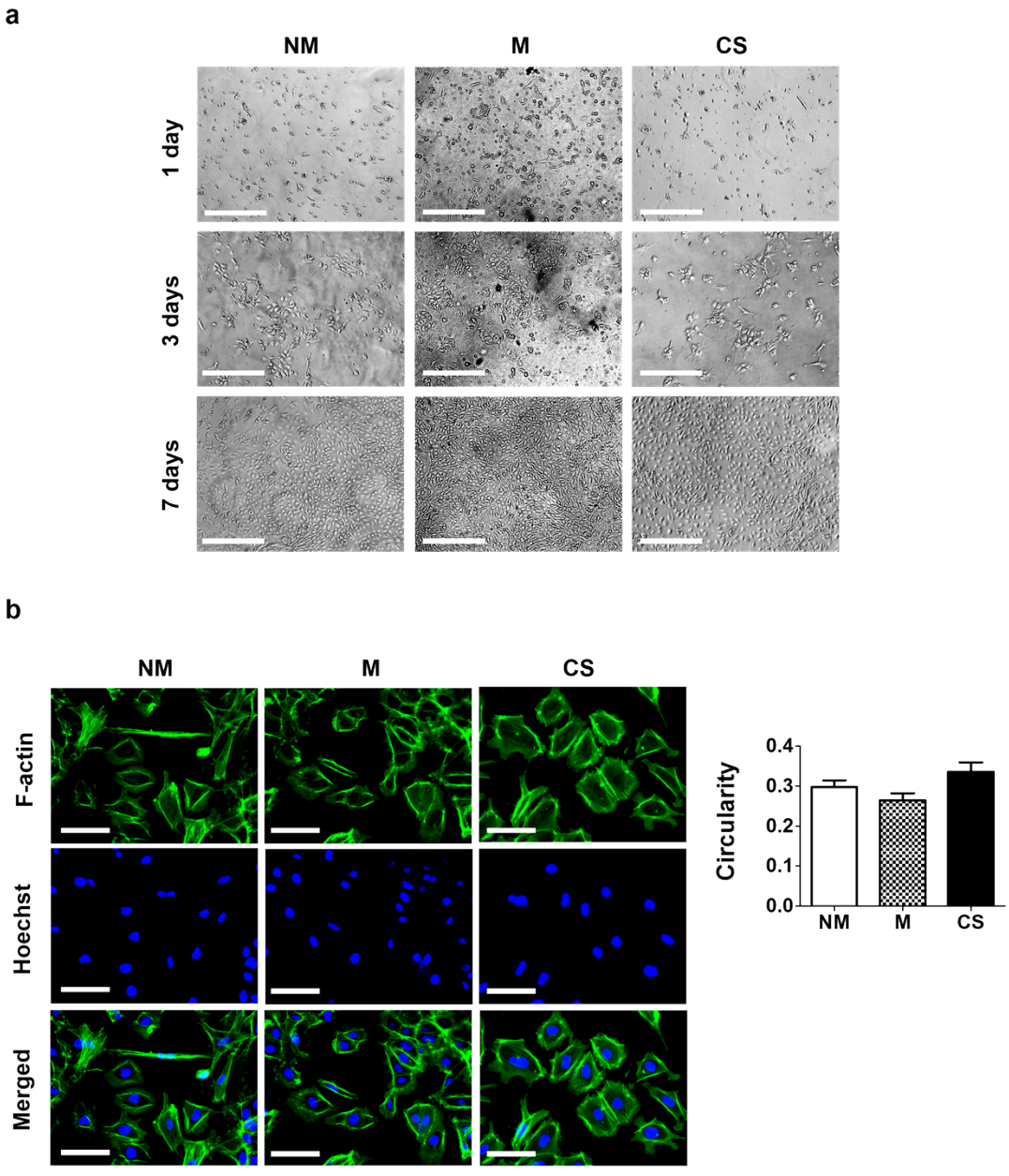
Supplementary Figure S2



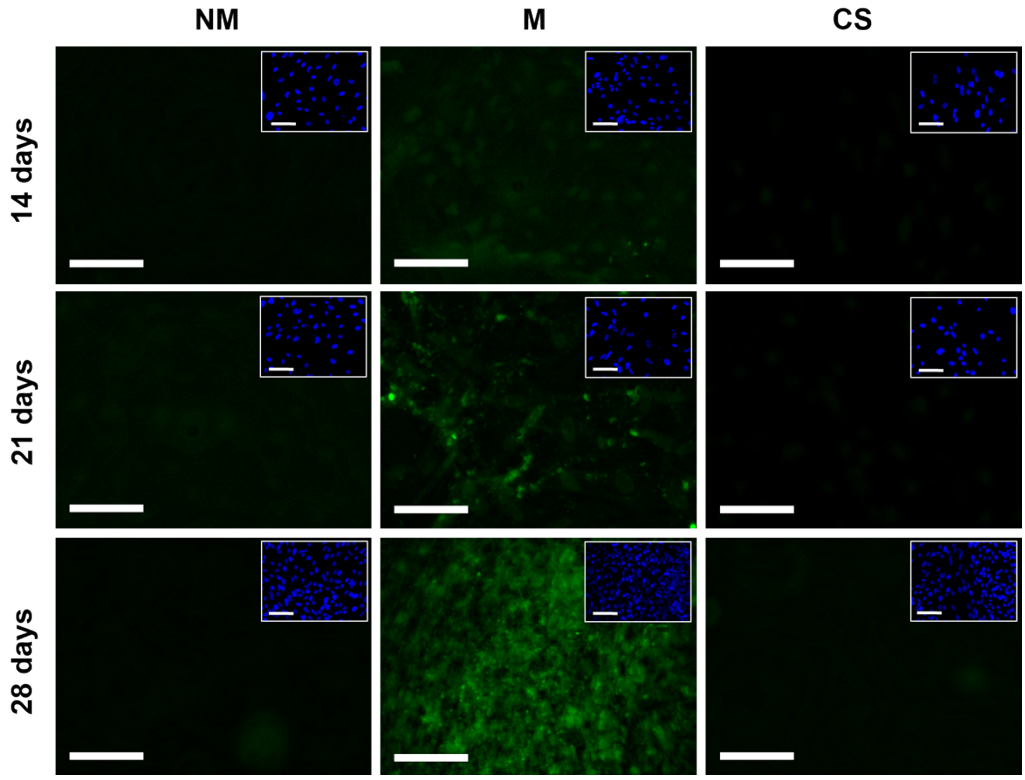
Supplementary Figure S3



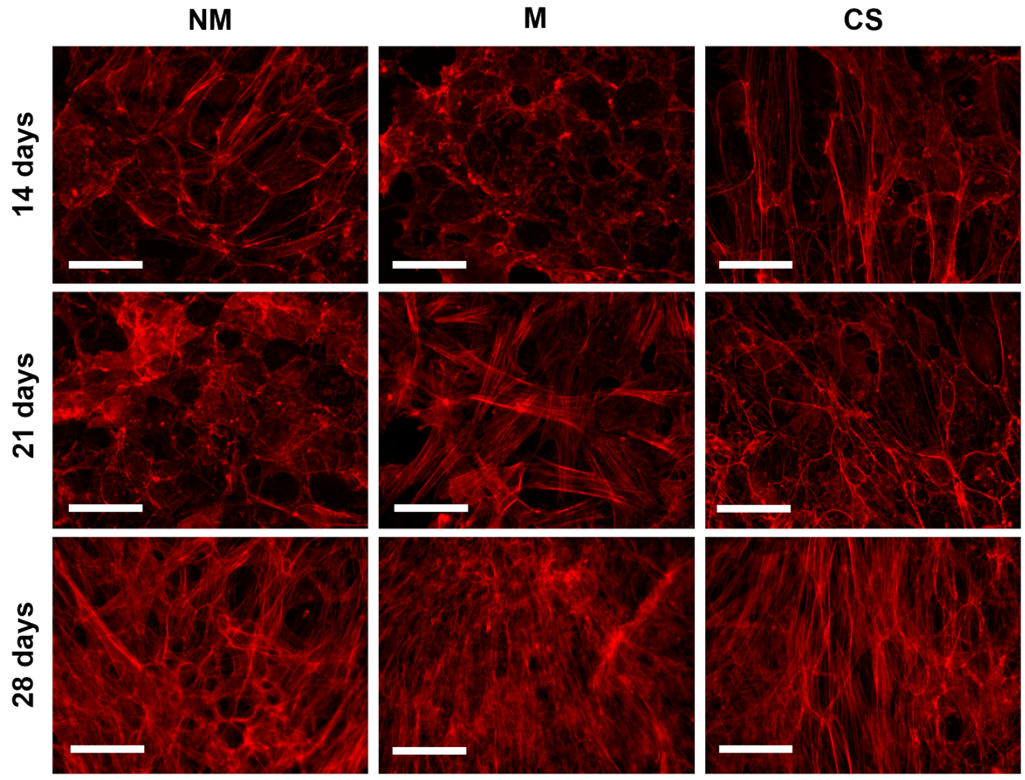
Supplementary Figure S4



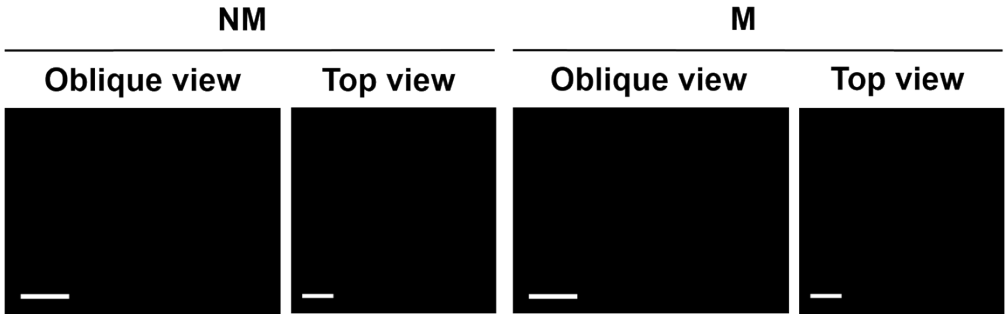
Supplementary Figure S5



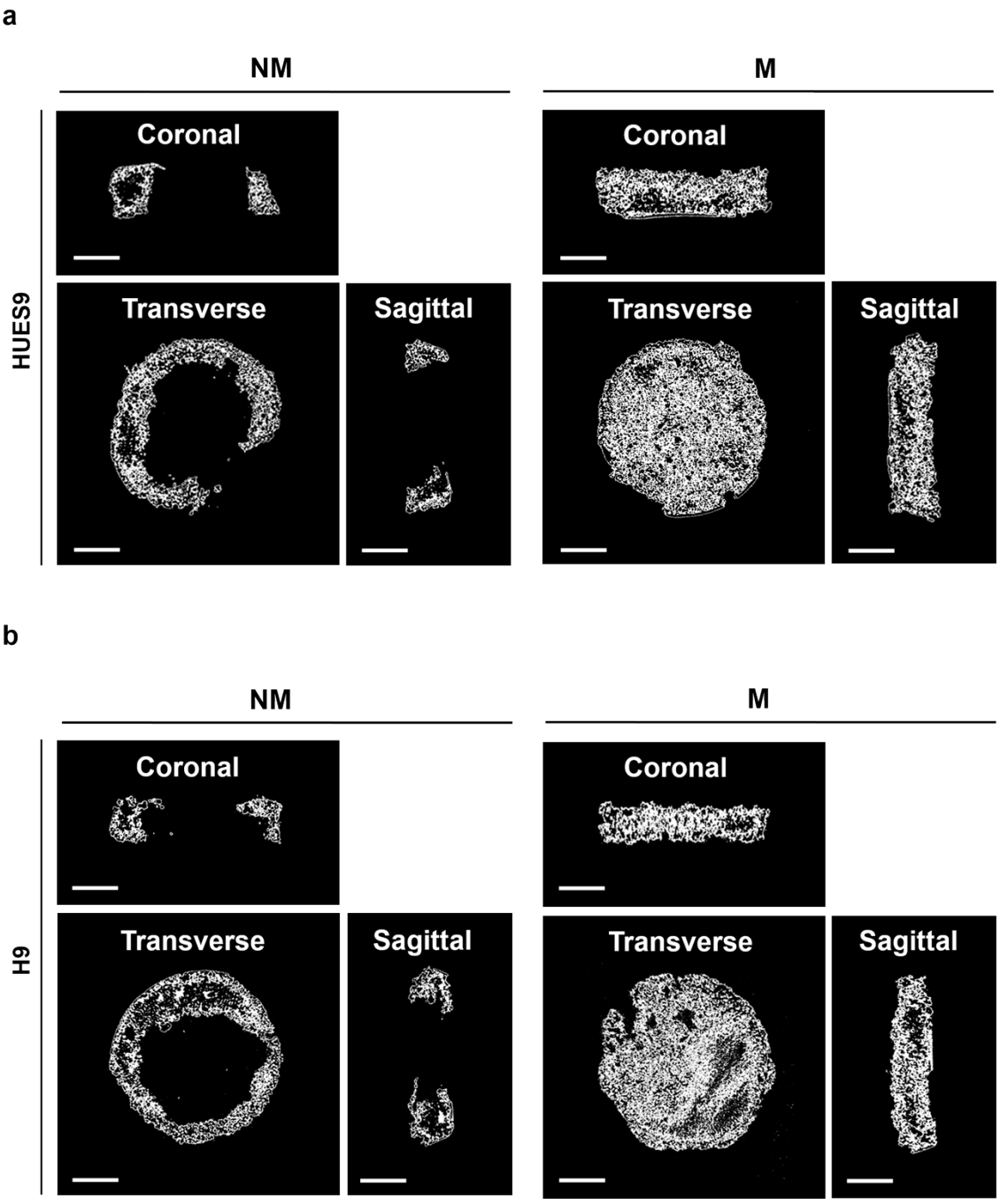
Supplementary Figure S6



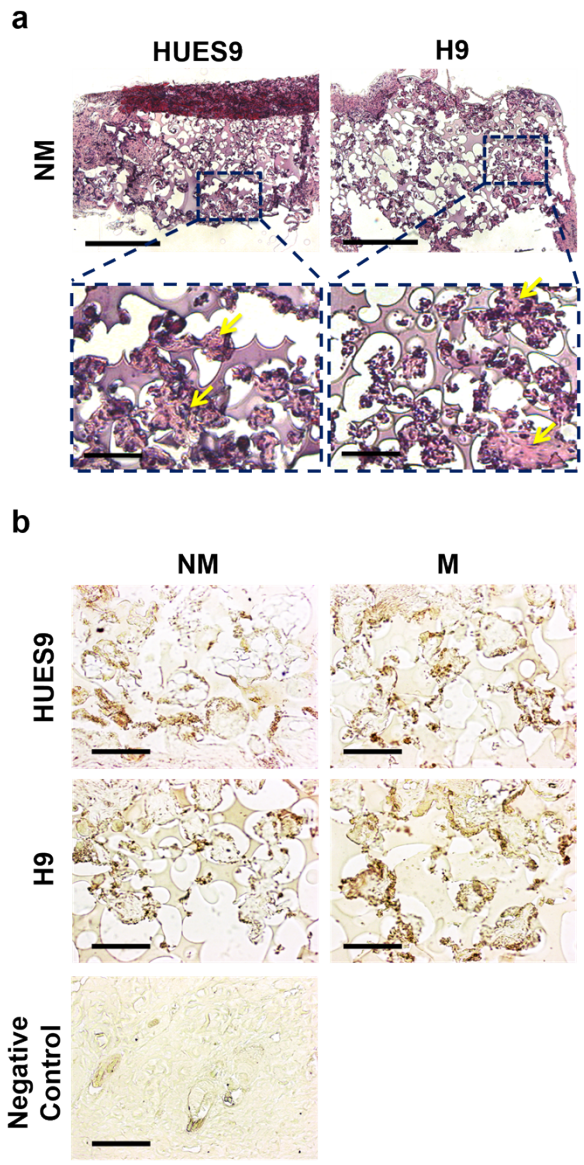
Supplementary Figure S7



Supplementary Figure S8



Supplementary Figure S9



Supplementary Table S1

Gene	Gene (Abbreviation)	Direction	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	NANOG	Forward	5’ GAT TTG TGG GCC TGA AGA AA 3’
		Reverse	5’ ATG GAG GAG GGA AGA GGA GA 3’