Supporting Information

A Biphasic Osteo-Vascular Biomimetic Scaffold for Rapid and Self-Sustained Endochondral Ossification

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Supplemental Tables

Table S1 – Sequences of primers used for qRT-PCR*

Gene	Forward (5' to 3')	Reverse (5' to 3')
ALP	GTTGCCAAGCTGGGAAGAACAC	CCCACCCCGCTATTCCAAAC
RUNX2	CGGTCTCCTTCCAGGATGGT	GCTTCCGGTCAGCGTCAACA
Osteocalcin (OCN)	GACAAAGCCTTCATGTCCAAG	AAAGCCGAGCTGCCAGAGTTT
Osteopontin (OPN)	CGAGGAGTTGAATGGTGCATAC	CATCCAGCTGACTCGTTTCATAA
Osterix (OSX)	TGAGCTGGAGCGTCATGTG	GGTGGTCGCTTCGGGTAAA
Collagen I	AACCCGAGGTATGCTTGATCT	CCAGTTCTTCATTGCATTGC
GAPDH	AGGAGTATATGCCCGACGTG	TCGTCCACATCCACACTGTT

* Primers were designed to specifically recognize human, but not murine, genes.

Table S2 – Antibodies used in the study

Antibody	Vendor	Cat number	Dilution*
Mouse anti-human Vimentin (V9)	Abcam	Ab8069	1:200 (IF)
Mouse anti-human Mitochondria	Abcam	ab92824	1:200 (IF)
Rat anti-mouse CD45	Abcam	ab25386	1:200 (IF)
Rabbit anti-Osteopontin (OPN)	Abcam	ab8448	1:200 (IF)
Rabbit anti-Osterix (OSX)	Abcam	ab22552	1:200 (IF)
Rat anti-mouse F4/80	Bio X Cell	CI:A3-1	1:50 (IF)
Mouse anti-human CD31 (JC70A, human specific)	Agilent	M082329-2	1:50 (IHC) 1:200 (IF)
Rat anti-mouse Ly-6G	Bio X Cell	1A8	1:50 (IF)
Rabbit anti-human Osteocalcin (OCN)	Proteintech	23418-1-AP	1:200 (IF)
Rat anti-human RUNX2/CBFA1 (232902)	R&D Systems	MAB2006	1:200 (IF)
Mouse anti-human Osteopontin (OPN)	R&D Systems	AF1433-SP	1:200 (IF)
Mouse anti-human Osteocalcin (OCN)	R&D Systems	MAB1419-SP	1:200 (IF)
Rabbit anti-α smooth muscle actin (1A4)	Sigma-Aldrich	A2547	1:300 (IF)

Rhodamine labeled Ulex Europaeus Agglutinin I (UEA I, human specific)	Vector Laboratories	RL-1062	1:100 (IF)
Texas Red -conjugated horse anti-mouse IgG	Vector Laboratories	TI-2000	1:200 (IF)
FITC-conjugated horse anti-mouse IgG	Vector Laboratories	FI-2000	1:200 (IF)
Peroxidase-conjugated horse anti-mouse IgG	Vector Laboratories	PI-2000	1:200 (IHC)
Texas Red-conjugated goat anti-rabbit IgG	Vector Laboratories	TI-1000	1:200 (IF)
FITC-conjugated goat anti-rabbit IgG	Vector Laboratories	FI-5000	1:200 (IF)

* IHC: immunohistochemistry staining; IF: immuno-histofluorescence staining.

Supplemental Figures

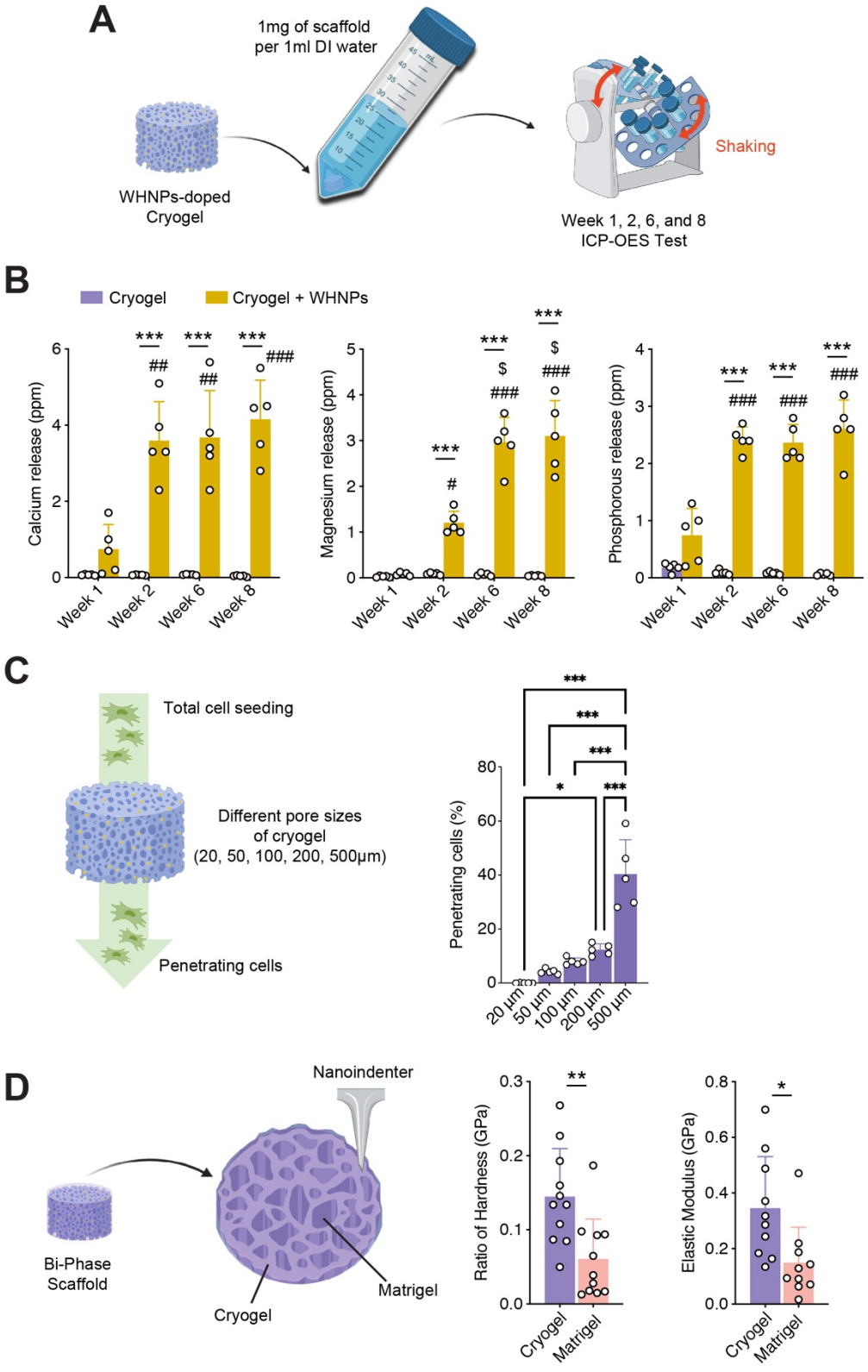

Figure S1. Scaffold material characterization. A) Schematic illustration depicting the assay to measure ion release from whitlockite nanoparticles (WHNPs) doped cryogel by inductively

coupled plasma atomic emission spectrometer (ICP-OES test). B) Total release of calcium, magnesium, and phosphorous ions quantified for both WHNPs-doped CS-cryogel (Cryogel + WHNPs) and control CS-cryogel (Cryogel). C) Schematic illustration depicting the penetration of seeded cells (MSCs) a through scaffold. Quantification of the percentage of penetrating cells for different pore size of scaffold. D) Schematic illustration depicting the measurement of mechanical properties of both the CS-cryogel and Matrigel phases of the scaffold using a nanoindenter. Quantification of both ratio of hardness (GPa) and elastic modulus (GPa) of CS-cryogel and Matrigel. In all quantitative panels, bars represent mean ± s.d. ($n \geq 4$). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ## $p < 0.01$, ## $p < 0.001$ compared to Cryogel + WHNPs at week 1. $ $p < 0.001$ compared to Cryogel + WHNPs at week 2.

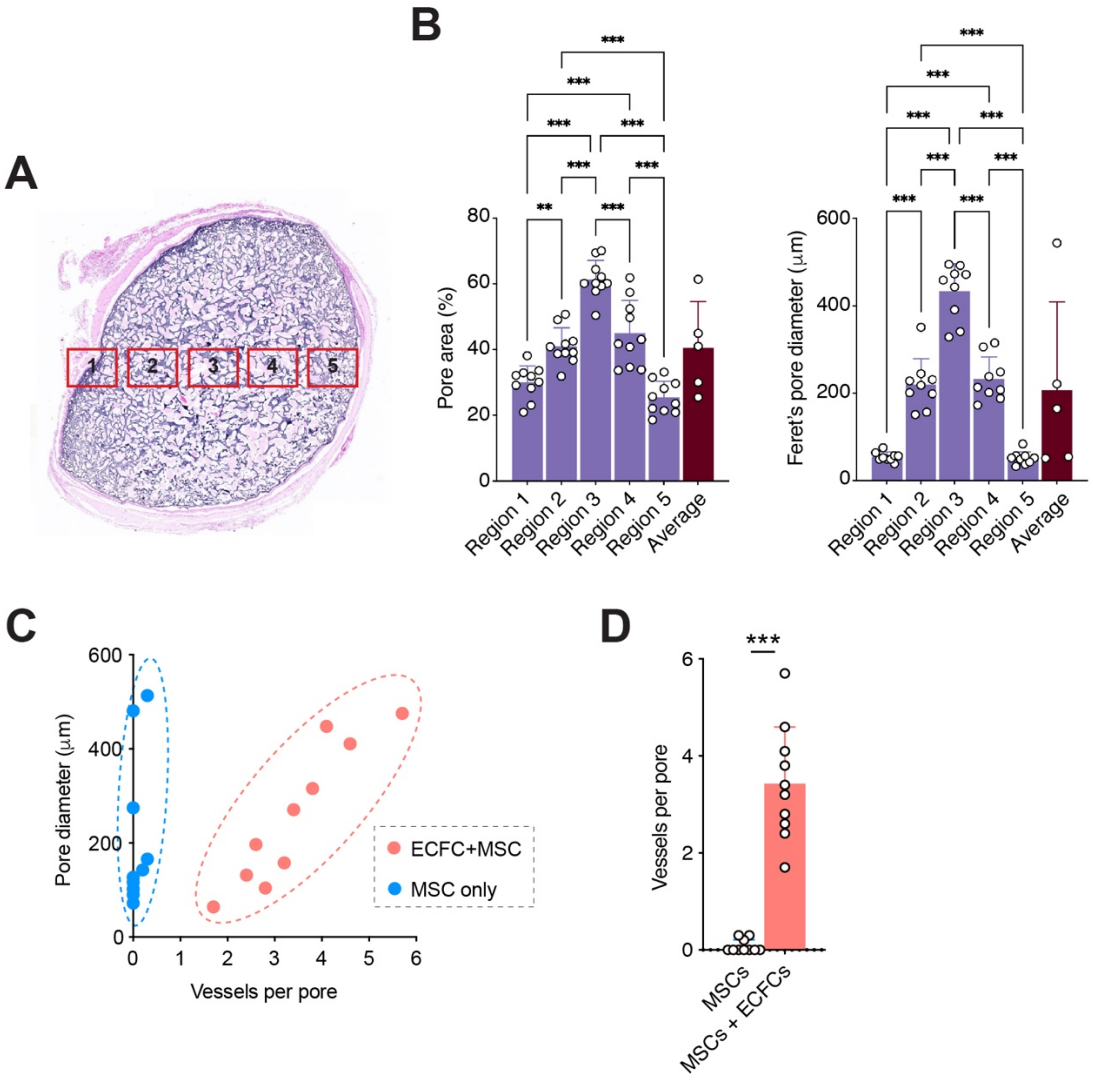

Figure S2. Pore size and average blood vessels per pore in composite scaffolds. A) Histological (H&E) staining of a representative acellular scaffold subcutaneously implanted into a nude mouse for 1 week (left panel). B) Pore area and diameter (Feret's diameter) quantified in explanted grafts by selecting 5 different regions (red squares in H&E) as well as the average value in all five regions (red bar). C) Spatial correlation between pore diameter and number of vessels per pore in graft seeded with MSCs and MSCs + ECFCs and explanted at week 1. Each dot represents 5 different pore areas within the scaffold. D) Average number of vessels per pore quantified in graft seeded with MSCs and MSCs + ECFCs and explanted at week 1. In all quantitative panels, bars represent mean ± s.d. ($n \geq 5$). ** $p < 0.01$, *** $p < 0.001$.

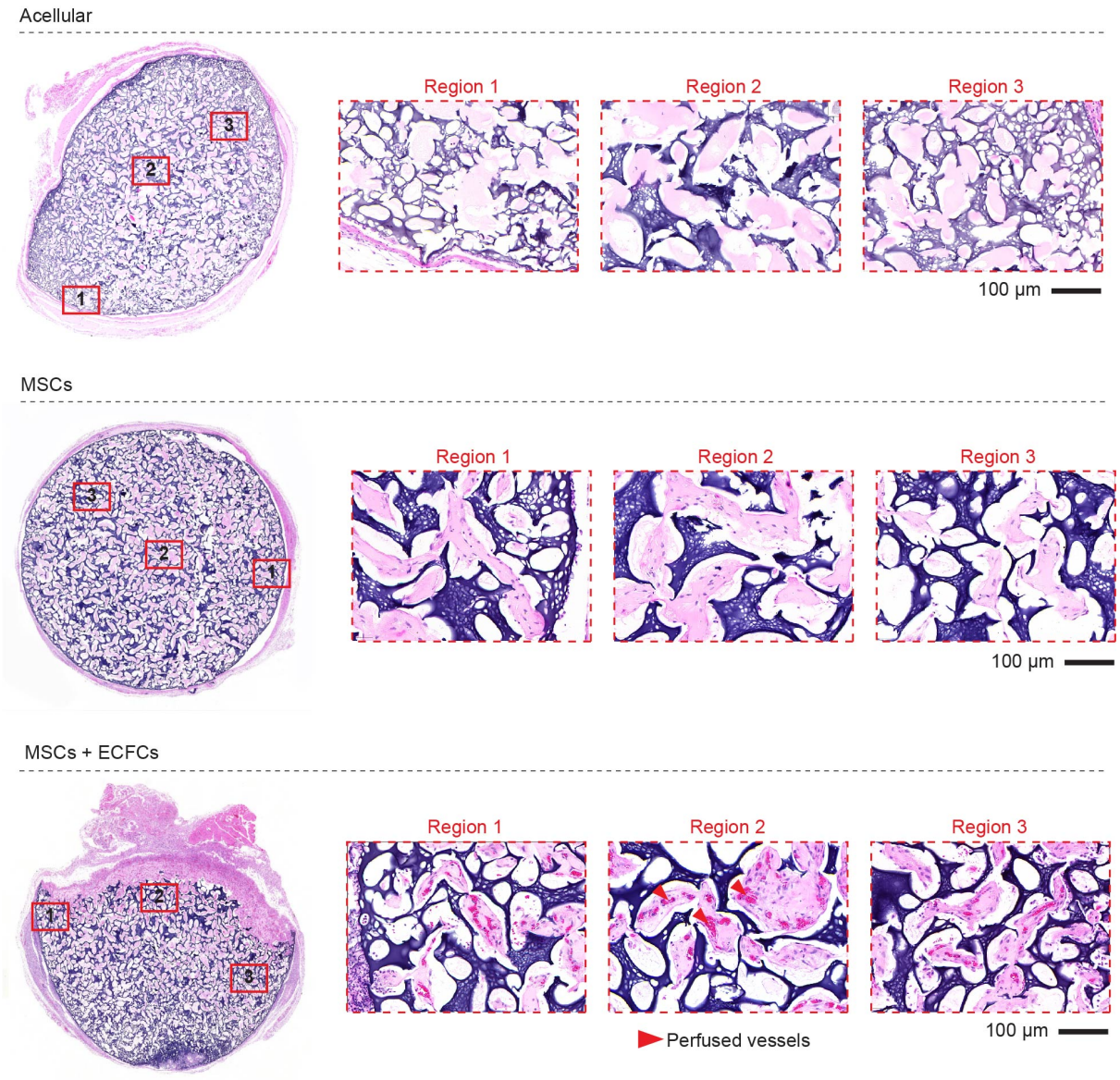

Figure S3. Vascularization of composite scaffolds. Histological (H&E) staining of representative acellular, MSCs, and MSCs + ECFCs seeded scaffolds implanted subcutaneously into nude mice for 1 week. Blue and pink colors represent the CS-cryogel and BM-hydrogel phases of the scaffold, respectively. Grafts seeded with MSCs + ECFCs exhibited numerous perfused blood vessels containing erythrocytes (red arrowheads). MSCs grafts were largely unperfused. Scale bars = 100 µm.

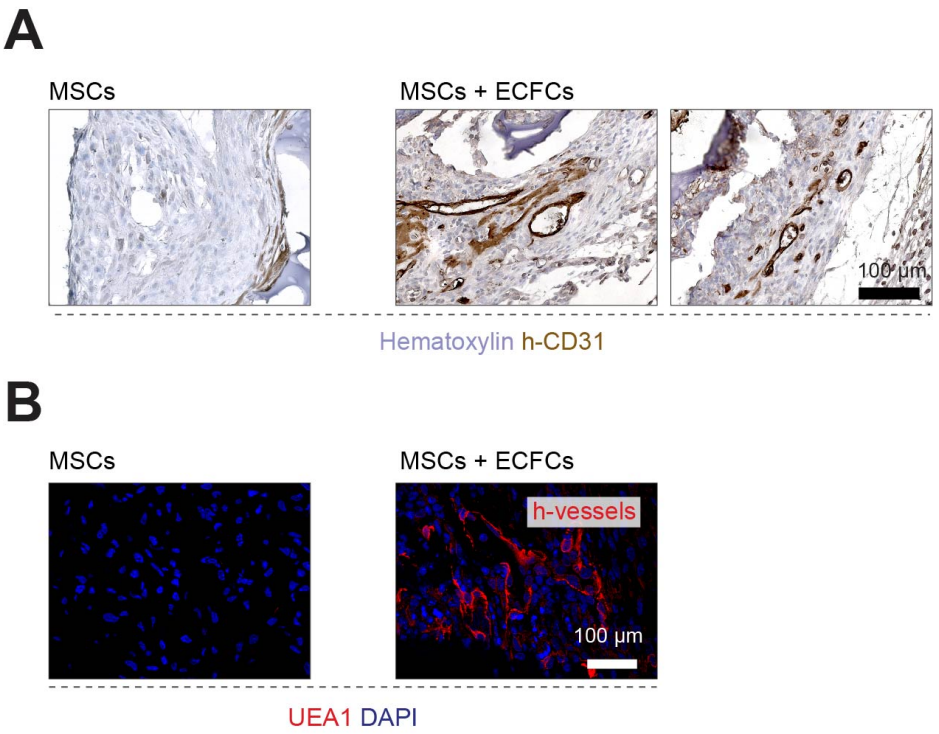

Figure S4. Identification of human-specific blood vessels. A) Human microvessels identified at week 1 in grafts seeded with MSCs + ECFCs by immunostaining for human-specific CD31 (h-CD31) and hematoxylin. B) Immunofluorescence staining showed the presence of human vessels (UEA-1^{+} lumens) at week 1 in grafts seeded with MSCs + ECFCs. MSCs grafts lack human vessels. Scale bars = 100 µm.

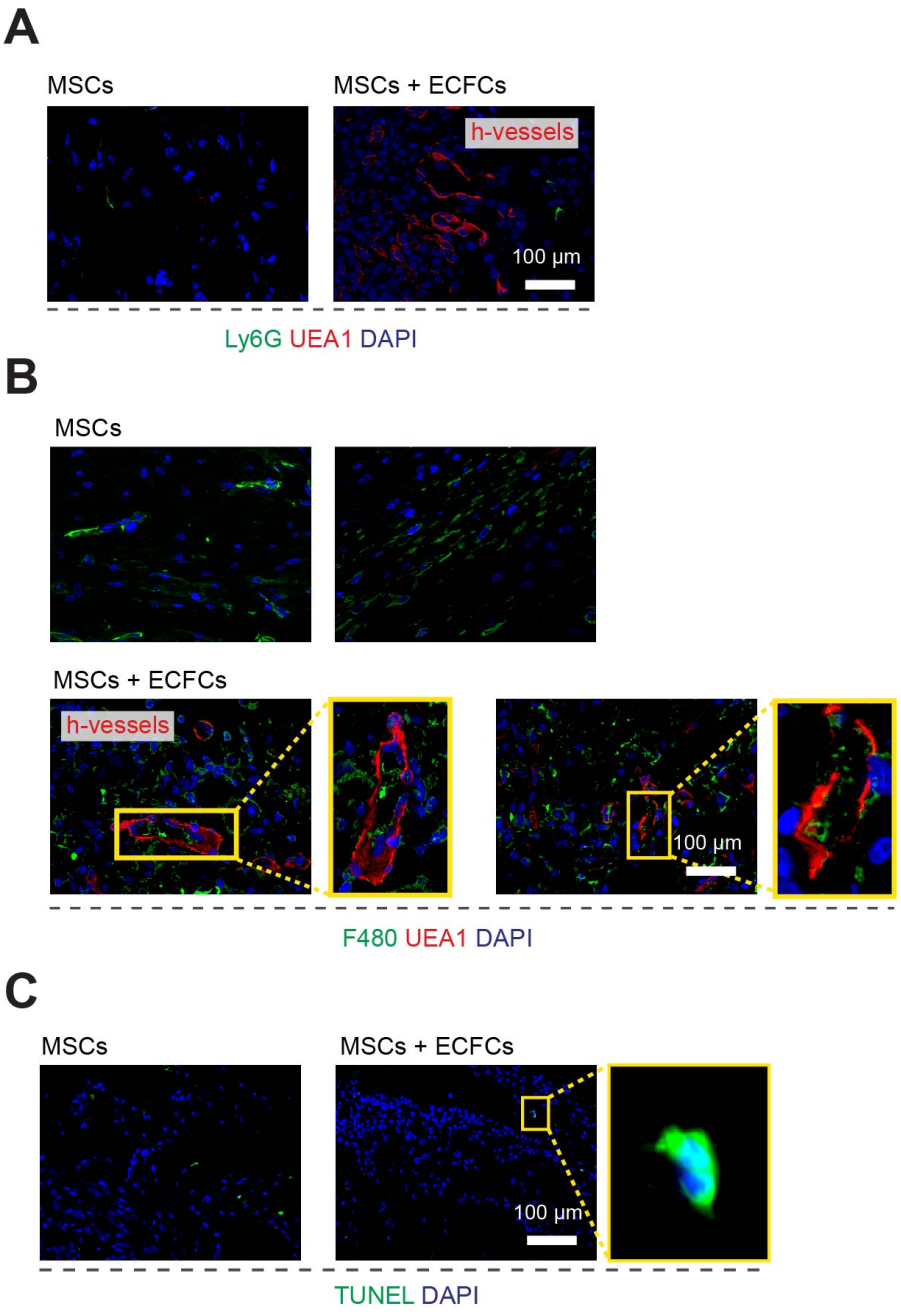

Figure S5. In vivo identification of inflammatory and apoptotic cells in the scaffold. Immunofluorescence staining of A) Ly6G^{+} neutrophils and B) F4/80^{+} macrophages in grafts seeded with MSCs and MSCs + ECFCs. Human cells visualized as UEA-1^{+} lumens (red). Nuclei stained with DAPI. C) Immunofluorescence staining for TUNEL (apoptosis) and DAPI in grafts seeded with MSCs and MSCs + ECFCs and explanted at week 1. Scale bars = 100 µm.

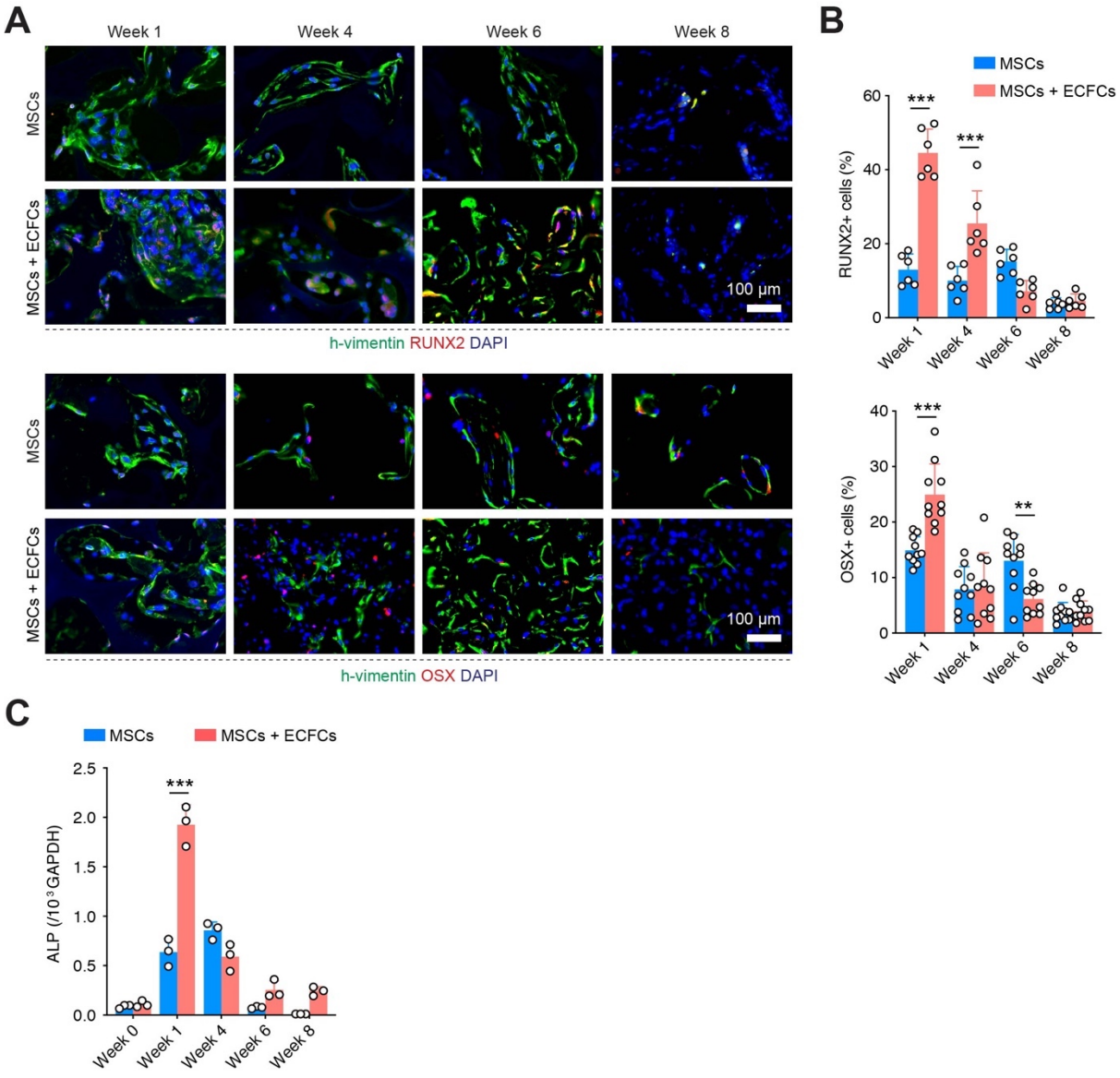

Figure S6. In vivo identification of pre-osteoblasts. A) Representative images of immunostaining for RUNX2 and OSX in grafts seeded with MSCs and MSCs + ECFCs and explanted at 1, 4, 6, and 8 weeks. Cells were also stained by h-vimentin and nuclei by DAPI. B) Total number of $RUNX2^+$ cells (top) and OSX^+ cells (bottom) quantified in grafts explanted at weeks 1-8. C) Gene expression profile of alkaline phosphatase (ALP) quantified in grafts seeded with MSCs and MSCs + ECFCs and explanted at 0, 1, 4, 6, and 8 weeks. In all quantitative panels, bars represent mean ± s.d. ($n \geq 3$). $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bars = 100 µm.

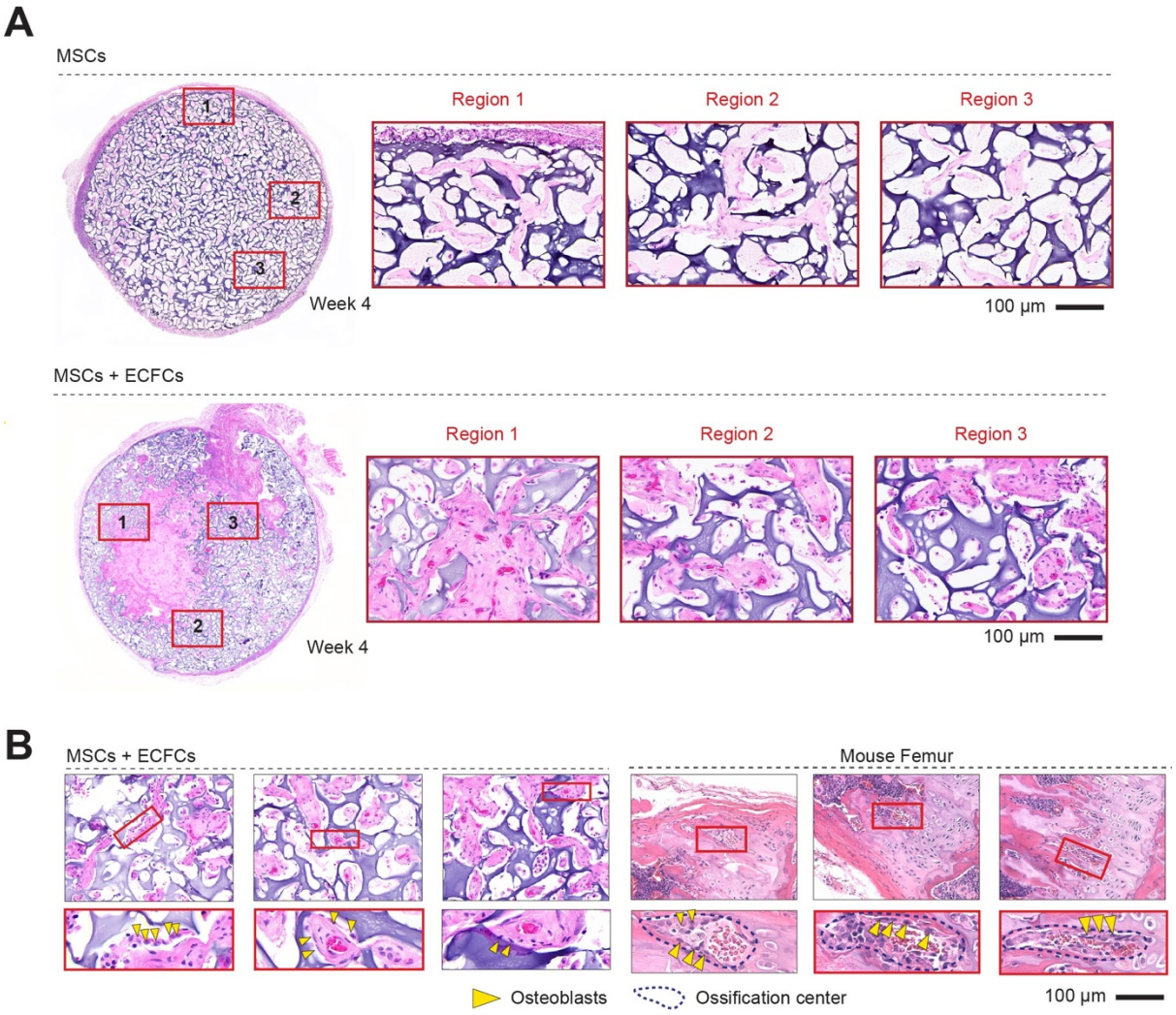

Figure S7. In vivo identification of ossification centers. A) Histological (H&E) staining of representative grafts seeded with MSCs and MSCs + ECFCs explanted at week 4. Only grafts with MSCs + ECFCs contained perfused blood vessels distributed within the porous phase of the scaffold. B) H&E staining of grafts seeded with MSCs + ECFCs revealed the presence of ossification centers containing perfused blood vessels surrounded by osteoblasts (yellow arrowheads). Staining of mouse femur served as control for ossification centers (dash lines). Scale bars = 100 µm.

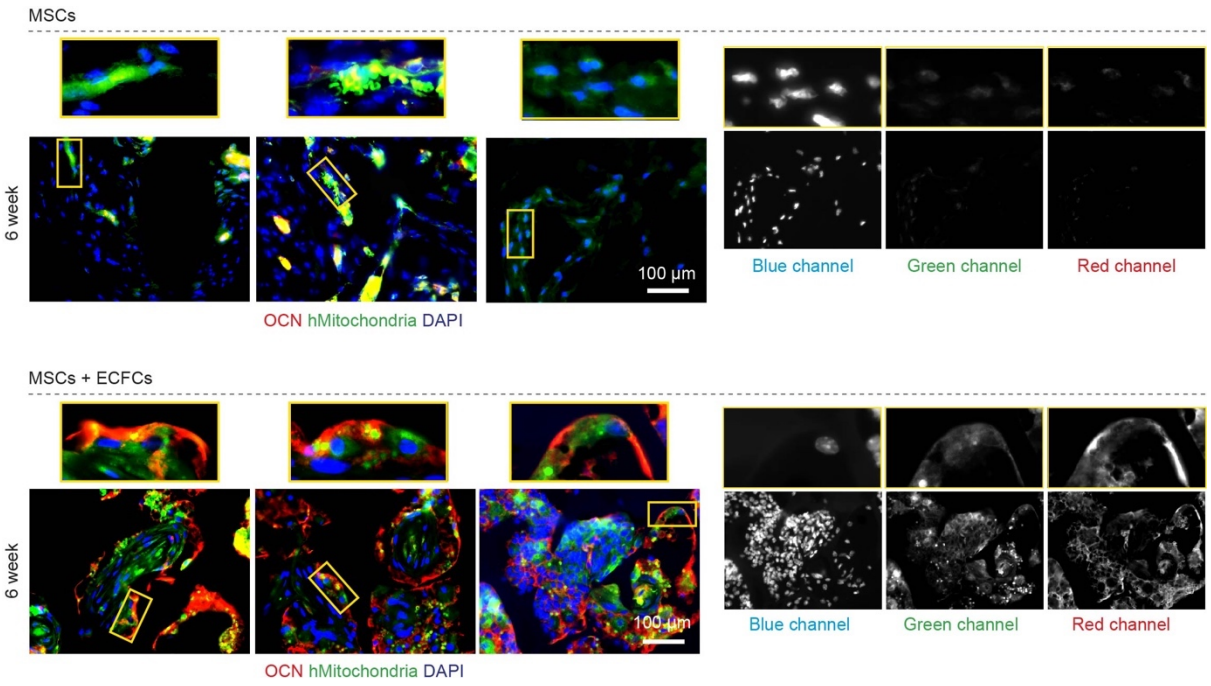

Figure S8. In vivo identification of human-specific osteoblasts. Representative images of immunostaining for OCN and human-specific mitochondria (hMitochondria) in grafts seeded with MSCs and MSCs + ECFCs and explanted at week 6. Nuclei stained by DAPI. Human osteoblasts were identified as OCN^{+} $hMitochondria^{+}$ cells and were only present in grafts with MSCs + ECFCs. Scale bars: 100 µm.

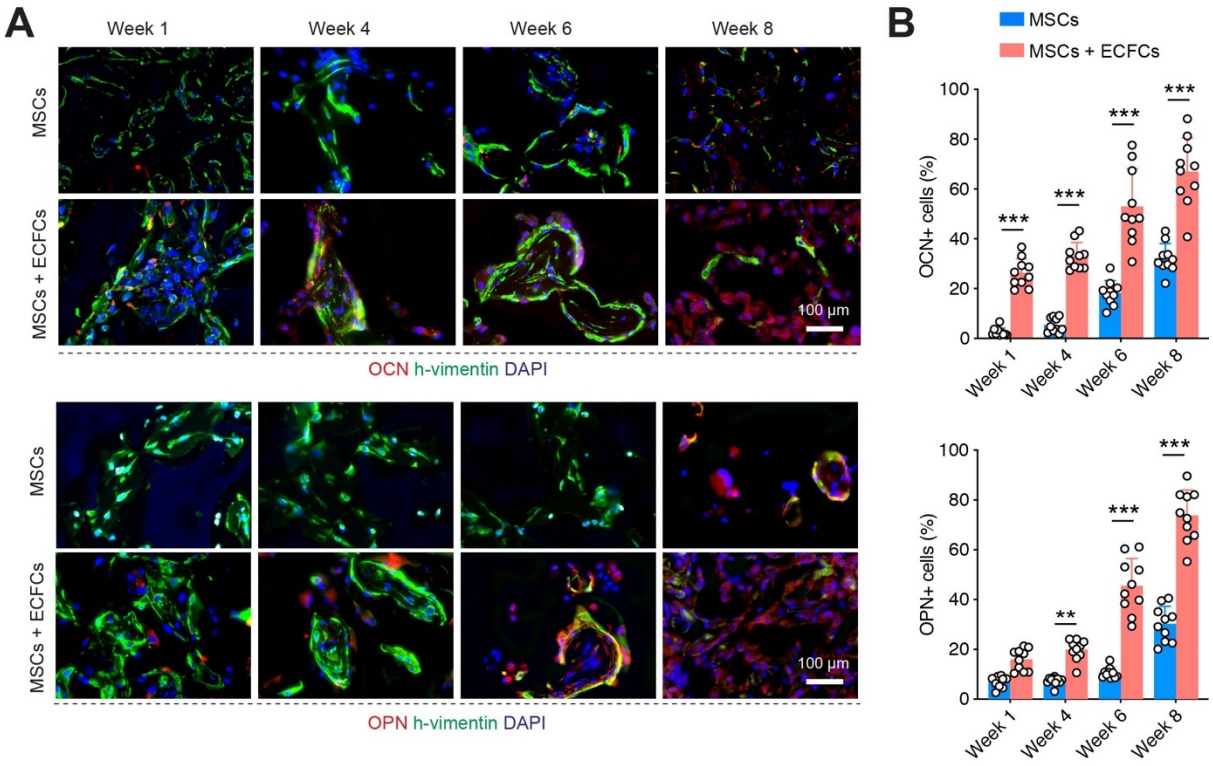

Figure S9. In vivo identification of mature osteoblasts. A) Representative images of immunostaining for OCN and OPN in grafts seeded with MSCs and MSCs + ECFCs and explanted at 1, 4, 6, and 8 weeks. Cells were also stained by h-vimentin and nuclei by DAPI. B) Total number of OCN^+ cells (top) and OPN^+ cells (bottom) quantified in grafts explanted at weeks 1-8. Bars represent mean ± s.d. ($n \geq 3$). $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bars = 100 µm.

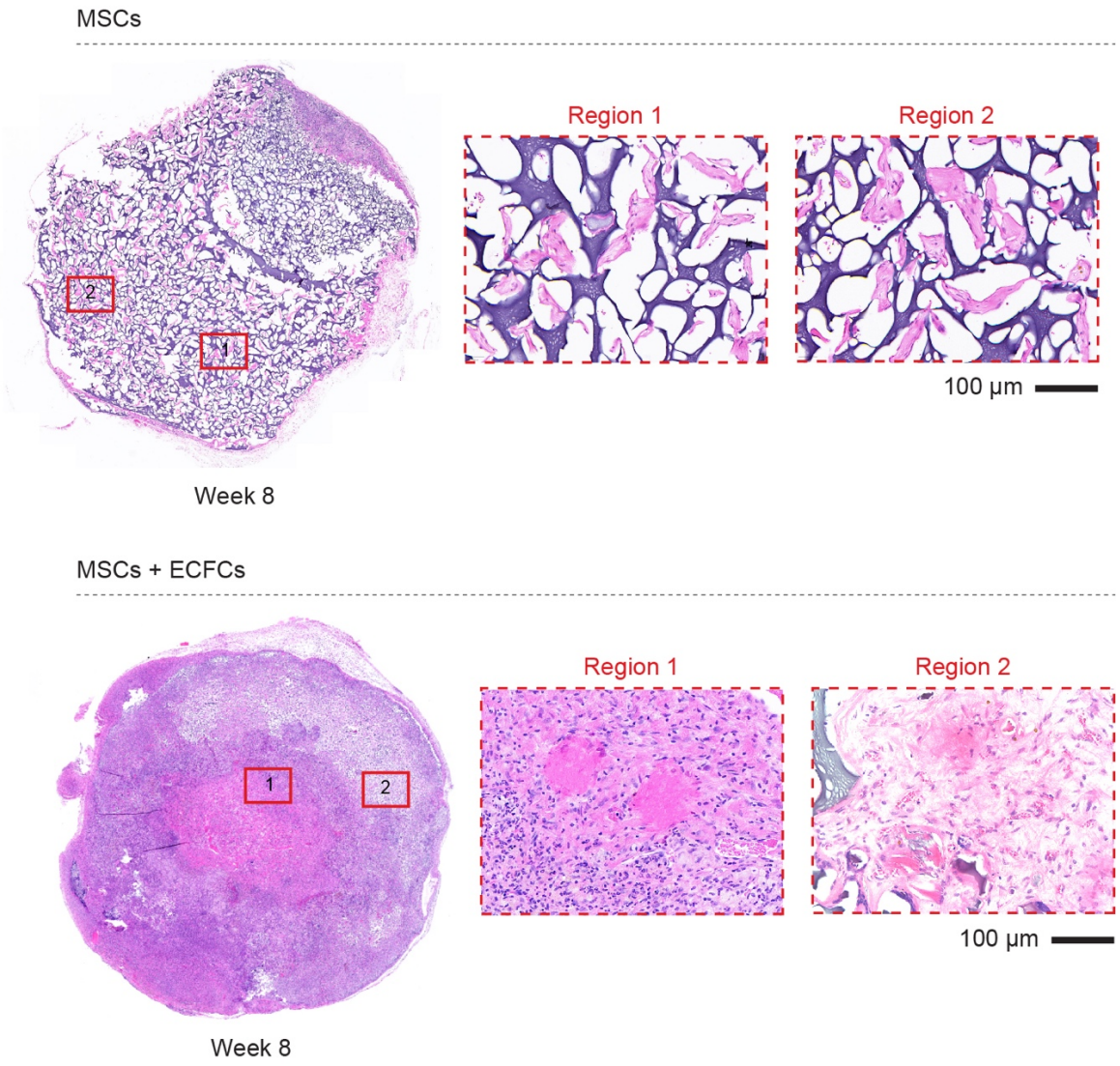

Figure S10. Histological identification of bone-like tissue fragments. Histological (H&E) staining of a representative graft seeded with MSCs + ECFCs and explanted at 8 weeks revealed the presence of numerous areas containing bone-like tissue fragments (dark pink). In contrast, graft seeded with only MSCs showed little evidence of bone tissue formation at week 8. Scale bar: 100 µm.

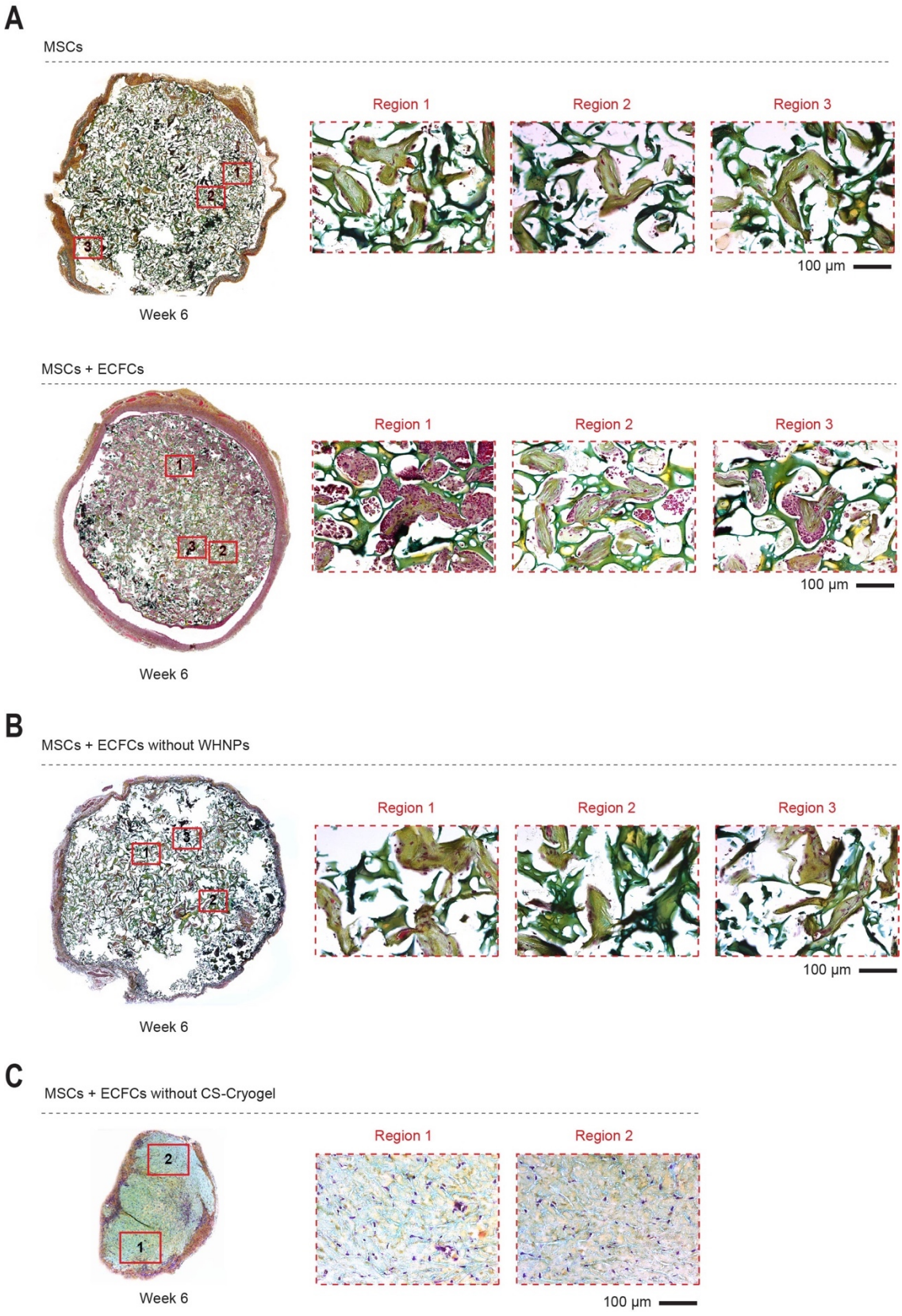

Figure S11. Deposition of extracellular matrix. A) Histological (Movat pentachrome) staining of representative grafts seeded with MSCs and MSCs + ECFCs and explanted at week 6. Colorimetric analysis revealed the cartilaginous CS-cryogel phase of the scaffold (green) and collagen deposition (yellow). B) Movat pentachrome staining at 6 weeks of a representative

graft seeded with MSCs + ECFCs in a composite scaffold containing CS-Cryogel and Matrigel but no WHNPs. C) Movat pentachrome staining at 6 weeks of a representative graft seeded with MSCs + ECFCs in a scaffold containing Matrigel and WHNPs but no CS-Cryogel. Scale bars: 100 μm.

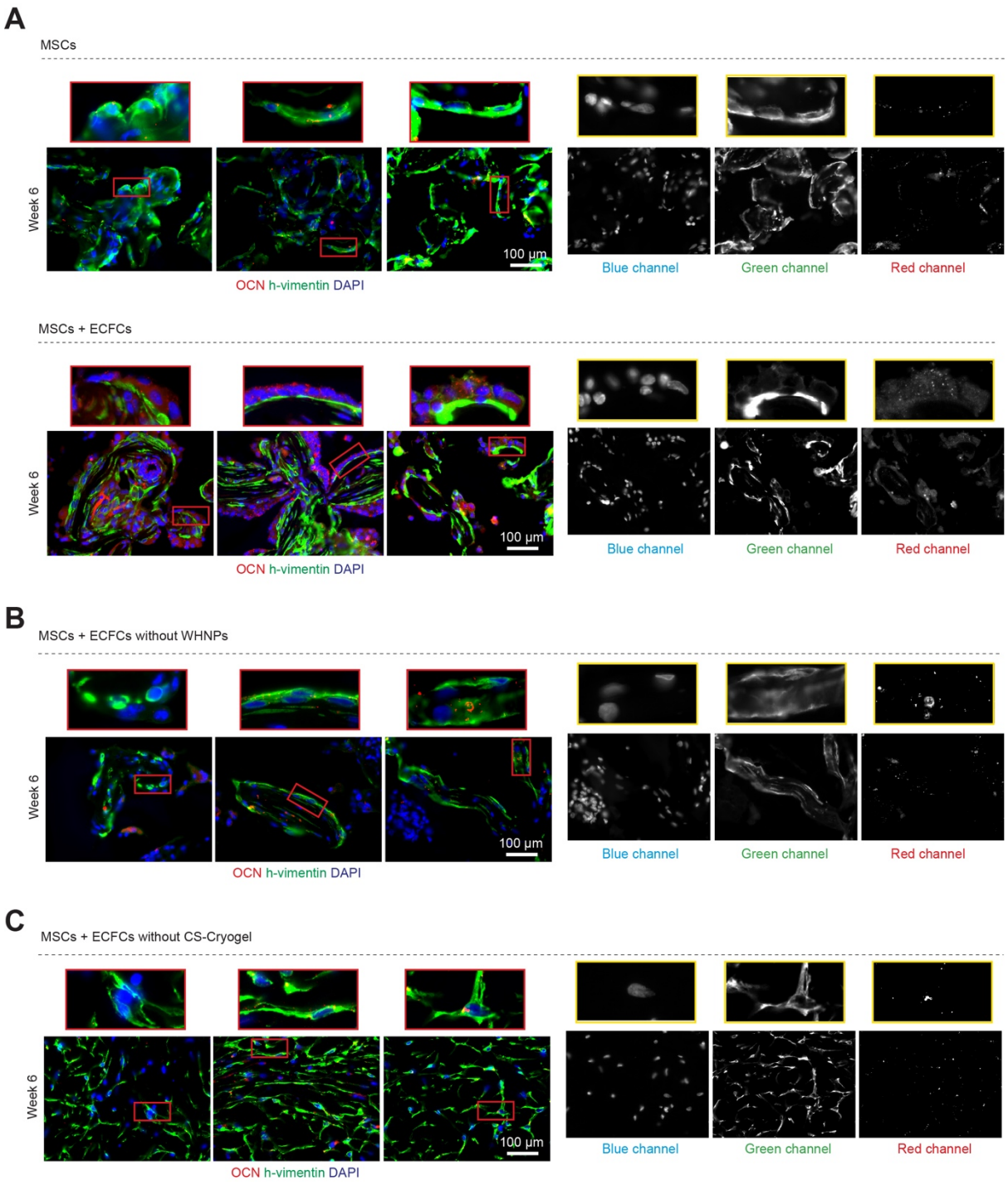

Figure S12. The effect of scaffold composition on the in vivo formation of mature osteoblasts. A) Representative images of immunostaining for OCN in grafts seeded with MSCs and MSCs + ECFCs into complete composite scaffolds and explanted at week 6. Mature osteoblasts were identified as OCN^+ cells. Cells were also stained by h-vimentin and nuclei by DAPI. B) Immunostaining for OCN and h-vimentin at 6 weeks of a representative graft seeded with MSCs + ECFCs in a composite scaffold containing CS-Cryogel and Matrigel but no WHNPs. C) Immunostaining for OCN and h-vimentin at 6 weeks of a representative graft seeded with

MSCs + ECFCs in a scaffold containing Matrigel and WHNPs but no CS-Cryogel. Scale bars = 100 μm.

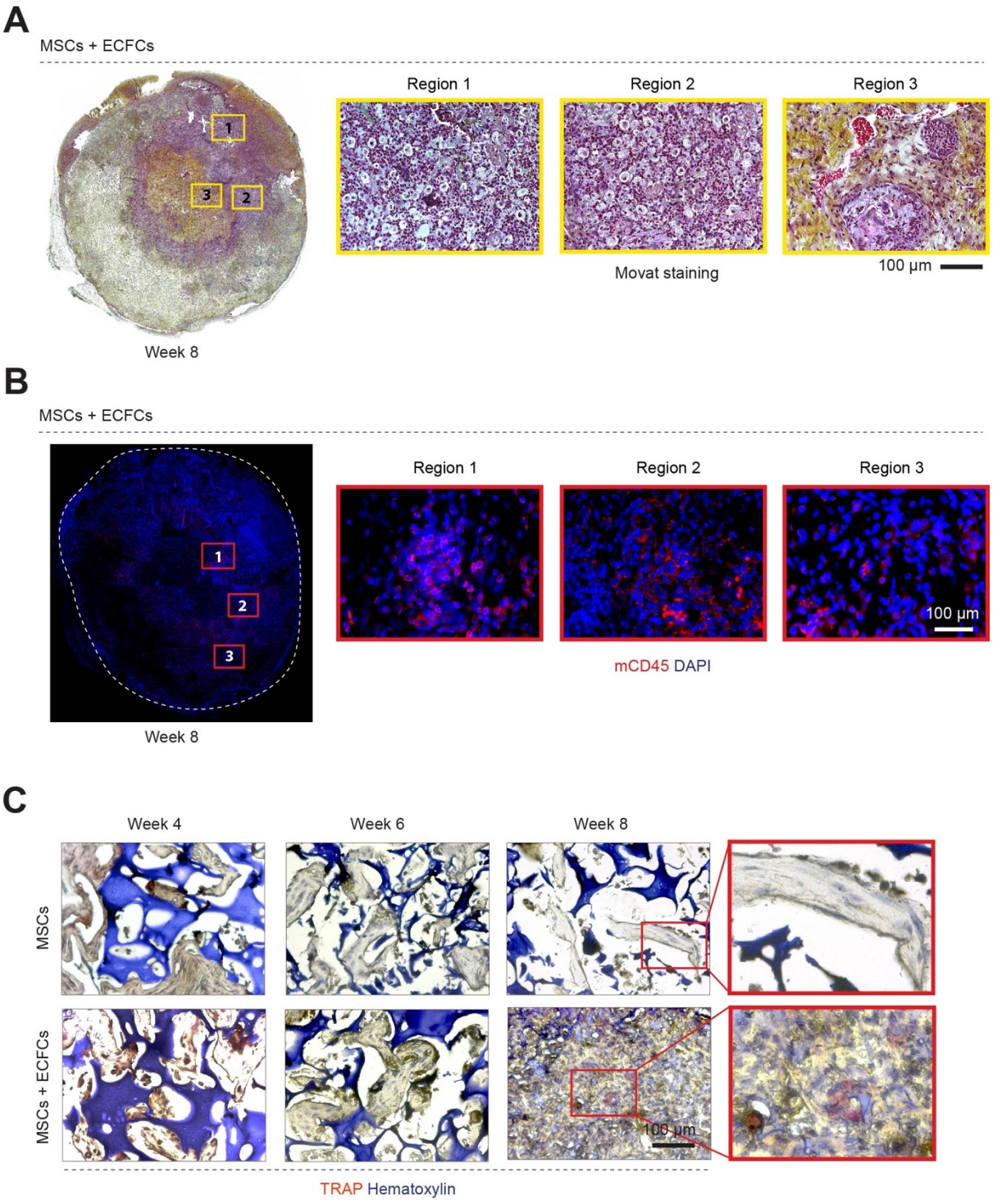

Figure S13. Identification of hematopoietic cells and osteoclast activity. A) Movat pentachrome staining of a representative graft seeded with MSCs + ECFCs and explanted at week 8. Identification of hematopoietic foci development in selected regions of the grafts neighboring the bone matrix (yellow). B) Immunofluorescent staining for mouse specific CD45 (mCD45) in a representative graft seeded with MSCs + ECFCs and explanted at week 8. Cell nuclei stained by DAPI. C) Histological (TRAP) staining of representative grafts seeded with MSCs + ECFCs and explanted at weeks 4-8. Osteoclast activity (red) detected at week 8 in selected regions neighboring the bone matrix. Scale bars: 100 µm.

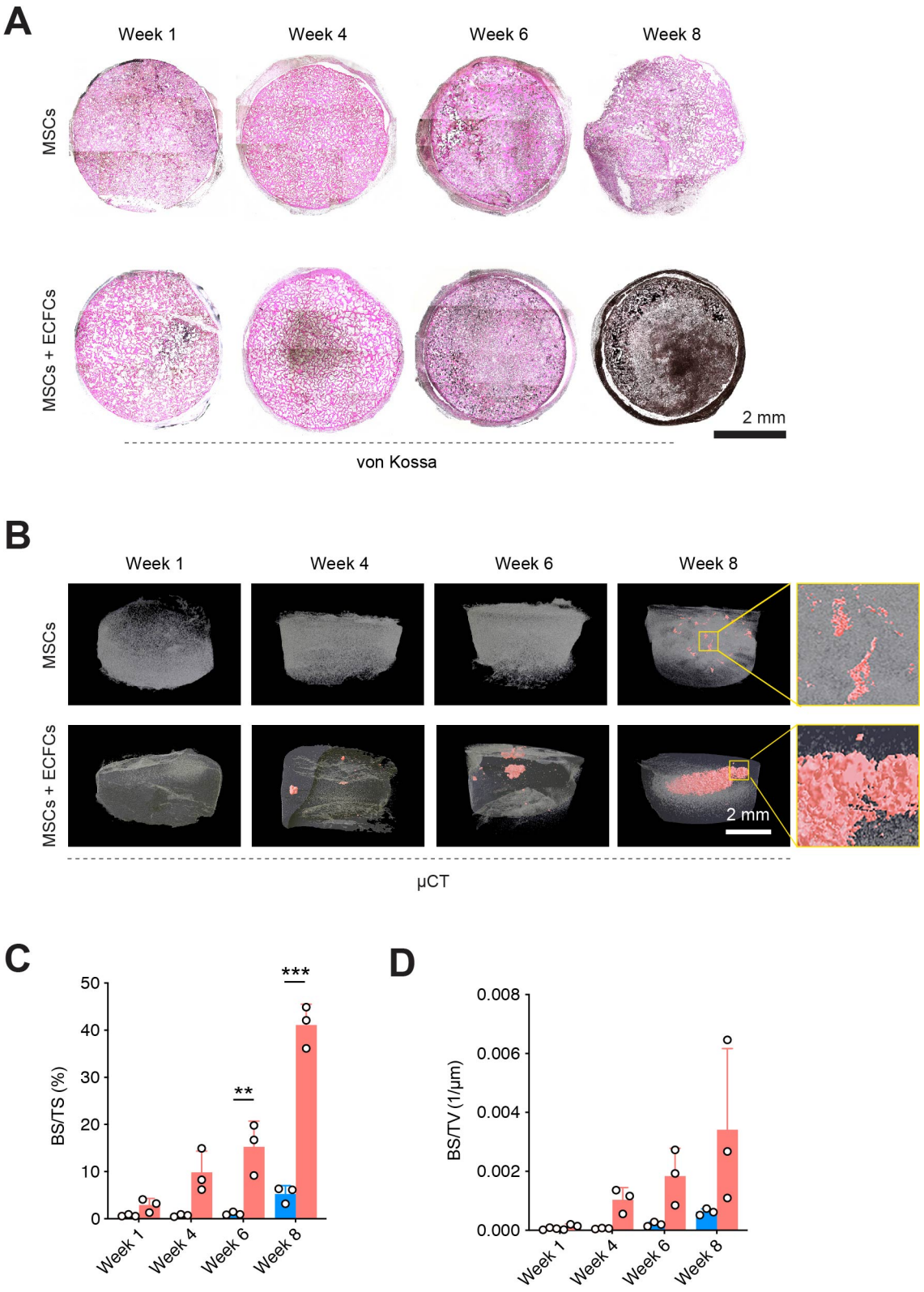

Figure S14. Identification of mineralized tissue. A) von Kossa staining of representative grafts seeded with MSCs and MSCs + ECFCs and explanted at weeks 1-8. Mineralized matrix identified as black. B) Quantitative microtomography (µCT) revealed a gradual appearance of mineralized tissues within the MSCs + ECFCs grafts at week 1, 4, 6, and 8. Red pseudo color represents mineralized tissue. Grafts with only MSCs exhibited minimal mineralization.

Quantitative analysis of C) bone surface area per total surface area (BS/TS) and D) bone surface density (i.e., bone surface area per total volume) (BS/TV) from the μCT imaging of the grafts. Bars represent mean ± s.d. (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bars: 2 mm.