SUPPLEMENTARY METHODS

Preparation of decellularized bone scaffolds

Bone scaffolds were prepared by using a previously established method (*16*). Trabecular bone blocks were cut out from the subchondral region of the knee joints of 2-week-old to 4-month-old cows. The blocks were placed on a lathe and formed into cylinders that were then milled into the desired geometry. After milling, the bovine trabecular bone into a desired shape, the bone was washed with high-velocity streams of water to remove the marrow from the pore spaces, and then treated on an orbital shaker in four steps, to remove any remaining cellular material: (i) PBS with 0.1% EDTA (w/v) for 1 h at room temperature; (ii) hypotonic buffer [10 mM Tris, 0.1% EDTA (w/v)] overnight at 4°C; (iii) detergent [10 mM Tris, 0.5% SDS (w/v)] for 24 h at room temperature; (iv) enzymatic solution (50 U/ml DNase, 1 U/ml RNase, 10 mM Tris) for 6 h at 37°C. After SDS treatment, 10 washes with PBS were performed, and it was confirmed that no more bubbles were seen in the PBS during washing. At the end of the process, decellularized bone blocks were freeze-dried. Prior to seeding, the scaffolds were submerged in PBS for 24 h and sterilized by wet autoclaving. As in our previous studies (*47*), we used the PicoGreen assay (Molecular Probes) to demonstrate that the decellularized bone is essentially free of DNA.

Perfusion bioreactor

The bioreactor implemented three major innovative features: a perfusion system for supporting large grafts with complex geometries, compatibility with CT imaging, and portability. The redesigned bioreactor system was suitable for large-scale tissue engineered bone graft manufacturing due to its simple and well-controlled perfusion (Fig. 1D). The culture chamber consisted of five components (Fig. 1B): (i) anatomical inner chamber, (ii) two halves of the polydimethylsiloxane (PDMS) block with incorporated channels, (iii, iv) two manifolds, and (v) an outer casing.

The PDMS block was designed as an impression of the anatomical RCU structure, and contained flow channels at both sides for the flow of culture medium in and out of the scaffold. The channel diameters and spacing were specifically designed in SolidWorks software with flow simulation module for each pig, to provide a desired interstitial flow velocity for a given shape and size of the anatomical RCU unit. The channels of various sizes were placed into the PDMS and the fluid flow was numerically simulated to provide the flow velocity at any point within the scaffold in the range 400-1000 μm/s that is optimal for bone development *in vitro* (*18*).

For each animal, an anatomical PDMS block with an array of channels (with specific diameters and spacing) was generated using a mold designed in SolidWorks and 3D-printed using WaterShed 11122XC material at ProtoCAM. The mold consisted of 2 casings and a positive anatomical structure with precise channels locations. Metal rods of the specific size (designed as described above for each animal) were inserted through the casing and into the positive mold. The two casings were clamped together (Fig. 1B), PDMS was poured into the mold, degased, and allowed to cure overnight at 60°C. The metal rods were then removed and the solid PDMS block was cut in half to leave a space with perfect anatomical match to the scaffold and perfusion channels.

To assemble the bioreactor, the scaffold was inserted into the void of the PDMS block that was sandwiched between two polycarbonate fluid-routing manifolds. A tubular enclosure sliding over the assembly provided compressive force to seal the components together (movie S1). The polycarbonate manifolds were designed with one single port on each side serving as a fluid inlet and outlet. The grafts were transported in the same bioreactor chamber where they were cultured.

Transportation of bone grafts

Engineered bone grafts were sterilely transported within their culture chambers from Columbia University (New York, NY, USA) where the grafts were grown to the animal facility at Louisiana State University (Baton Rouge, LA, USA) where they were implanted. Each bioreactor was disconnected from the pump and media reservoir, and the tubing was sterile-assembled into a close loop. The bioreactor was then wrapped in sterile aluminum foil, placed into a plastic storage bag, and packaged into a foam box with ice packs, within a protective hard case. The total time for transportation was < 10 h. Upon arrival at the animal facility, the whole bioreactor apparatus was sterilely reassembled by connecting the chamber to the reservoir, and the system was placed into tissue culture incubator with perfusion until implantation (<48 h).

Finite element analysis to determine oxygen concentration, the limiting supplement for cell survival inside the scaffold, was performed for the duration of graft transport (10 h), using the Comsol Multiphysics program. The computation and constants were based on previously reported approaches while accounting for differences in geometry of the 3D model and the governing diffusion equation:

$$\frac{\partial c}{\partial t} + \nabla \cdot (-D\nabla c) = R$$

where c is O_2 concentration (mol/m^3), D is the diffusion coefficient of O_2 (m^2/s), and R is the O_2 consumption rate by the cells. The O_2 diffusion coefficient in media and PDMS are 3.29×10^{-9} m^2/s (*43*) and 3.55×10^{-9} m^2/s (*44*), respectively. The initial O_2 concentration and the O_2 concentration at the boundary of PDMS with air were assumed constant (0.21 mol/m^3).

The O_2 consumption rate, R, was assumed to follow the Michaelis-Mentin kinetics:

$$R = -\rho_{cell}\frac{Q_m c}{C_m + c}$$

where ρ_{cell} is the cellular density based on DNA quantitation (4.04×10^{13} cells/m^3), Q_m is the maximum oxygen consumption rate, and C_m is the oxygen concentration at half-maximum oxygen consumption. The physiologic rate of oxygen consumption (1.86×10^{-18} mol/cell/s) was used (*18, 45*). Under hypothermic condition (<15°C), the oxygen consumption rate can be reduced by more than 10-fold (*46*). Thus, we assumed a 10-fold reduction in oxygen consumption rate during transport. The time-dependent model was run for a period of 10 h.

DNA quantitation

The amount of DNA in tissue engineered bone graft was analyzed using the PicoGreen assay (Molecular Probes, OR) at 3 days (n = 7), 3 weeks (n = 7), and after transportation to surgical site (n = 7). Values were normalized to the wet weight. Samples were cut,

weighted and stored at -20°C in digestion buffer (10 mM Tris, 1 mM EDTA, 0.1% Triton-X, and 0.1 mg/ml proteinase K). For analysis, samples were thawed and digested at 56°C overnight prior to the assay.

RT-PCR analysis of gene expression

RT-PCR was conducted to assess gene expression at 3 days (post-seeding) and after 3 weeks (post–bioreactor cultivation). Samples were stored at -40°C in TriZol reagent (Invitrogen), and RNA were extracted according to the manufacturer's protocol. Equal amounts of RNA samples were reverse-transcribed using cDNA reverse transcriptase (Invitrogen). PCR amplification of cDNA was carried out using SYBR green. Porcine osteogenic markers (*CBFa1, ALP, Col1a1, BSP, Osterix, ON*), osteogenic growth factors (*BMP2, BMP4, PDGF,* and *TGFb2*), and inflammatory cytokines (*IGF, IL1b,* and *TNFa*) were analyzed (table S1). The forward and reverse primers were designed and verified with GeneBank. Single-peak melting curve tests were performed on all samples to ensure specificity of the primers. The expression was normalized to the day-3 values.

Histology and immunohistochemistry

Bone grafts were analyzed prior to implantation (3 days and 3 weeks of bioreactor culture) and following implantation (3 months and 6 months). Bone grafts from bioreactor cultures were fixed in 4% formalin for 3 days, decalcified in immunocal for 2 days, dehydrated, paraffin embedded, and sliced at 5 μm thick. The whole RCUs harvested from the animals were fixed in formalin for 1 week and decalcified for 8-10 weeks in EDF™ decalcifying solution (StatLab, TX, USA). The ramus was cut transversely and the condyle head was cut sagittally using a reciprocal saw (Stryker, MI, USA). The samples were cut in half if they were too large for the embedding cassette (29mm x 25mm x 5mm), embedded in paraffin and sectioned (5 μm thick).

Samples were stained with H&E and antibodies for type I collagen and bone sialoprotein. Primary and secondary antibody controls were performed to show the specificity of the antibody binding. Movat's pentachrome staining was performed to assess tissue structure. CD31 immunohistochemistry and Prussian blue staining were performed to assess vasculature. Low magnification images (4.2x) were taken on the whole sections using the Olympus FSX100 (Olympus) and merged in Adobe Photoshop using photo-merge and auto-blend layer functions.

Quantitation of histological images

Histological evaluation was blinded to eliminate any bias in assessing the tissue outcomes. For measurements of bone formation, resorption and vascularization, n=4 separate RCUs per group were analyzed. The total numbers of view fields (10X magnification, 52.74 mm^2 area) were n=56 for the acellular scaffold group, and n=90 for the tissue engineered bone group.

The amount of scaffold material, perimeter length, and resorbing surface were quantified using Movat's pentachrome stain, by built-in features of ImageJ. For each

sample, the images were pooled, randomized, and blindly quantified. The scaffold contour was drawn and the scaffold area and perimeter of each image were determined using ImageJ. The area of the resorbing scaffold was determined by connecting the outermost bite marks in the vicinity of osteoclasts, and the length of the perimeter was measured in ImageJ and normalized by the image area. The percent scaffold area was obtained as a ratio of the area of the remaining scaffold and the total image area. The percent resorbing surface was calculated for each image as a ratio of the resorbing surface and the total scaffold perimeter.

The number of vascular lumens was counted from CD31 immunohistochemistry images. The same numbers of view fields (n = 56 for acellular scaffolds, n=90 for tissue-engineered bone) were randomly selected in each of the 4 samples per group and evaluated at 10X magnification using ImageJ.

SUPPLEMENTARY FIGURES

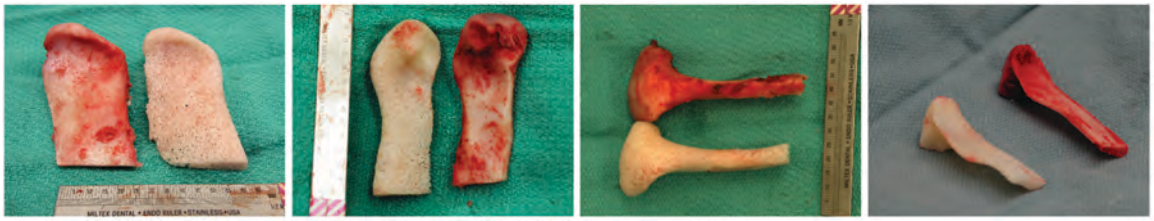

Figure S1. Structural comparison of tissue engineered graft and native explant. Tissue engineered bone grafts (off-white tissue) anatomically matched the native RCU explants (tissues with blood). The four images obtained from four animal subjects are representative of implanted grafts. Images from left to right show the lateral, medial, caudal, and angle view.

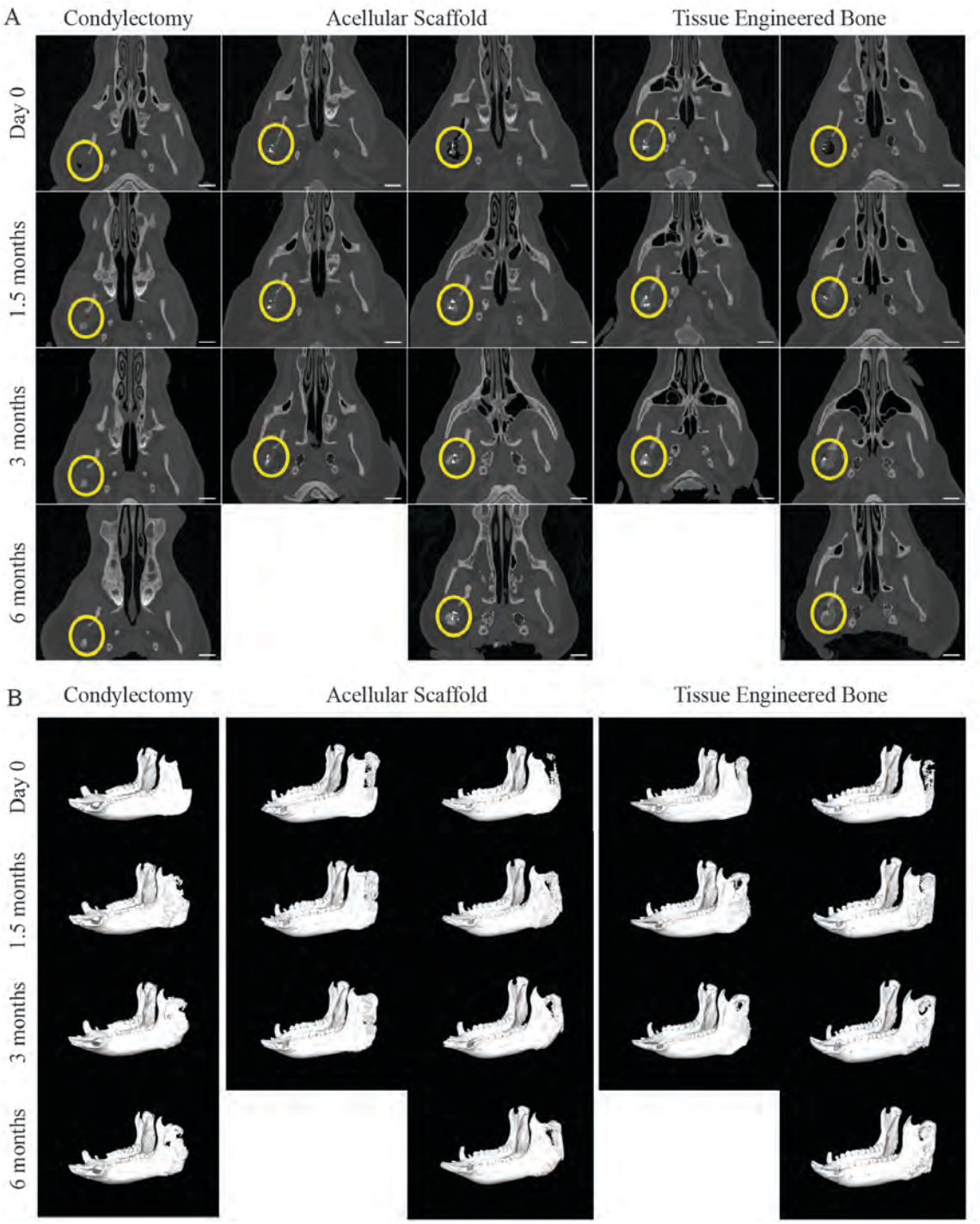

Figure S2. Regeneration of ramus condyle unit. (A) CT slice images show the defect region (yellow circle) over 3 to 6 months post implantation. Images are representative of condylectomy control group (n=2 animals) and the two experimental groups (n=6 animals each). The images are shown with the proximal end at the top, and distal end at the bottom. Scale bars: 2 cm. (B) Additional sequential 3D reconstructed images of porcine mandible for all groups.

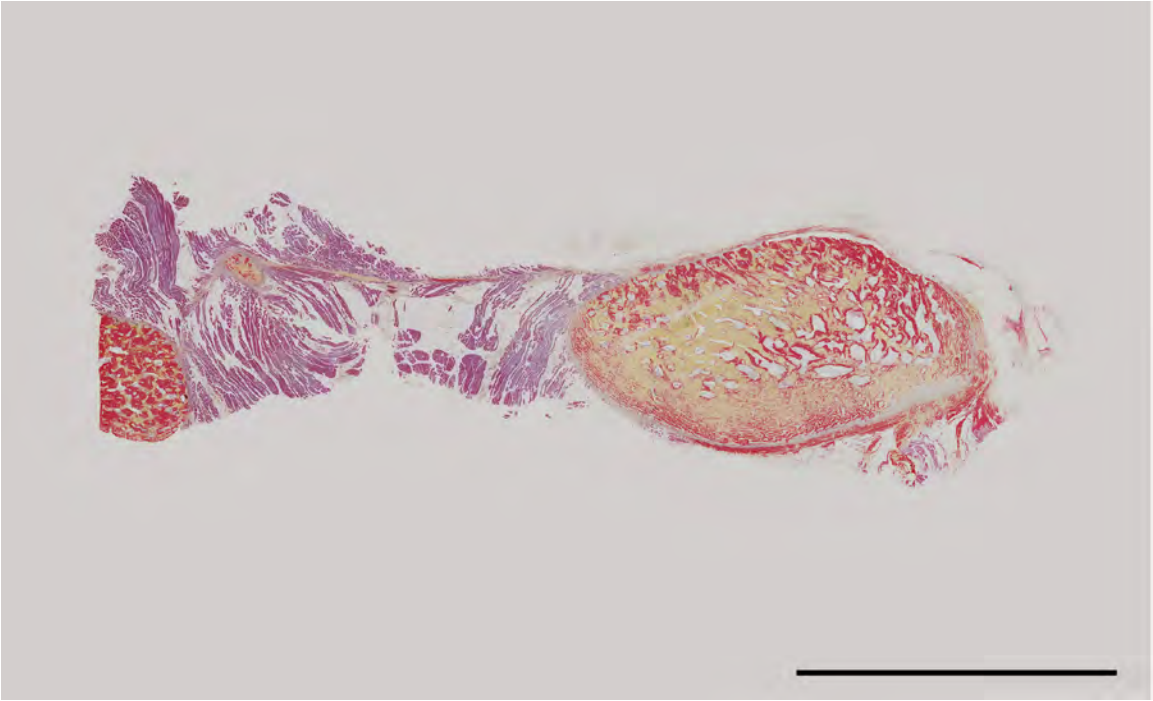

Figure S3. High-resolution Movat's pentachrome stain of the ramus in the condylectomy group at 6 months. Representative image is shown (n = 2 animals). Scale bar: 1 cm.

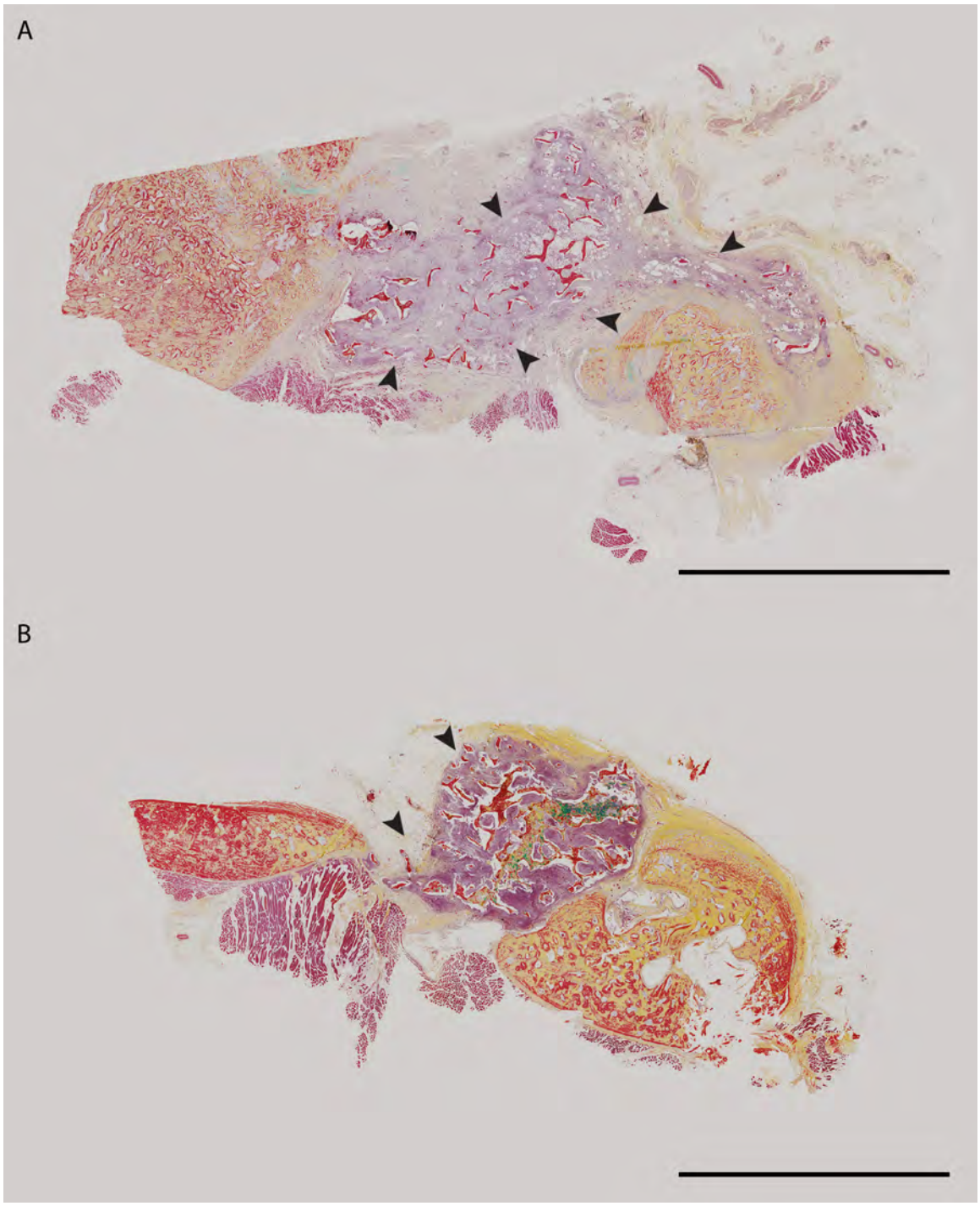

Figure S4. High-resolution Movat's pentachrome stain of the ramus in the acellular scaffold group. Representative images are shown at (**A**) 3 months post implantation (n=2 animals) and **(B)** 6 months post implantation (n=4 animals). Arrows indicate the resorbed scaffold. Scale bars, 1 cm.

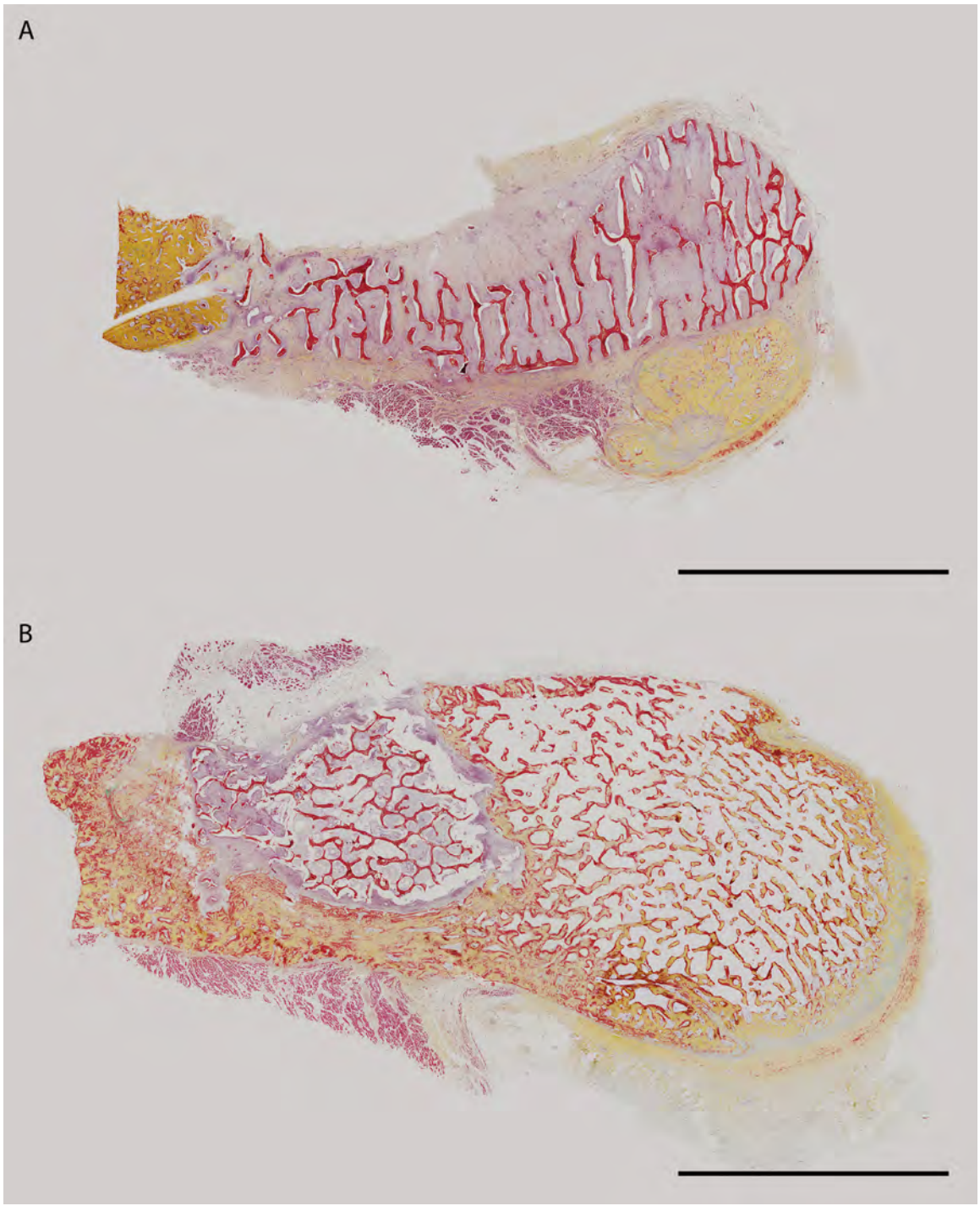

Figure S5. High-resolution Movat's pentachrome stain of the ramus in the tissue engineered bone group. Representative images are shown at (**A**) 3 months post implantation (n=2 animals) and **(B)** 6 months post implantation (n=4 animals). Arrows indicate the resorbed scaffold. Scale bars, 1 cm.

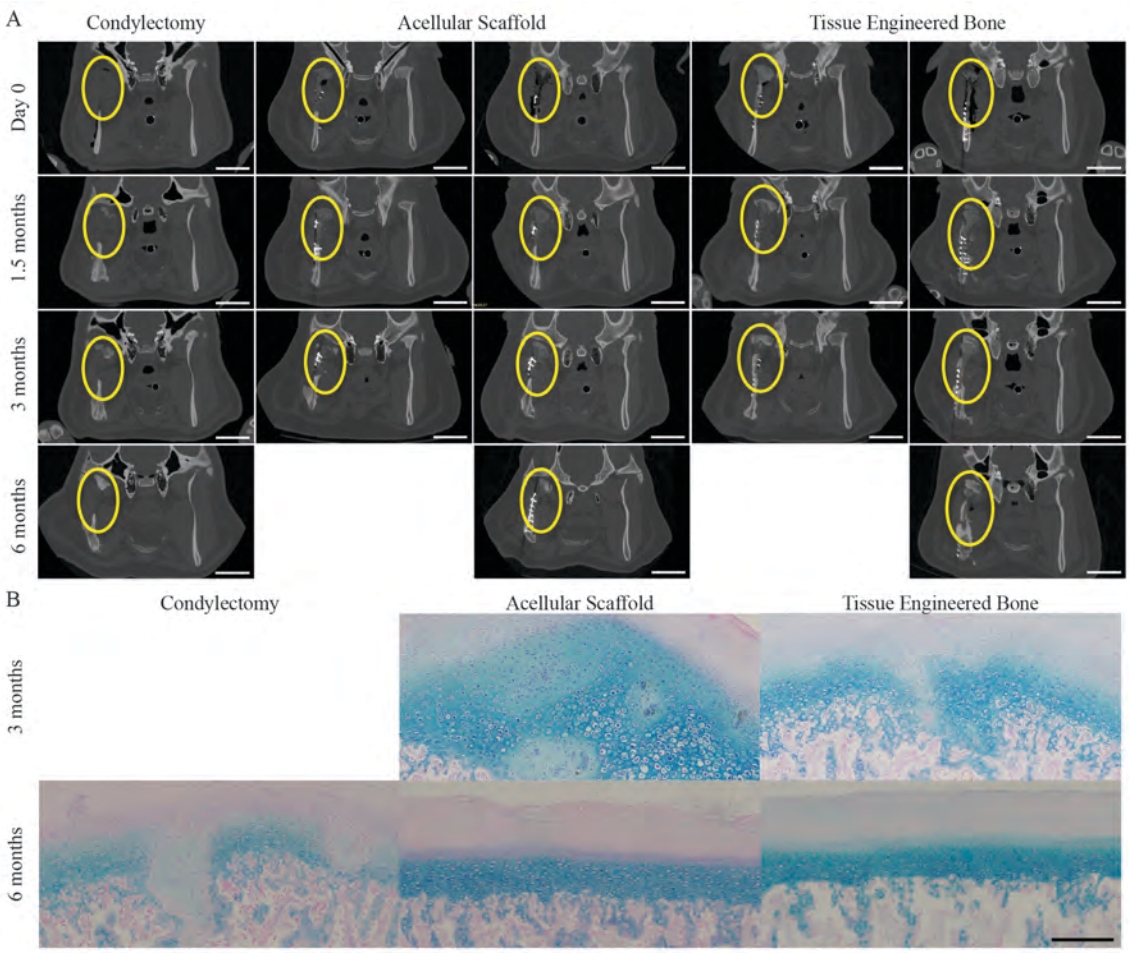

Figure S6. Condyle regeneration (posterior view and histologies). (**A**) CT images of the defect region (yellow circle) over 3 or 6 months of the study. Representative images are shown for the condylectomy control group (n=2 animals) and the two experimental groups (n=6 animals each). (**B**) Alcian blue staining of the cartilage region in a representative tissue from each group at 3 and 6 months post implantation. Scale bars: 4 cm (**A**) and 250 μm (**B**).

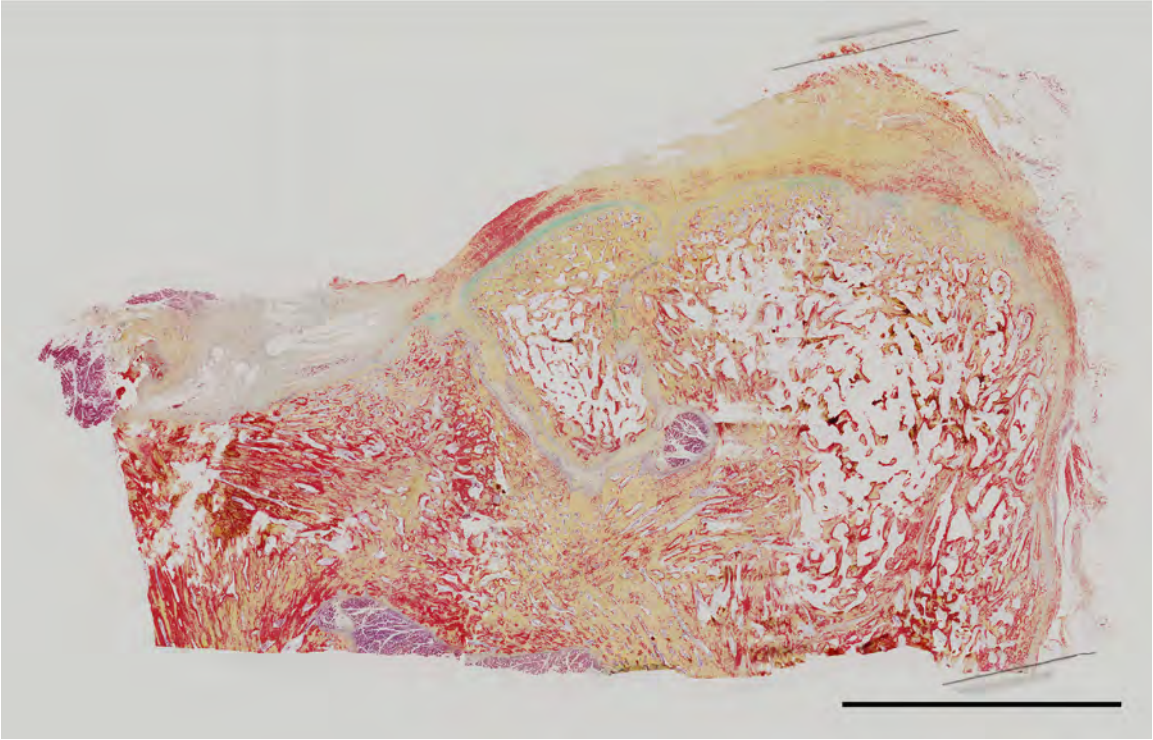

Figure S7. High-resolution Movat's pentachrome stain of the condyle in the condylectomy group at 6 months. Image is representative of n = 2 animals. Scale bar: 1 cm.

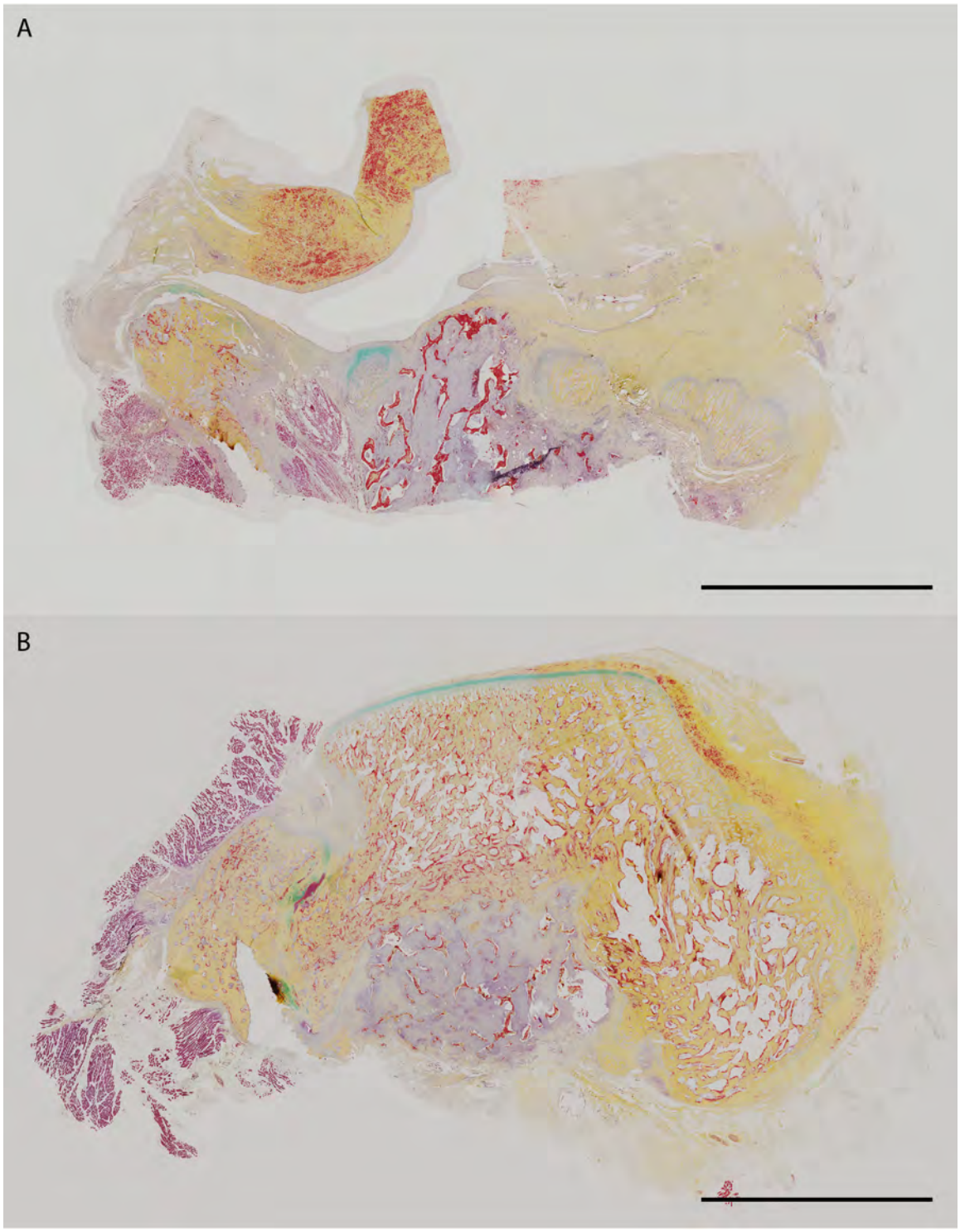

Figure S8. High-resolution Movat's pentachrome stain of the ramus in the acellular scaffold group. (A) 3 months. Image is representative of n = 2 animals. **(B)** 6 months. Image is representative of n = 4 animals. Scale bars: 1 cm.

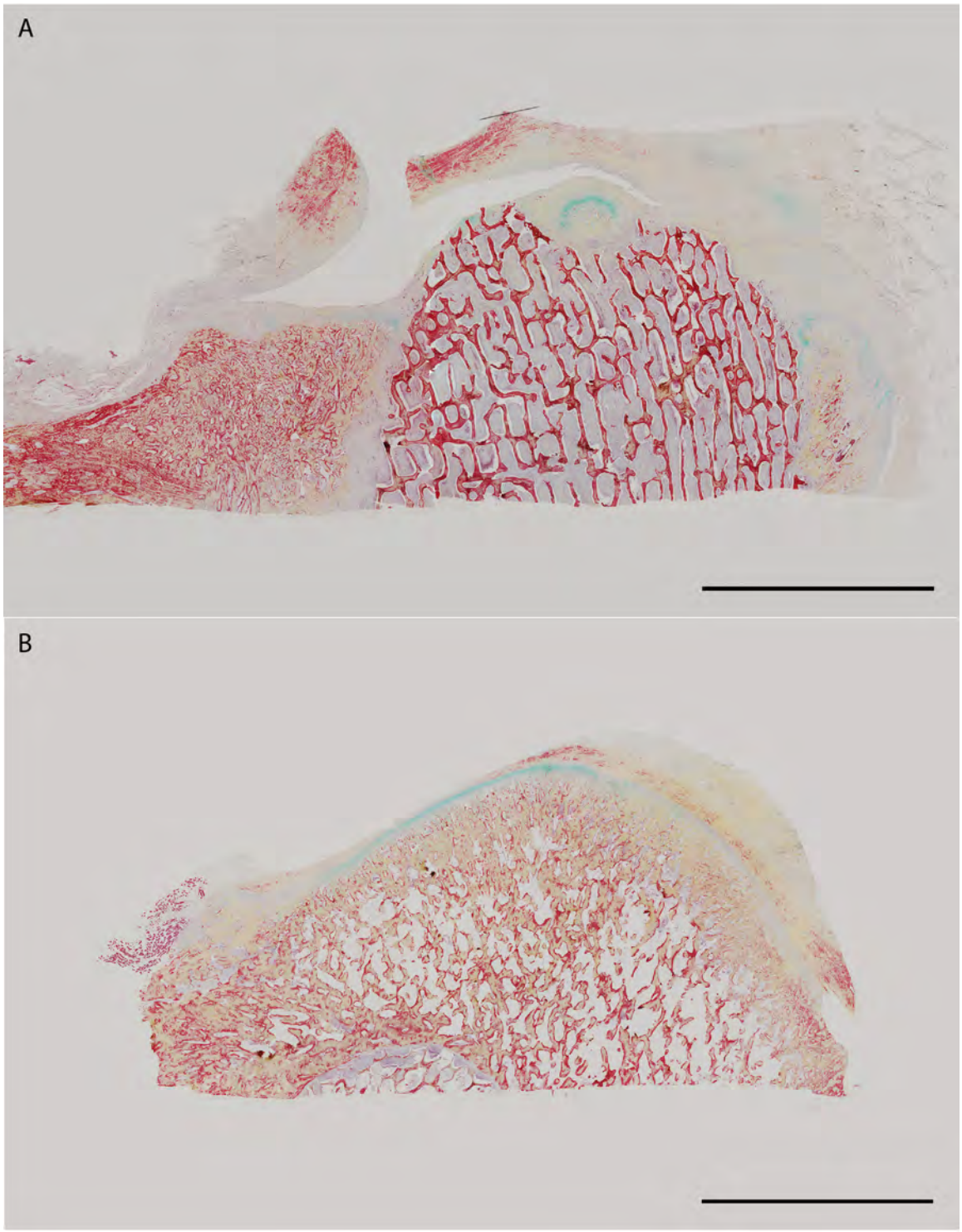

Figure S9. High resolution Movat's pentachrome stain of the ramus in the tissue engineered bone group. (A) 3 months. Image is representative of n = 2 animals. **(B)** 6 months. Image is representative of n = 4 animals. Scale bar: 1 cm.

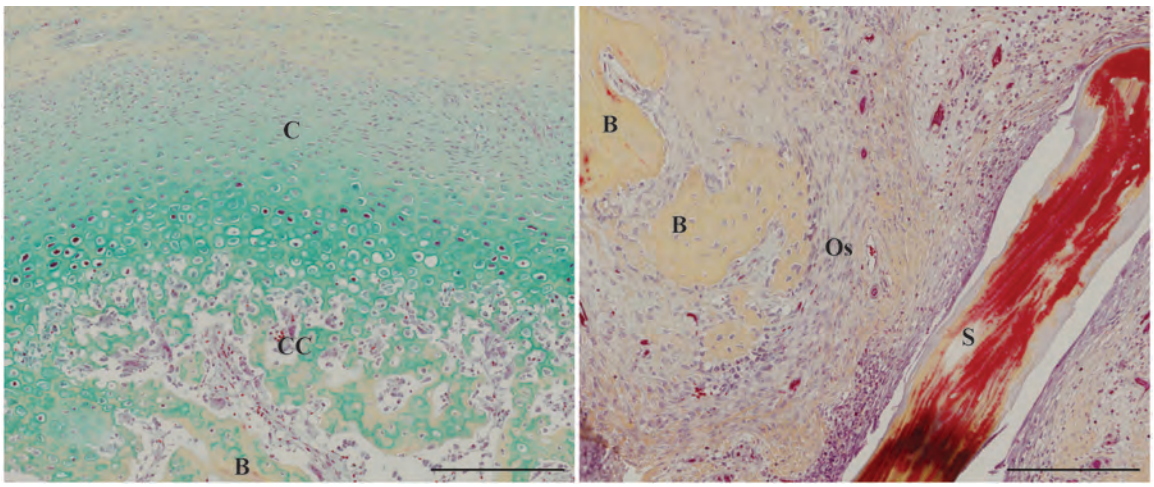

Figure S10. Ossification patterns in the condyle after tissue-engineered bone regeneration. At the articular surface of the reconstructed RCU (left), osteogenesis progressed through endochondral ossification where cartilage (C) preceded calcified cartilage (CC) to form subchondral bone (B). At the ramus region of the reconstructed RCU (right), evidence of intramembranous ossification into the graft and scaffold (S) region was observed. Cells deposited extracellular matrix and underwent ossification (Os) to form new bone (B). Scale bars: 200 μm.

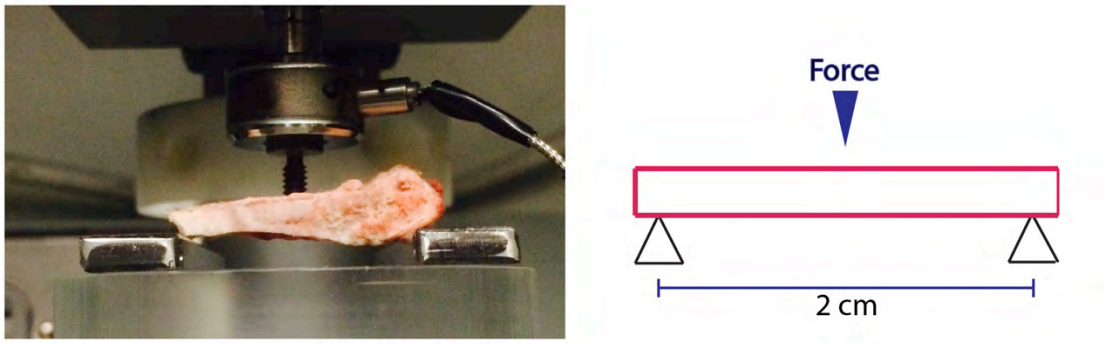

Figure S11. Experimental setup for the three-point bending mechanical testing. Three-point bending was conducted to evaluate the mechanical integrity of the graft-host bone interface. Samples were cut along dorsal plane into approximately 1-cm wide (ventral-dorsal) and 3-cm long (cranial-caudal). The samples were placed on two supports that were 25 mm apart. An actuator applied a load in the exact middle of the two supports onto the samples. A preload of 25 g was applied for 900 s prior to the test. The samples were subjected to a 300-μm displacement at a loading rate of 3 μm/s and then held in place for 1800 s. Force was measured throughout the period of loading.

SUPPLEMENTARY TABLES

Table S1. Porcine RT-PCR primers.

Function	Symbol	Forward Primer (5'->3'	Reverse (5'->3')
Housekeeping	*Gapdh*	GCTTTGCCCCGCGATCTAATGTTC	GCCAAATCCGTTCACTCCGACCTT
Osteogenesis	*Cbfa1*	GAGGAACCGTTTCAGCTTACTG	CGTTAACCAATGGCACGAG
	Alpl	ATGAGCTCAACCGGAACAA	GTGCCCATGGTCAATCCT
	Col1a1	AGAAGAAGACATCCCACCAGTCA	CCGTTGTCGCAGACACAGAT
	Ibsp	GCACGCCTACTTCTATCCTC	CGGCCTCGGAGTCTT
	Osterix	CGATGAGCTGGTCACCGATT	CTTCGGATCTGCGGAACTTCT
	Sparc	TCCGGATCTTTCCITTGCTTTCTA	CCTTCACATCGTGGCAAGAGTTTG
Growth factors	*Bmp2*	TCACCTGAACTCCACGAATC	GTGCTAACGACACCCACAAC
	Bmp4	CAACAGGCTTTGGGGATAC	GAATGACTGGATTGTGGCTC
	Pdgf	AACGTCCTCCTGGCACAAGG	ACTTCACCGGGAGGAAAGTA
	Tgfb2	TCCAAAGATTTAACATCTCCAACC	TCCCACTGTTTTTTTCCTAGTGG
Inflammatory	*Igf*	TGGAGAGATTGCAGATGGC	TGAAGACTCCGTCCTTGAGG
	Il1b	GGCCGCCAAGATATAACTGA	GGACCTCTGGGTATGGCTTTC
	Tnfa	CAGCCTCTTCTC CTTCCT	CGATGATCTGAG TCCTTGG

SUPPLEMENTARY VIDEOS

Movie S1. Assembly of bioreactor. Scaffold is inserted between the two halves of the PDMS mold and sandwiched between fluid-routing manifold. The whole assembly is sealed by inserting into the tightly fitting tubular case..

Movie S2. Finite element modeling of oxygen concentration. Modeling is shown for more than 10 hours of transport of tissue-engineered bone graft without medium perfusion. The minimum oxygen concentration at 10 hours was 0.14 mol/m^3.