Supplementary Materials and Methods

Mice and rats

3-month-old male C57BL/6J (WT) mice and Lewis rats were purchased from Charles River. The anterior cruciate ligament was transected to induce abnormal mechanical loading-associated osteoarthritis of the left knee. Sham operation was done by opening the joint capsule and then suturing the incision in the left knee of independent rodents. We divided the mice into 3 groups: sham-operated, vehicle-treated and halofuginone treated groups (n=30 per group, 6 on day 0, 14, 30 and 60 separately; with another 6 in each group specifically for angiography). Beginning the second day after surgery in mice, halofuginone (sc-211579, Santa Cruz Biotechnology) of 1 mg/kg body weight or equivalent volume of vehicle (DMSO and PBS) was injected intraperitoneally every other day for 30 d. Mice were sacrificed at 14, 30 and 60 d post operation. For rat experiments, after the ACLT surgery, Halofuginone (2.5 ng) embedded in an alginate bead was implanted in subchondral bone canal with the depth of 2 mm made in the medial plateau using a 20G needle. The canal was closed with bone wax. Rats were euthanized 3 months after surgery (n=6 per group). All mice and rats were maintained in the Animal Facility of the Johns Hopkins University School of Medicine. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University, Baltimore, MD, USA.

Western blot and Real-time PCR

Mouse adult MSCs were acquired from the Texas A&M Health Science Center College of Medicine Institute (College Station). MSCs were cultured (Passage 3-5) in Iscove’s Modified
Dulbecco’s Medium (Invitrogen), supplemented with 10% FBS (Atlanta Biologicals), 10% horse serum (Thermo Scientific) and 1% penicillin-streptomycin (Mediatech) to a cell density of 1.8 x 10^5. Cultured MSCs were treated with halofuginone for 4 h (10, 100 or 1000 nM), 8 h (100 nM) and 12 h (100 nM) and then exposed to TGF-β1 for 30 min after 4h starvation and processed for Western Blot. Cells were lysed and centrifuged. Cell lysates supernatants were separated using SDS-PAGE and blotted on polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). Proteins were analyzed for Smad2 (Cell Signaling Technology Inc., 1:1,000, 3103) and pSmad2 (Cell Signaling Technology Inc., 1:1,000, 3108) and visualized by an enhanced chemiluminescence kit (Amersham Bioscience). For real-time PCR, total RNA was extracted from articular cartilage in different groups with TRIzol reagent (Invitrogen). cDNA synthesis was performed with TaqMan Reverse Transcription reagent (Applied Biosystems, Foster, CA). PCR was performed with TaqMan Gene Expression assays (Applied Biosystems). The following primers were used: Actin: forward 5’- AGAAAATCTGGCACCACACC-3’ and reverse 5’-CAGAGGCGTACAGGGGATAGC-3’; Collage type II: forward 5’-CACACTGGTAAGTGGGGCAACCGG-3’ and reverse 5’-GGATTGTGTTGTTTCAGGGTTCGGG-3’; Aggrecan: forward 5’-CAGTGCGATGCAGGCTGGCT-3’ and reverse 5’-CCTCCGGCACTCGTTGGCCTG-3’; ADAMTS 5: forward 5’-GGCATCATTATGCTGACACC-3’ and reverse 5’ –CGAGTACTCAGGCAATG-3’. Values were normalized to actin mRNA levels.

**Histochemistry, immunohistochemistry and histomorphometry analysis**

Knee joints of mice and rats were dissected, fixed in 10% buffered formalin for 48 h, and decalcified in 10% EDTA (pH 7.4) for 3 weeks. Specimens were embedded in either paraffin or optimal cutting temperature (OCT) compound (Sakura Finetek). 4-µm-thick longitudinal-oriented
sections of the knee joint medial compartment were cut and processed for H&E and Safranin O and fast green staining. For the measurement of the thickness of the calcified cartilage, we used 10x modified images. Hyaline cartilage was separated from calcified cartilage by the tidemark line. We measured the distance from the tidemark to subchondral bone plate (SBP) as the thickness of calcified cartilage, and the distance from the tidemark to articular cartilage surface as the thickness of hyaline cartilage. Tartrate-resistant acid phosphatase (Trap) staining was conducted using a standard protocol (Sigma-Aldrich). Sagittal sections of knee joint medial compartment were incubated with primary antibodies against CD4 (Santa Cruz Biotechnology Inc., 1:50, sc-19642), IL-17 (Santa Cruz Biotechnology Inc., 1:50, sc-7927), RANKL (Santa Cruz Biotechnology Inc., 1:50, sc-7628), nestin (Aves Labs, Inc., 1:300, lot NES0407), osterix (Abcam, 1:600, ab22552), pSmad2/3 (Santa Cruz Biotechnology Inc., 1:50, sc-11769), CD31 (Abcam, 1:100, ab28364), endomucin (Santa Cruz, V.7C7, 1:50), Ki67 (Novus Biologicals, NB500-170, 1:50), MMP-2 (Santa Cruz Biotechnology Inc., 1:50, sc-10736), MMP13 (Abcam, 1:40, ab3208), collagen X (Abcam, 1:80, ab58632) and lubricin (Santa Cruz Biotechnology Inc., 1:50, sc-98454) at 4 °C overnight. For immunohistochemical staining, we used a horseradish peroxidase-streptavidin detection system (Dako) and counterstained with hematoxylin (Dako) or methyl green (Sigma-Aldrich). For immunofluorescence staining, second antibodies conjugated with fluorescence were incubated for 1 h at room temperature (RT) while avoiding light. We then microphotographed slices to perform histomorphometric measurements on the entire area of the tibia subchondral bone (Olympus DP71). The number of positively stained cells were counted in the whole tibia subchondral bone area per specimen and five sequential specimens per mouse in each group were measured. Quantitative analysis was conducted in a blinded fashion with OsteoMeasureXP software (OsteoMetrics, Inc.). Osteoarthritis Research Society International-modified Mankin
criteria (OARSI) scores were calculated as previously described.45

Flow cytometry
C57BL/6J mice were assigned into 3 groups: (1) sham-operated, (2) ACLT-operated + vehicle-treated (DMSO and PBS) and (3) ACLT-operated + halofuginone-treated (sc-211579, Santa Cruz Biotechnology). Two weeks and 1 month post-surgery, the mice were euthanized and the tibial subchondral bone marrow and blood were harvested. Red blood cells were lysed using commercial ammonium-chloride-potassium lysis buffer (Quality Biological, Inc.) and centrifuged at a velocity of 1,200 r.p.m. for 5 min at room temperature. Cell pellets were resuspended and fixed with 4% paraformaldehyde. Cells were washed with 0.1% BSA in PBS and counted, recovering 3 x 10^6 cells on average per specimen for both bone marrow and blood. We then permeabilized the cells in 0.1% Triton X-100 before blocking in 3% FACS buffer (PBS, 3% FBS and 0.1% NaN₃ sodiumazide) for 30 min on ice. All staining was performed with fluorophore-conjugated primary and isotype control antibodies. The cells were incubated with Alexa Fluor 488-conjugated antibody against CD4 (eBioscience Inc., 1:100, 53-0041) and eFluor 660-conjugated antibody against IL-17 (eBioscience Inc., 1:50, 50-7177) or isotype control antibody for 1 h at 37 °C in a dark room and then washed twice with 0.1% BSA in PBS. The cells were analyzed immediately after washing with 3% FACS buffer. Data were obtained using CellQuest software on a FACS Calibur flow cytometer (Becton Dickinson). Data were analyzed and all flow cytometry contour plots (with outliers) were generated using FlowJo software (TreeStar).

Micro-CT analysis
Intact knee joints were dissected free of soft tissue and fixed in 70% ethanol overnight. Specimens
were scanned using high-resolution micro-CT (SkyScan 1172) and reconstructed (NRecon v1.6). The data were analyzed using data analysis software (CTAn v1.9) and three-dimensional model visualization software (μCTVol v2.0). We set the scanner at a voltage of 50 kVp, current of 200 μA and resolution of 5.8 μm per pixel. We chose a threshold of 50 based on visual interpretation. Three-dimensional histomorphometric analysis was performed using longitudinal images of the tibial subchondral bone. We defined the region of interest to cover the whole subchondral bone medial compartment. Three-dimensional structural parameters analyzed included: TV (total tissue volume; contains both trabecular and cortical bone), BV/TV (trabecular bone volume per tissue volume), Tb.Th (trabecular thickness), Tb.Sp (trabecular separation), SMI (structure model index), Conn.Dn (connectivity density) and Tb.Pf (trabecular pattern factor).

**CT-based microangiography**

Angiography of microphil-perfused bones was performed to image blood vessels in bone. After the mice were euthanized, the vascular system was flushed with 0.9% normal saline solution containing heparin sodium (100U ml⁻¹) through a needle inserted into the left ventricle. We then pressure fixed the specimen with 10% neutral buffered formalin and washed with heparinized saline solution. Radiopaque silicone rubber compound containing lead chromate (Microfil MV-122, Flow Tech) was then injected. Specimens were stored at 4°C overnight for contrast agent polymerization. We then dissected and harvested the mouse knee joint and soaked them in 10% neutral buffered formalin for 4 d to ensure complete tissue fixation. The bone specimens were decalcified in a formic acid-based solution (Cal-Ex II) for 48 h to facilitate image threshold of the vasculature from the surrounding tissues. Images were acquired using a high-resolution micro-CT imaging system (Skyscan 1172). The scanner was set at a resolution of 9 μm isotropic voxel size.
We chose a threshold of 100 based on visual interpretation of threshold two-dimensional tomograms.

**Statistical analysis**

Data are presented as mean ± s.d. One-way analysis of variance (ANOVA) was used for multifactorial comparisons in this study. Homogeneity of variance was tested first and then the differences between groups were assessed by *post hoc* multiple comparisons. Specifically, if no heterogeneity was observed, the Bonferroni test was used to assess the differences between groups. However, if heterogeneity did exist, the Welch test was used to test the equality of means and the Dunnett’s T3 was used to assess the differences between groups. The investigators were blinded to allocation during experiments and outcome assessment. The level of significance was set at P < 0.05 and indicated by “*” for the comparison between vehicle-treated group and sham group, or “**” for the comparison between halofuginone-treated group and vehicle-treated group; P<0.01 was indicated by “***” for the comparison between vehicle-treated group and sham group, or “###” for the comparison between halofuginone-treated group and vehicle-treated group. All data analysis was conducted with SPSS 22.0 analysis software (SPSS Inc).
**Supplementary Figure 1.** Doses screened of halofuginone in ACLT mice to identify the optimal dose. Safranin O and fast green staining of sagittal views of tibial medial subchondral bone 2 month post-surgery: sham operated (A), ACLT-operated treated with vehicle (B), ACLT-operated treated with halofuginone 0.2 mg/kg (C), 0.5 mg/kg (D), 1 mg/kg (E), 2.5 mg/kg (F). Scale bar, 50µm.

**Supplementary Figure 2.** Treatment with halofuginone protects articular cartilage in ACLT mice. (A) Immunostaining of collagen II (Col II) (left), Aggrecan (middle) and ADAMTS 5 (right) in articular cartilage 30 d post-operation. Scale bar, 100 µm. (B-D) Quantitative analysis of Col II⁺ cells (B), Aggrecan⁺ cells (C) and ADAMTS 5⁺ cells (D) 30 d post-operation. Sham = sham-operated. Vehicle = ACLT-operated treated with vehicle. HF = ACLT-operated treated with halofuginone. n = 6 per group. *P < 0.05 compared to the sham-operated group; **P < 0.01 compared to the sham-operated group; ¤P < 0.05 compared to the ACLT-operated treated with vehicle group; ¤¤P < 0.01 compared to the ACLT-operated treated with vehicle group.

**Supplementary Figure 3.** Alteration of mRNA expression in articular cartilage of ACLT mice using RT-PCR. (A-C) Fold changes of mRNA expression regarding Collagen type II (Col II) (A), Aggrecan (B) and ADAMTS 5 (C) in different group and different time points. Sham = sham-operated. Vehicle = ACLT-operated treated with vehicle. HF = ACLT-operated treated with halofuginone. n = 6 per group. *P < 0.05; **P < 0.01.

**Supplementary Figure 4.** Local administration of halofuginone in subchondral bone attenuates articular cartilage degradation in rats after ACLT surgery. (A) Safranin O and fast green staining
(top) and three dimensional micro-CT images (bottom) of sagittal views of rat tibial medial subchondral bone 3 months post operation. (B-E) Quantitative analysis of subchondral bone microarchitecture parameters of trabecular pattern factor (Tb.pf) (B), connectivity density (Conn. D) (C), subchondral bone plate thickness (SBP Th) (D) and total tissue volume (TV) (E). Sham = sham-surgery. Vehicle = ACLT-surgery treated plus locally embedded alginate beads with vehicle. HF = ACLT-surgery plus locally embedded alginate beads with halofuginone. 1D11 = ACLT-surgery plus locally embedded alginate beads with TGF-β antibody. n = 6 per group. *P < 0.05 compared to the sham-operated group; **P < 0.05 compared to the sham-operated group; #P < 0.05 compared to the ACLT-operated treated with vehicle group; ##P < 0.01 compared to the ACLT-operated treated with vehicle group.
**Supplementary Figure 5.** Immunostaining of protein expression alterations in rat tibial medial subchondral bone 3 months post-operation: MMP 13 (A-Left), Collagen X (Col X) (A-Right). Scale bar, 100 µm. (B-C) Quantitative analysis for MMP13 (B) and Col X (C) positive cells in rat tibial subchondral bone 3 months post operation. (D) Osteoarthritis Research Society International (OARSI)-modified Mankin scores of rat knee joint 3 months after surgery. Sham = sham-surgery. Vehicle = ACLT-surgery treated plus locally embedded alginate beads with vehicle. HF = ACLT-surgery plus locally embedded alginate beads with halofuginone. 1D11 = ACLT-surgery plus locally embedded alginate beads with TGF-β antibody. n = 6 per group. *P < 0.05 compared to the sham-operated group; #P < 0.05 compared to the vehicle-treated ACLT group.

**Supplementary Figure 6.** Alteration of CD4^+IL17^+ cells in the subchondral bone during the onset of OA in ACLT mice. (A, B) Flow cytometry analysis of CD4^+IL17^+ cells in subchondral bone marrow and peripheral blood 2 weeks post-operation. No significant differences were viewed. (C, D) Flow cytometry analysis of CD4^+IL17^+ cells in subchondral bone marrow and peripheral blood 1 month post operation. No significant differences were viewed. Sham = sham-operated. Vehicle = ACLT-operated treated with vehicle. HF = ACLT-operated treated with halofuginone. n = 6 per group.
Supplementary Figure 7. Time-dependent alteration of Th17 cells in subchondral bone during the onset of OA in ACLT mice. (A) Representative immunofluorescence double staining for CD4 (green) and IL17 (red) 0, 14, 30 and 60 days post-surgery. CD4^+IL-17^+ co-localization represented in merged image by yellow. Scale bar, 50 µm. (B) Quantitative analysis of the number of CD4^+IL17^+ (Th17) cells per bone marrow area (mm^2). (C) Flow cytometry analysis of double staining Th17 (CD4^+IL17^+) cells in subchondral bone marrow 2 weeks and 1 month post operation. Sham = sham-surgery. Vehicle = ACLT-surgery treated with vehicle. HF = ACLT-surgery treated with halofuginone. n = 6 per group. ^p < 0.05 compared to the sham-operated group.

Supplementary Figure 8. Representative immunofluorescence staining for MMP-2 (red) in ACLT mice 30 days after surgery. Scale bar, 50 µm. Sham = sham-surgery. Vehicle = ACLT-surgery treated with vehicle. HF = ACLT-surgery treated with halofuginone.