EXTENDED REPORT

Halofuginone attenuates osteoarthritis by inhibition of TGF-β activity and H-type vessel formation in subchondral bone

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ABSTRACT
Objectives Examine whether osteoarthritis (OA) progression can be delayed by halofuginone in anterior cruciate ligament transection (ACL) rodent models.

Methods 3-month-old male C57BL/6J (wild type; WT) mice and Lewis rats were randomised to sham-operated, ACLT-operated, treated with vehicle, or ACLT-operated, treated with halofuginone. Articular cartilage degeneration was graded using the Osteoarthritis Research Society International (OARSI)-modified Mankin criteria. Immunostaining, flow cytometry, RT-PCR and western blot analyses were conducted to detect relative protein and RNA expression. Bone micro CT (μCT) and CT-based microangiography were quantitated to detect alterations of microarchitecture and vasculature in tibial subchondral bone.

Results Halofuginone attenuated articular cartilage degeneration and subchondral bone deterioration, resulting in substantially lower OARSI scores. Specifically, we found that proteoglycan loss and calcification of articular cartilage were significantly decreased in halofuginone-treated ACLT rodents compared with vehicle-treated ACLT controls. Halofuginone reduced collagen X (Col X), matrix metalloproteinase-13 and A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS 5) and increased lubricin, collagen II and aggrecan. In parallel, halofuginone-attenuated uncoupled subchondral bone remodelling as defined by reduced subchondral bone tissue volume, lower trabecular pattern factor (Tb.pf) and increased thickness of subchondral bone plate compared with vehicle-treated ACLT controls. We found that halofuginone exerted protective effects in part by suppressing Th17-induced osteoclastic bone resorption, inhibiting Smad2/3-dependent TGF-β signalling to restore coupled bone remodelling and attenuating excessive angiogenesis in subchondral bone.

Conclusions Halofuginone attenuates OA progression by inhibition of subchondral bone TGF-β activity and aberrant angiogenesis as a potential preventive therapy for OA.

INTRODUCTION

Osteoarthritis (OA), characterised by articular cartilage degeneration, joint pain and functional impairment, affects nearly 27 million people in the USA alone.1–2 There is no effective disease-modifying treatment for OA until the end stage of disease necessitating joint replacement.3–4 Despite the identified risk factors, for example, mechanical, metabolic or genetic, the exact pathogenesis of OA is still unclear5 and remains an active area of investigation as targets for preventive and disease-modifying therapies are greatly needed.

The aetiology of OA is multifactorial and includes intrinsic and extrinsic factors that propagate a multitude of cellular responses.6 The resultant phenotype includes articular cartilage degeneration, subchondral bone sclerosis and oedema, osteochondral angiogenesis, inflammation and osteophyte formation.6–8 Changes in the subchondral bone microarchitecture have been described to precede articular cartilage damage in OA.9–13 Articular cartilage and subchondral bone form a functional unit in the joint.14,15 Articular cartilage acts as a bearing, while subchondral bone acts as a structural girder and shock absorber.16 Subchondral bone, separated by the cement line from the calcified zone of the articular cartilage, consists of the subchondral bone plate (SBP) and the subarticular spongiosa.17 The architecture of subchondral bone and plate adapt via modelling and remodelling in response to mechanical stress.8,17 Coupled bone remodelling ensures the integrity of the subchondral bone, where osteoclast and osteoblast activity are temporally and spatially regulated. Specifically, osteoclasts resorb bone and generate a bone marrow microenvironment that coordinates the migration and differentiation of cells to support angiogenesis and osteogenesis for subsequent osteoblast bone formation.18–21

Following an acute injury, such as anterior cruciate ligament tear, osteoclast bone resorption dramatically increases.22–24 The subchondral bone marrow microenvironment changes substantially and results in woven bone and angiogenesis. We have previously found that excessive activation of TGF-β1 by elevated osteoclast bone resorption uncouples bone resorption and formation, contributing to the sclerotic phenotype in the subchondral bone in OA animal models.16,21 Specifically, high levels of TGF-β result in erroneous recruitment of mesenchymal/stromal stem cells (MSCs) and formation of osteoid islands. The progression of OA could be attenuated, but not completely abrogated, by inhibiting TGF-β1 signalling.25 Vascularisation and innervation of articular cartilage have also been noted in OA, with blood vessels and nerves originating from subchondral bone and breaching the

tide mark in the early stages. A specific subtype of vessels, termed H-type vessels and defined by high costaining for CD31 and endomucin (CD31 hiEmcn hi), has been identified to couple angiogenesis and osteogenesis. A therapy that is able to target the multiple pathological changes in subchondral bone would be desired.

The small molecule halofuginone (HF) is an analogue of febrifugine, which was isolated from the plant Dichroa febrifuga in ancient Chinese herbal medicine for the treatment of malarial fever. HF has shown therapeutic promise in clinical trials for fibrotic diseases, such as scleroderma and chronic graft-versus-host disease by inhibiting phosphorylation of Smad2/3 and TGF-β-mediated collagen type I synthesis. HF has also been reported to inhibit the differentiation of the CD4 + T helper cell subset, Th17, elucidating beneficial effects in an autoimmune arthritis mouse model. Th17 functions as an osteoclastogenic CD4+ helper T cell subset that links T cell activation and bone destruction. Th17 cells produce interleukin (IL) 17, inducing the expression of receptor activator of nuclear factor-κB ligand (RANKL) to promote osteoclastogenesis. TGF-β is essential for the initiation of Th17 differentiation. HF has also been shown to induce antiangiogenic effects in preclinical studies at several essential stages of angiogenesis, largely through inhibition of matrix metalloproteinase-2 (MMP-2). As increased CD4 + T cell subsets, high TGF-β concentrations and angiogenesis have been shown to be involved in the pathogenesis of OA, we investigated the potential effect of HF as a preventive treatment for OA. We found that HF could attenuate progression of OA by delaying articular cartilage degeneration and subchondral bone sclerosis in rodent anterior cruciate ligament transection (ACLT) models by inhibiting Th17 differentiation, TGF-β-dependent Smad2/3 phosphorylation and angiogenesis.

**MATERIALS AND METHODS**

Three-month-old male C57BL/6J (WT) mice and Lewis rats were purchased from Charles River. Rodents were randomised to sham-operated, ACLT-operated, treated with vehicle or ACLT-operated, treated with HF. We performed histological analysis using Safranin O-fast green and H&E staining and graded articular cartilage degeneration using the Osteoarthritis Research Society International (OARSI)-modified Mankin criteria. Immunostaining, flow cytomert, RT-PCR and western blot analyses were conducted to detect relative protein expression. We quantitated bone micro CT (μCT) and CT-based microangiography parameters to detect the alterations of micro-architecture and vasculature in tibial subchondral bone. A detailed description of the Material and methods can be found in the online supplementary text.

**RESULTS**

HF attenuates progression of OA in ACLT mice

To investigate the effects of HF on disease activity and progression in OA, we administered HF intraperitoneally in mice after ACLT. The optimal dose (1 mg/kg body weight (mg/kg)) was identified using multiple concentrations of HF (0.2, 0.5, 1 or 2.5 mg/kg) injected every other day for 1 month post surgery (see online supplementary figure S1). Lower concentration (0.2 or 0.5 mg/kg) had minimal effects on subchondral bone and higher concentration (2.5 mg/kg) induced proteoglycan loss in articular cartilage. Specifically, Safranin O staining demonstrated retention of proteoglycan and decreased thickness of calcified cartilage after anterior cruciate ligament transection (ACLT) models by inhibiting Th17 differentiation.

![Figure 1](http://ard.bmj.com/annrheumdis-2015-207923.onlin...2015.10.1136/annrheumdis-2015-207923)
cartilage zone (from the tidemark line to SBP) in HF-treated ACL T mice (1 mg/kg) relative to vehicle-treated ACL T controls (figure 1A and table 1). HF normalised expression of lubricin, MMP-13 and collagen X (Col X), collagen II, aggrecan and A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS 5) as assessed by immunostaining and RT-PCR in the HF-treated ACL T mice relative to sham controls (figure 1B–E and online supplementary figures S2 and S3). OARSI scores were improved in HF-treated ACL T mice relative to vehicle-treated ACL T controls, whereas no difference was noted in HF versus sham controls (figure 1F).

HF sustains coupled subchondral bone remodelling

The effect of HF on the structure of tibial subchondral bone was analysed by μCT. HF significantly reduced the tibial subchondral bone tissue volume (TV), lowered trabecular pattern factor (Tb.pf) and increased SBP thickness post ACLT relative to vehicle treatment (figure 2A–D). There was no statistically significant difference in TV, Tb.pf or SBP thickness between the HF-treated ACL T mice and sham controls. Consistently, the number of tartrate-resistant acid phosphatase-positive osteoclast cells and osteoprogenitor osterix-positive cells increased after ACLT (vehicle vs sham) (figure 2E–H). The increase in both cell populations was abrogated by HF treatment (figure 2E–H). Notably, in the vehicle-treated ACL T mice, the majority of osterix-positive cells were found in clusters in subchondral bone marrow compared with localisation predominately on the bone surface in the HF-treated ACL T mice (figure 2G,H).

We also examined the effect of local administration of HF on OA progression in ACL T rats by embedding HF containing alginate beads directly into the tibial subchondral bone. Similar to administration of TGF-β neutralising antibody (1D11) in the

Table 1 Cartilage thickness changes in different group and time-points (10× magnified images; mean±SD; unit: mm)

<table>
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<th>Time (days)</th>
<th>HC Sham</th>
<th>Vehicle</th>
<th>Halofuginone</th>
<th>CC Sham</th>
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<th>Halofuginone</th>
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<td>0.45±0.18*</td>
<td>0.76±0.112*</td>
<td>0.32±0.186</td>
<td>0.65±0.19*</td>
<td>0.35±0.113*</td>
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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.

CC, calcified cartilage; HC, hyaline cartilage.

Figure 2 Halofuginone normalises subchondral bone after anterior cruciate ligament transection (ACLT). (A) Representative three dimensional micro-CT images of sagittal views of subchondral bone medial compartment at 30 and 60 days after sham operation or ACLT surgery. Scale bar, 500 μm. (B–D) Quantitative micro-CT analysis of tibial subchondral bone of total tissue volume (TV) (B), trabecular pattern factor (Tb.pf) (C) and subchondral bone plate thickness (D). (E and F) Tartrate-resistant acid phosphatase (TRAP) staining (E) and quantitative analysis (F) at 14 days after surgery. Scale bar, 50 μm. (G and H) Immunohistochemical staining (G) and quantification (H) of osterix-positive cells (brown) in subchondral bone 30 days after surgery. Scale bar, 100 μm. Sham=sham-surgery. Vehicle=ACLT-surgery treated with vehicle. HF=ACLT-surgery treated with halofuginone. n=6 per group. *p<0.05 compared with sham or as denoted by bar, **<0.01 compared as denoted by bar; †p<0.05 compared with the vehicle.
HF suppresses osteoclastogenesis by decrease of Th17 cells in the subchondral bone of mice

We examined whether HF inhibits osteoclastogenesis through modulation of Th17 cells. Immunofluorescence staining of Th17 specific markers (CD4 and IL17) revealed a significant increase of Th17 cells in the subchondral bone marrow of vehicle-treated mice as early as 2 weeks post surgery, whereas HF-treated mice had equivalent Th17 cells compared with sham controls (figure 3A,B). Consistently, a significant increase of Th17 cells (CD4+IL17+) in the bone marrow 14 days post surgery in vehicle-treated mice was also observed using flow cytometry analysis. HF-treated ACL T mice had significantly decreased Th17 cells, similar to the level of sham controls (figure 3C,D). The number of CD4+IL17+ cells in bone marrow and peripheral blood remained unchanged at different time-points (see online supplementary figure S6). Changes in bone marrow Th17 cell numbers were time-dependent, with Th17 cell numbers decreasing to baseline (0 day) in the ACLT-vehicle treated mice 2 months post surgery (see online supplementary figure S7A-G). No difference in the number of Th17 cells in peripheral blood was noted regardless of vehicle or HF-treated mice relative to sham controls (figure 3C,D).

In parallel with the increased aggregation of Th17 cells in the subchondral bone marrow of vehicle-treated mice, an increase in expression of RANKL was observed in the subchondral bone compared with sham controls (figure 3E,F). HF treatment significantly attenuated RANKL expression with no significant difference noted relative to sham controls (figure 3E,F). The results indicate HF inhibits osteoclastogenesis by decreasing Th17 cells and RANKL expression in the subchondral bone.

HF inhibits Smad2/3-dependent TGF-β signalling pathway in bone marrow MSCs

Immunofluorescence staining of nestin showed that HF significantly attenuated the increase in number of MSCs in the subchondral bone post ACLT relative to vehicle (figure 4A,B). There was no statistical difference between the number of nestin-positive cells in HF-treated ACLT mice relative to sham controls (figure 4A,B). Furthermore, nestin-positive cells were dispersed throughout the bone marrow in vehicle-treated mice compared with the closer proximity to the bone surface in the HF-treated mice (figure 4A). As high active TGF-β recruits MSCs in the subchondral bone marrow, we investigated whether HF could directly inhibit TGF-β signalling in MSCs. Western blot analysis of MSCs revealed that phosphorylation of Smad2 (pSmad2) was inhibited by HF in both a time-dependent and dose-dependent manner.
dependent manner (figure 4C). Immunohistochemistry staining of pSmad2/3 further validated HF inhibition of TGF-β signal-
ing in subchondral bone cells. Specifically, pSmad2/3-positive cells in the subchondral bone of vehicle-treated ACL T mice were significantly increased and attenuated with HF to levels comparable with sham mice (figure 4D,E).

HF abrogates aberrant blood vessel formation in subchondral bone

Finally, we examined the potential effects of HF on subchondral bone angiogenesis. Using CT-based angiography in microphil perfusion, we found the number and volume of blood vessels were significantly increased in the subchondral bone of vehicle-treated ACL T mice. HF inhibited the increase of vessel number and volume in subchondral bone relative to vehicle treatment, retaining vessel number and volume similar to sham controls (figure 5A–C). We further analysed the type of vessels inhibited by HF by performing double immunofluorescence staining for CD31 and endomucin, recently described as H-type vessels.29 CD31hiEmcnhi blood vessels were significantly increased in the subchondral bone of vehicle-treated ACL T mice. HF restored CD31hiEmcnhi blood vessels similar to sham controls (figure 5D,F). Changes in H-type vessels correlated with a similar pattern in endothelial cell proliferation as evaluated by immunostaining and quantification of the number of endomucin-positive cells that costained positive for Ki67 (figure 5E,G). Additionally, MMP-2 levels were statistically increased in vehicle-treated ACL T mice, whereas the HF-treated ACL T mice had similar MMP-2 levels compared with sham controls (figure 5H and online supplementary figure S8).

DISCUSSION

We have shown that HF preserves the subchondral bone micro-
architecture to prevent articular cartilage degeneration by inhibition of Th17-induced osteoclastogenesis, excessive TGF-β activity and H-type vessel formation in recruitment of MSCs for aberrant bone formation. Particularly, the protection of articular cartilage by local administration of HF in the subchondral bone post ACL T in rats further suggests that maintaining the micro-
structural integrity of subchondral bone provides an essential physiological environment for articular cartilage.8 Biochemical and biomechanical interplay between subchondral bone and articular cartilage mediates effects on articular cartilage.14

CD4+ T cell subsets are infiltrated and involved in the patho-
genesis of OA,35 36 including Th17 cells.37 Th17 cells produce IL-17, which, in bone, induces the expression of RANKL from osteoclastogenesis-supporting mesenchymal cells to promote osteoclastogenesis.38 39 Therefore, Th17 can be regarded as an osteoclastogenic helper cell, linking T cell activa-
tion with bone destruction.39 We observed, during OA develop-
ment, a significant increase of Th17 cells in the subchondral bone marrow of vehicle-treated mice as early as 2 weeks post surgery by immunofluorescence staining and flow cytometry.
with no significant changes in peripheral blood. We found the increase of Th17 cells in bone marrow was time-dependent, with Th17 cells numbers decreasing by 1 month and returning almost to baseline (0 day) by 2 months post surgery. We observed significantly increased expression of RANKL in the bone marrow in a similar time distribution. These results support that Th17 cells are involved in the onset of OA by promoting osteoclast bone resorption. The increase of Th17 cells (CD4+IL17+) and RANKL expression in bone marrow was inhibited by HF, indicating that HF inhibited osteoclastogenesis. HF is known to inhibit Th17 differentiation via inhibition of TGF-β signalling and activating the amino acid response pathway. TGF-β can directly and indirectly initiate Th17 differentiation. In intestinal cells, TGF-β has been shown to increase expression of Runx1, which is necessary for Th17 differentiation. TGF-β can also block the signalling pathways that promote Th1 and Th2 differentiation. TGF-β can also inhibit Th17 differentiation via activating the amino acid response pathway by suppressing prolyl-transfer RNA synthetase (ProRS) to induce uncharged tRNA accumulation within cells. HF directly binds onto two different binding sites of ProRS via an ATP-dependent mechanism.

Abnormal TGF-β signalling-induced uncoupled subchondral bone remodelling precedes articular cartilage degeneration in ACLT OA mice. HF likely maintains coupled bone remodelling through modulation of TGF-β activity. During coupled bone remodelling, TGF-β is released and activated during osteoclast bone resorption. Smad2/3-dependent TGF-β signalling pathway induces migration of MSCs from their perivascular niche to the bone surface for osteoblast differentiation. However, during OA development, excessive release and activation of TGF-β will interrupt coupled bone remodelling, recruiting MSCs to form aberrant osteoid islets in bone marrow as opposed to bone resorption pits for coupled bone resorption. We found in vehicle-treated ACLT mice that the number of nestin-positive MSCs increased and clustered in bone marrow, indicating uncoupled bone remodelling. HF reduced MSCs numbers and relocated osteoprogenitors from the bone marrow to bone surface, re-establishing coupled bone remodelling. We speculate a combination of two mechanisms elicit this effect. The reduction in osteoclast bone resorption by HF likely reduces TGF-β release from bone matrix.

Abnormal vascular congestion in subchondral bone is a known pathological feature of OA. OA is thought to progress by osteochondral angiogenesis where blood vessels breach the tidemark at the osteochondral junction. HF inhibits angiogenesis through indirectly inhibiting MMP-2-dependent tubular network formation. MMP-2 can degrade structural extracellular matrix by cleaving type IV collagen, the protein backbone of the endothelial basement membrane, then promote

Figure 5  Halofuginone attenuates aberrant angiogenesis in subchondral bone of anterior cruciate ligament transection (ACLT) mice. (A–C) Three dimensional CT-based microangiography of medial tibial subchondral bone (A) 30 days post surgery, with a quantification of vessel volume relative to tissue volume (VV/TV) (B) and vessel number (VN) (C). Scale bar, 500 μm. (D and F) Representative immunofluorescence double staining (D) and quantification (F) of CD31 (green) and endomucin (red) positive cells 1 month after surgery. Scale bar, 50 μm. (E and G) Immunofluorescence double staining (E) and quantification (G) of Ki67 (green) and endomucin (red) positive cells 1 month after surgery. Scale bar, 50 μm. (H) Quantification of MMP-2 positive cells in subchondral bone marrow (BM) 1 month post surgery. Sham=sham-surgery. Vehicle=ACLT-surgery treated with vehicle. HF=ACLT-surgery treated with halofuginone. n=6 per group. *p<0.05 compared with sham and #p<0.05 compared with vehicle.
angiogenesis. The significantly increased expression of MMP-2 in vehicle-treated ACLT mice was normalised with HF. TGF-β signalling in endothelial progenitor cells can also induce angiogenesis. We have previously shown that TGF-β inhibition can reduce angiogenesis in subchondral bone in ACLT OA mice. The normalisation of vessel volume and numbers in ACLT mice treated with HF was thus likely secondary to indirect suppression of MMP-2, direct inhibition of TGF-β signalling or other additional unexplored mechanisms. We further investigated whether the increase in vasculature is from H-type vessels. H-type vessels, defined by high costing for CD31 and endomucin (CD31Emcn), is a specific subtype of vessels that couples angiogenesis with osteogenesis. Building on our prior findings that demonstrated the formation of an ‘osteoid islet’ during OA development, we found the increase in vasculature within the ‘osteoid islet’ was H-type vessels. H-type vessels increased after ACLT, but were equivalent to sham-operated controls when ACLT-mice were treated with HF. These results suggest that HF can attenuate OA progression by prevention of pathological angiogenesis.

The immune system has also been implicated in the pathogenesis of OA. HF has been shown to decrease nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 mitogen-activated protein kinases (MAPK) in activated T cells in vitro with anticipated downstream signalling effects, such as lower interleukin (INF)-γ and tumour necrosis factor α concentrations. This may be an additional mechanism, particularly as HF has shown beneficial effects in an autoimmune arthritis mouse model. More detailed studies are required to comprehensively understand the effects of HF on the immune system, particularly adaptive immunity.

Febirufine has been used in Chinese herbal medicine for more than 2000 years. The small molecule HF, a derivative of febirufine, has been granted orphan drug status for scleroderma and Duchenne muscular dystrophy (DMD). HF has shown therapeutic promise in clinical trials for scleroderma and chronic graft-versus-host disease, and is currently being investigated in effectiveness of reversing muscle fibrosis in DMD. Our findings broaden the potential clinical application of HF. We found that HF-attenuated OA progression by targeting three subchondral bone pathological features in the early OA in rodent ACLT models. HF prevented subchondral bone changes, including reduced Th17-induced osteoclast bone resorption, reduced aberrant bone formation through inhibition of TGF-β signalling and abrogated H-type blood vessel formation. Most importantly, articular cartilage degeneration was attenuated, suggesting that targeting of subchondral bone changes in early OA may be an effective preventive strategy.

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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 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’-CAGAGGCGTACAGGGATAGC-3’; Collage type II: forward 5’-CACACTGTTAAGTGCAAGACCG-3’ and reverse 5’-GGATTGTGTTGTTTCAGGGTTCGGG-3’; Aggrecan: forward 5’-CAGTGCAGTGACGGTGAGTGGGGACAGACC-3’ and reverse 5’-GGATTGTGTTGTTTCAGGGTTCGGG-3’; ADAMTS 5: forward 5’-GGCATCATCTCATGTGACACC-3’ and reverse 5’–CGAGTACTCAGGCGTACAGGGATAGC-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% NaN_3 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 (μ CTVol 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 (mm²). (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.
Supplementary Fig. 2

A  

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B  

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C  

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D  

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<tr>
<td>HF</td>
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Supplementary Fig. 3

A. Relative Col II mRNA expression

B. Relative Aggrecan mRNA expression

C. Relative Adamts 5 mRNA expression
Supplementary Fig. 4

A  Sham  Vehicle  HF  1D11

B  C  D  E

Tb.pf (mm$^{-3}$)  Conn.D (mm$^{-3}$)  SBP Th. (mm)  TV (mm$^3$)

*  #  #  #  *

Sham  Vehicle  HF  1D11  Sham  Vehicle  HF  1D11  Sham  Vehicle  HF  1D11  Sham  Vehicle  HF  1D11
Supplementary Fig. 5

A. MMP13\(^+\) (\%)  
B. COL\(X\) (\%)  
C. OARSI score
Supplementary Fig. 6

A

B

CD4 IL17+ cells (%)

0.0 0.1 0.2 0.3 0.4 0.5

Bone marrow  Blood

CD4 IL17+ cells (%)

0.0 0.1 0.2 0.3 0.4 0.5

Bone marrow  Blood
Supplementary Fig. 7

A

CD4  IL-17  Merge

0 d

14 d

30 d

60 d

B

C

CD4+IL17+/BM (Per mm²)

Th17 cells (%)

Time (d)

Time (d)
Supplementary Fig. 8

MMP-2  DAPI  Merge

Sham

Vehicle

HF

50 µm
Supplementary Table 1

Table 1 Cartilage thickness changes in different group and time-points (10x magnified images; mean ± sd; unit: mm)

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<td>Sham Vehicle Halofuginone</td>
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<td>14 d</td>
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<td>30 d</td>
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<td>60 d</td>
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<td>0.32 ± 0.186 0.65 ± 0.19* 0.35 ± 0.113#</td>
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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.