ORIGINAL ARTICLE

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Positive-Feedback Regulation of Subchondral H-Type Vessel Formation by Chondrocyte Promotes Osteoarthritis Development in Mice

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ABSTRACT

Vascular-invasion-mediated interactions between activated articular chondrocytes and subchondral bone are essential for osteoarthritis (OA) development. Here, we determined the role of nutrient sensing mechanistic target of rapamycin complex 1 (mTORC1) signaling in the crosstalk across the bone cartilage interface and its regulatory mechanisms. Then mice with chondrocyte-specific mTORC1 activation (*Tsc1* CKO and *Tsc1* CKO^{ER}) or inhibition (*Raptor* CKO^{ER}) and their littermate controls were subjected to OA induced by destabilization of the medial meniscus (DMM) or not. DMM or *Tsc1* CKO mice were treated with bevacizumab, a vascular endothelial growth factor (VEGF)-A antibody that blocks angiogenesis. Articular cartilage degeneration was evaluated using the Osteoarthritis Research Society International score. Immunostaining and Western blotting were conducted to detect H-type vessels and protein levels in mice. Primary chondrocytes from mutant mice and ADTC5 cells were treated with interleukin-1β to investigate the role of chondrocyte mTORC1 in VEGF-A secretion and in vitro vascular formation. Clearly, H-type vessels were increased in subchondral bone in DMM-induced OA and aged mice. Cartilage mTORC1 activation stimulated VEGF-A production in articular chondrocyte and H-type vessel formation in subchondral bone. Chondrocyte mTORC1 promoted OA partially through formation of VEGF-A–stimulated subchondral H-type vessels. In particular, vascular-derived nutrients activated chondrocyte mTORC1, and stimulated chondrocyte activation and production of VEGF, resulting in further angiogenesis in subchondral bone. Thus a positive-feedback regulation of H-type vessel formation in subchondral bone by articular chondrocyte nutrient-sensing mTORC1 signaling is essential for the pathogenesis and progression of OA. © 2018 American Society for Bone and Mineral Research

KEY WORDS: OSTEOARTHRITIS; MTORC1; CHONDROCYTE; H-TYPE VESSELS; ANGIOGENESIS

Introduction

Osteoarthritis (OA) is a chronic, incurable, and destructive joint disease and a major cause of pain and disability in the aging population.[1–3] Age, gender, and genetic predisposition are major intrinsic risk factors for OA. The OA disease process affects all joint tissues, and is characterized by progressive degeneration of articular cartilage, vascular invasion of the articular surface, subchondral bone remodeling, osteophyte formation, and synovial inflammation.[1,4] There is no effective medical therapy for OA because of limited understanding of its pathogenesis.[5,6]

Chondrocytes are the only cells found in healthy cartilage, and their primary function is to maintain cartilage homeostasis.[7] During OA development, chondrocytes show aberrant proliferation, hypertrophy, activation, and senescence, accompanied by extracellular matrix degeneration in articular cartilage.[7–9] Although OA was initially thought to be driven only by cartilage degradation,[10,11] pathological processes in subchondral bone and

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synovium are now also considered to be critical for its development. During OA, subchondral bone undergoes sclerosis, angiogenesis, microfracture, and the formation of cysts and osteophytes. Some of these changes are thought to occur in the early stages of OA.[12,13] Progressive cartilage degradation and subchondral bone sclerosis are widely observed in OA. Synovial inflammation is detected in very early OA, while vessel formation is overexpressed in early OA.[1] Furthermore, it has been reported that synovitis and subchondral bone cysts are respectively present in up to 50% and 57% of OA patients.[14,15] However, the mechanisms underlying the activation of normally quiescent articular chondrocytes and the changes in subchondral bone are not fully understood.

Emerging evidence demonstrates that interactions between articular chondrocytes and subchondral bone are essential for the pathological changes during OA development.[16,17] The close physical association and the presence of connections (microfractures, fissures, vascular channels, and blood vessels) between subchondral bone and articular cartilage suggests the biochemical and molecular crosstalk across the interface between healthy and osteoarthritic joints.[18,19] Vascular invasion into articular surface is a hallmark of advanced OA.[20] The formation of new blood vessels not only supplies oxygen and nutrients but also mediates the molecular crosstalk across the bone cartilage interface.[21–23] Emerging evidence demonstrates that perfusion abnormalities in subchondral bone are associated with OA and avascular necrosis.[24] It has been shown that OA hypertrophic chondrocytes potentially express a phenotype promoting angiogenesis and the development of the hypertrophic chondrocyte phenotype in vitro requires the presence of serum (nutrients),[25,26] suggesting that chondrocyte hypertrophy and cartilage angiogenesis are interdependent.[27,28] However, the exact mechanism by which articular chondrocytes sense nutrients and biochemical factors and promote angiogenesis during OA progression is unknown.

Mechanistic target of rapamycin complex (mTORC)1 is a master regulator of cellular growth, proliferation, and metabolism in response to nutrients, growth factors, and stresses.[29] mTORC1 activation may play a vital role in chondrocyte metabolism and OA pathophysiology.[30,31] Both cartilage-specific deletion of mTOR and pharmacological inhibition of mTORC1 decrease the severity of experimental OA in mouse models.[32–34] We recently showed that mTORC1 coordinates chondrocyte proliferation and differentiation during endochondral bone development, and mTORC1 activation stimulates articular chondrocyte aberrant proliferation and differentiation to initiate OA in mice.[35,36] However, how chondrocyte mTORC1 is activated during OA development and whether chondrocyte mTORC1 contributes to osteoarthritic angiogenesis are unknown.

In this study, we demonstrated that vascular-derived nutrients activated chondrocyte mTORC1, and stimulated chondrocyte proliferation and hypertrophic differentiation, and production of vascular endothelial growth factor (VEGF), resulting in further angiogenesis. Thus, we established a novel crosstalk between articular chondrocytes and osteoarthritic subchondral blood vessels via a nutrient-sensing, signaling-mediated, positive feedback mechanism.

Materials and Methods

Animals

All animal experiments were approved by the Southern Medical University Animal Care and Use Committee. The Col2a1-cre mouse line was a generous gift from Dr. Xiao Yang (Academy of Military Medical Sciences, Beijing, China). *Tsc1*$^{fl/fl}$ mice, *Raptor*$^{fl/fl}$ mice, and inducible Col2a1-Cre/ERT mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA; Jax no. 005680, Jax no. 013188, and no. 006774, respectively). Other conditional KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA): floxed *Tsc1* mice (Jax#005680), and floxed Raptor mice (Jax#013188), inducible Col2a1-Cre/ERT transgenic mice (Jax#006774). Six-week-old male C57BL/6 mice were purchased from the Laboratory Animal Centre of Southern Medical University. To generate chondrocyte-specific *Tsc1* deletion mice (*Tsc1*$^{fl/fl}$; Col2a1-Cre), *Tsc1*$^{fl/fl}$ mice were hybridized with Col2a1-cre mice, as reported.[35,36] We generated inducible chondrocyte-specific *Tsc1* deletion mice in the same way. Mice with Col2a1-specific deletion of *Tsc1* were defined as *Tsc1* CKO mice and mice with inducible Col2a1-specific deletion of *Tsc1* were defined as *Tsc1* CKO^{ER} mice. Eight-week-old *Tsc1* CKO^{ER} mice were treated with tamoxifen (100 μg/g body weight) by intraperitoneal injection for 5 days, knocking out the *Tsc1* gene in the chondrocytes. Genotyping was performed on genomic DNA from tail biopsies, and primers used were as follows: loxP *Tsc1* loxP primer up, 5′-GTC ACG ACC GTA GGA GAA GC-3′, and down, 5′-GAA TCA ACC CCA CAG AGC AT-3′; Col2a1-Cre forward, 5′- GCA TCG ACC GGT AAT GCA GGC-3′, and reverse, 5′-GAG GGT CCA GCC CGA GCT ACTT-3′; Col2a1-Cre/ERT forward, 5′-CAC TGC GGG CTC TAC TTC AT-3′, and reverse, 5′-ACC AGC AGC ACT TTT GGA AG-3′ (both from Sangon Biotech, China).

Mice model

Eight-week-old control mice and *Tsc1* CKO mice were subjected to DMM to induce OA. We quantified articular cartilage degeneration by the Osteoarthritis Research Society International (OARSI) scoring system. Collagenase-induced osteoarthritis (CIOA) or anterior cruciate ligament transection (ACLT) was induced in mice as described.[37,38] Antigen-induced arthritis (AIA) was induced rheumatoid arthritis (RA) in mice, using methylated bovine serum albumin (mBSA). Briefly, mice were sensitized subcutaneously with an emulsion containing mBSA (500 μg) and Freund's complete adjuvant (2 mg/mL of inactivated *Mycobacterium tuberculosis*) (both from Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1 in phosphate buffered saline (PBS). Booster injections of mBSA dissolved in Freund's incomplete adjuvant (without *M. tuberculosis*) were given 7 and 14 days after the first immunization. Sham-immunized mice received similar injections but without the antigen.[39]

Cells and cartilage explants

The prechondrocyte cell line, ATDC5 (Tsukuba, Japan), was maintained in DMEM/F12 (Gibco, Grand Island, NY, USA) with 10% FBS (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate (Life Technologies, Inc., Grand Island, NY, USA), at 37°C with 5% CO_2. We dissected rib cartilage from newborn mice (24–72 hours) under a stereo light microscope to harvest primary chondrocytes. After trypsin digestion for 30 min, primary chondrocytes were separated and purified, and digested in 0.1% collagenase type II (Sigma) with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate at 37°C for 4 to 6 hours. Primary chondrocytes were resuspended and seeded in a 24-well plate and cultured in DMEM/F12 with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate at 37°C with 5% CO_2. Femoral heads were isolated from 8-week-old mice. Explants cultured in DMEM/F12 were treated

with 10mM PBS for 1 hour, followed by treatment with 50 ng/mL recombinant mouse or human interleukin (IL)-1β in PBS/0.1% BSA, or with PBS/0.1% BSA alone. The explants were harvested and the medium was collected after 3 days in culture.

Drug treatment

Cells were treated with IL-1β (50 ng/mL; PeproTech, Rocky Hill, NJ, USA) for 24 hours to mimic OA in vitro. Two-week-old *Tsc1* CKO mice were treated with rapamycin (1 mg/kg/day; Sigma) by intraperitoneal injection for 4 weeks. Bevacizumab (25 mg/kg/week; Roche, USA) was administered to 4-week-old *Tsc1* CKO mice by intraarticular injection for 4 weeks before the mice were killed. Similarly, 8-week-old control mice and *Tsc1* CKO mice after surgery were treated with or without bevacizumab for 5 weeks (Supporting Fig. 1).

In vitro tube formation assay

The 96-well plates were coated with 20 μL growth-factor-depleted Matrigel (BD Biosciences, Australia). Matrigel solidified for 45 min at 37°C, and human umbilical vein endothelial cells (HUVECs) were seeded at a density of 50,000/well. One hundred microliters (100 μL) of conditional cell suspension was added and photomicrographs were obtained 9 hours later. Tube length was measured by Image-Pro Plus 6.0.

Elisa

Cell supernatant was analyzed by Mouse VEGF-A ELISA Kit (#EEL-M0050; Elabscience Biotechnolog, China).

Preparation of decalcified sections, histochemistry, immunostaining, and immunohistochemistry

Knee joints freshly dissected from mice were fixed for 24 hours in 4% paraformaldehyde at 4°C then decalcified for 30 days in 14% EDTA (pH 7.4) at 25°C. Tissues were embedded in paraffin and sectioning continuously (3 μm thick). Safranin-O/Fast Green staining was performed as described.[35] Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay was performed with the In Situ Cell Death Detection Kit TMR Red (Roche). For immunofluorescence and immunohistochemistry, sections were treated with 0.1 mg/mL proteinase K (Sigma) for 10 min at 37°C or soaked in citrate buffer (10mM citric acid, pH 6.0) for 16 to 18 hours at 60°C to unmask antigen after deparaffinization and rehydration. For immunohistochemistry we added 3% hydrogen peroxide for 10 min. Sections were blocked with 1% sheep serum at 37°C for 1 hour and incubated with primary antibodies (in 1% BSA, 0.1% Triton X-100) at 4°C overnight. Sections were incubated with secondary antibodies at 37°C for 1 hour. Species-matched antibodies labeled with Alexa Fluor 488 and Alexa Fluor 594 or horseradish peroxidase were used (1:100 in 1% BSA) as described.[40] Nuclei were labeled with 4′,6-diamidino-2-phenylindole (Thermo Fisher, USA) before imaging. For immunohistochemistry, we used 3,3′-diaminobenzidine to observe chromogen and hematoxylin to counterstain. In situ hybridization with digoxin-labeled probes was performed using a CXCL9 mRNA (#MK3237; Boster, China).

Western blotting

Lysis buffer was prepared with 10% glycerol, 2% sodium dodecyl sulfate (SDS), 10mM dithiothreitol, 10mM Tris–HCl (pH 6.8), 1mM phenylmethylsulfonyl fluoride, and 10% β-mercaptoethanol. Tissues and cells were lysed by lysis buffer at 98°C for 10 min. Samples were separated by SDS-PAGE for 90 min. Samples were blotted onto nitrocellulose membranes for 1 hour and incubated with primary antibodies (in 5% BSA, 0.2% NaN_3) at 4°C overnight. Samples were incubated with secondary antibodies at 37°C for 1 hour.

Antibodies

The following antibodies were used: rabbit anti-p S6 (S235/236) (1:1,000 for Western blotting [WB], 1:100 for immunohistochemistry/immunofluorescence [IHC/IF]; clone D57.2.2E, 4858; Cell Signaling Technology, Beverly, MA, USA), mouse anti-S6 (1:3000 for WB; Santa Cruz Biotechnology, Santa Cruz, CA, USA; clone C-8, sc-74459), rabbit anti-Col X (1:100 for IF; Abcam, Cambridge, MA, USA; ab58632), rabbit anti-Lc3 (1:100 for IF; Cell Signaling Technology; 2275S), rabbit anti-MMP-13 (1:100 for IF; Abcam; ab39012), rabbit anti-Runx2 (1:100 for IF; Abclone, Australia; A2851), rabbit anti-VEGF-A (1:100 for IHC; Proteintech Group, USA; #19003-1-AP), rat anti-endomucin (1:50 for IF; Santa Cruz Biotechnology; sc-65495), goat anti-CD31 (1:100 for IF; R&D Systems, Minneapolis, MN, USA; AF3628), Anti-Rabbit IgG Light Chain (1:100 for IHC; Abbkine, USA; A25022), horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG H&L (1:1000 for WB; 1:100 for IHC; Jackson ImmunoResearch, West Grove, PA, USA; 111-035-003), HRP-labeled Goat Anti-Mouse IgG H&L (1:3000 for WB; Jackson ImmunoResearch; 115-035-003), Alexa Fluor 594 labeled Goat Anti-Rabbit IgG H&L (1:500 for IF; Abcam; ab150080), Alexa Fluor 488 labeled Goat Anti-Rabbit IgG H&L (1:500 for IF; Abcam; ab150077), and Alexa Fluor 488 labeled Goat Anti-Mouse IgG H&L (1:500 for IF; Abcam; 150113).

Statistical analysis

Experiments were performed three times. All data are presented as mean ± SD using SPSS version 19.0 software (IBM Corp., Armonk, NY, USA) and curve analysis was determined using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The data among different groups were analyzed using multiple factorial analysis of variance. The data in each group were analyzed using unpaired, two-tailed Student's *t* test. The level of significance was set at $p < 0.05$.

Results

H-type vessels are increased in subchondral bone in DMM and aged mice

H-type vessels are a specific subtype defined by high co-staining for CD31 and endomucin ($CD31^{hi}Emcn^{hi}$), which has been identified to couple angiogenesis and osteogenesis.[41,42] To determine whether H-type vessels in subchondral bone changed during OA progression, we performed double immunofluorescence staining for CD31 and endomucin in a DMM model. $CD31^{hi}Emcn^{hi}$ blood vessels were significantly increased in the subchondral bone of DMM mice (Fig. 1*A–D*). We observed more H-type vessels in the injured meniscus, but fewer vessels in the intact meniscus in the DMM model (Supporting Fig. 2). Age is the most prominent risk factor for the development of OA. A previous study has shown H-type vessels are drastically reduced in the long bones of aged mice, contributing to decreased osteogenesis and osteoporosis.[41] We also observed pronounced reduction of H-type vessels in the

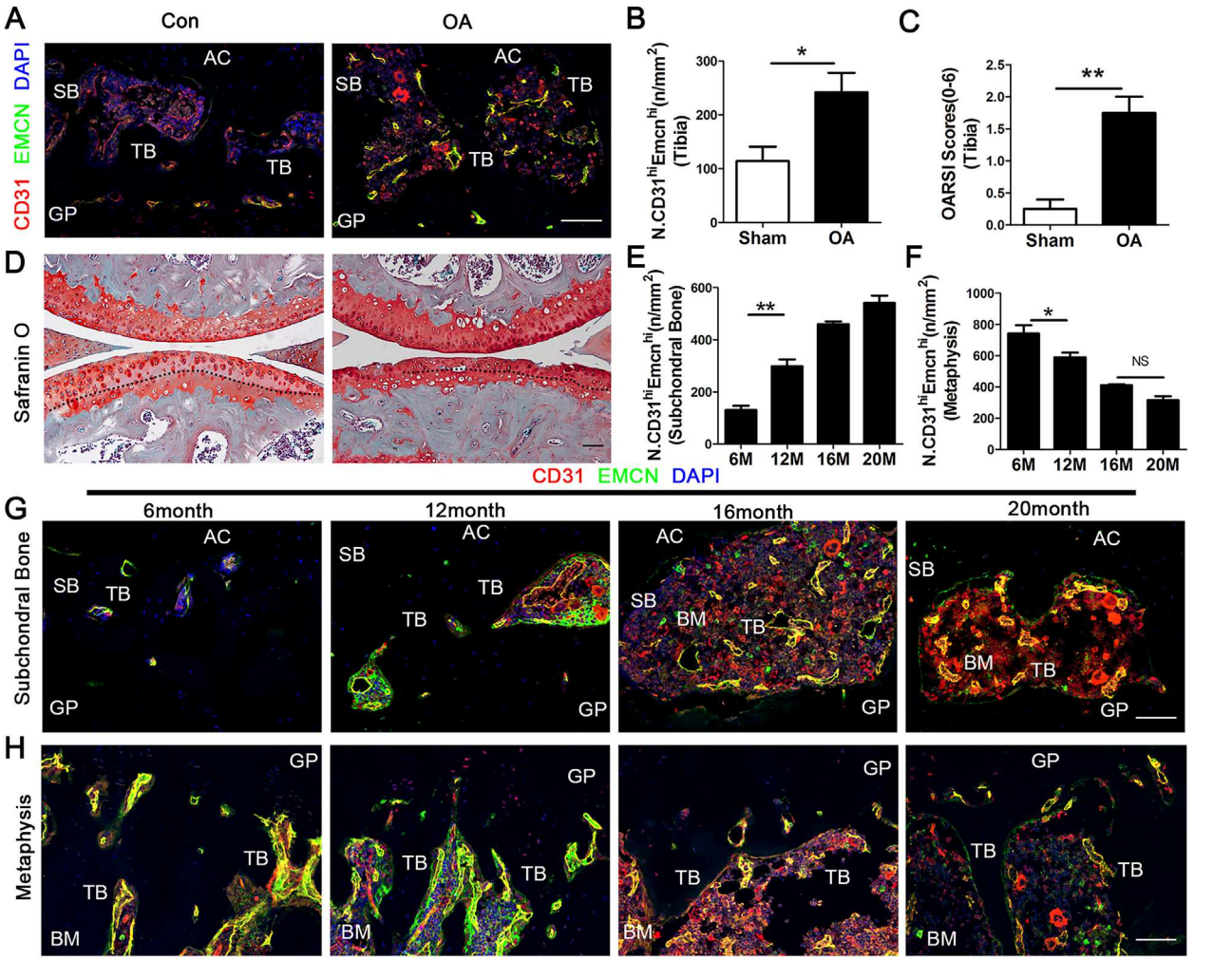

Fig. 1. H-type vessel formation was increased in subchondral bone of DMM and aging mice. (*A*, *B*, *E–H*) Representative immunofluorescence double staining (*A*, *G*, *H*) and quantification (*B*, *E*, *F*) of cells positive for CD31 (green) and endomucin (red) in mice after DMM surgery for 5 weeks and in aging mice. Scale bars = 50 μm. (*C*) OARSI score was evaluated in sham and OA mice. (*D*) Cartilage degradation assessed by Safranin O and fast green staining. The dotted lines represent the tide line. Scale bars = 100 μm. ($n \geq 4$), NS, not significant. $^{*}p < 0.05$, $^{**}p < 0.01$. AC = articular cartilage; TB = trabecular bone; SB = subchondral bone; GP = growth plate; BM = bone marrow cavity; Sham = sham surgery.

metaphyses in aged mice (Fig. 1*E–H*). Surprisingly, H-type vessels were markedly increased in subchondral bone of aged mice (Fig. 1*E–H*), suggesting that distinct microenvironments or mechanisms may control H-type vessel formation in subchondral bone. All these results indicate that H-type vessel formation in subchondral bone is strongly correlated with OA development.

Articular chondrocyte mTORC1 activation stimulates angiogenesis in subchondral bone

OA hypertrophic chondrocytes may stimulate vascular formation via releasing angiogenic factors such as VEGF.[43] We previously generated mice with chondrocyte-specific deletion of tuberous sclerosis (TSC)1, an upstream inhibitor of mTORC1 (*Tsc1*$^{fl/fl}$Col2-Cre mice were referred to as *Tsc1* CKO; *Tsc1*$^{fl/fl}$ mice were referred to as control). We found that articular chondrocyte mTORC1 activation stimulated aberrant proliferation of chondrocytes, and hypertrophic differentiation to initiate OA.[35,36] We also examined mTORC1 signaling in articular chondrocyte in CIOA and ACLT OA models and in AIA rheumatoid arthritis mouse model. Thus, mTORC1 activation may be a general mechanism of joint tissue response damage in mice (Supporting Fig. 3).

We examined whether chondrocyte mTORC1 regulated vascular formation in subchondral bone. mTORC1 was hyperactivated in articular chondrocytes of *Tsc1* CKO mice, as manifested by high levels of p-S6(S235/236) in these cells (Fig. 2*A*, *B*). Subchondral bone of the mutant mice was highly vascularized, and CD31^{+} and CD31^{hi}Emcn^{hi} blood vessels were markedly increased in subchondral bone of 8-week-old *Tsc1* CKO mice (Fig. 2*C–F*). These mice also developed severe OA (Fig. 2*G*, *H*). Importantly, rapamycin treatment inhibited mTORC1 and reversed the angiogenesis and OA phenotype of *Tsc1* CKO mice (Fig. 2*A–H*). Furthermore, chondrocyte mTORC1 activation also promoted H-type vessel formation in the synovium of *Tsc1* CKO mice (Supporting Fig. 4).

To duplicate the process of angiogenesis during OA development, we generated *Tsc1* CKO^{ER} mice and *Raptor* (a specific component of mTORC1) CKO^{ER} mice and specifically

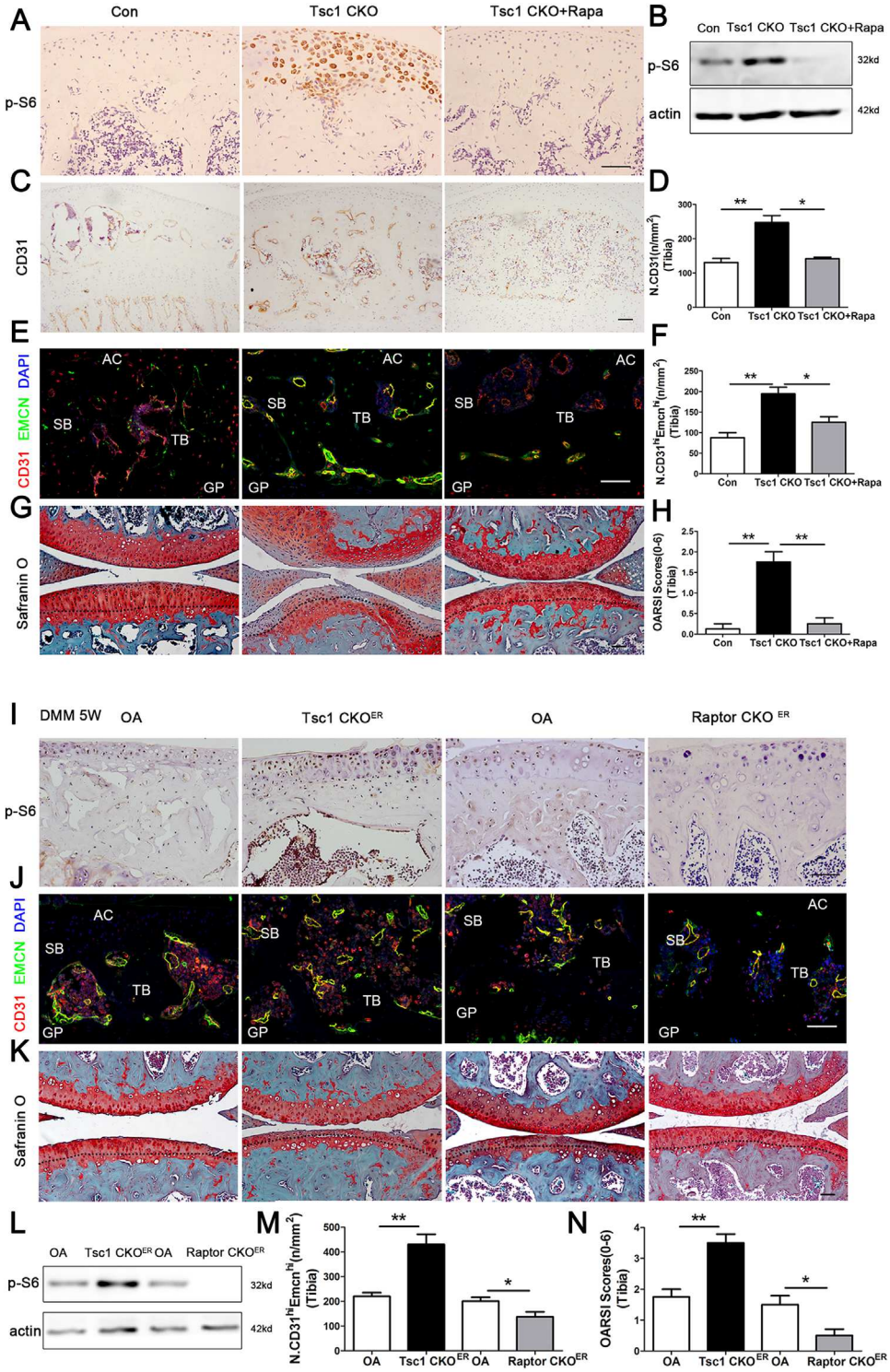

Fig. 2. Chondrocyte mTORC1 activation stimulates H-type vessel formation in subchondral bone. (*A*, *B*, *I*, *L*) p-S6 (S235/236) levels were detected by immunohistochemistry (*A*, *I*) and Western blotting (*B*, *L*) in articular chondrocytes. Scale bars = 50 μm. (*C*–*F*, *J*, *M*) Immunostaining and quantitative analysis of CD31-positive cells (*C*, *D*), and cells positive for CD31 (green) and endomucin (red) (*E*, *F*, *J*, *M*) in subchondral bone. Scale bars = 100 μm and 50 μm. (*G*, *K*) Safranin O and fast green staining. Scale bars = 100 μm. (*H*, *N*) OARSI was evaluated in mice. ($n \geq 4$), $^{*}p < 0.05$, $^{**}p < 0.01$. AC = articular cartilage; TB = trabecular bone; SB = subchondral bone; GP = growth plate; BM = bone marrow cavity; Sham = sham-surgery; Rapa = rapamycin.

activated or disrupted chondrocyte mTORC1 at adult stages. Eight-week-old mice were injected with tamoxifen or PBS for 5 days and subjected to DMM surgery (*Tsc1*$^{fl/fl}$ Cre/ERT and *Raptor*$^{fl/fl}$ Cre/ERT were injected with PBS and referred to as control, respectively). Five weeks postsurgery, the targeting efficiency and specificity were confirmed by mTORC1 activation or inhibition in articular chondrocytes of *Tsc1* CKO^{ER} and *Raptor* CKO^{ER} mice (Fig. 2*I*, *L*). Importantly, H-type vessels were

significantly increased in the subchondral bone of *Tsc1* CKO^{ER}, but decreased in that of *Raptor* CKO^{ER} mice (Fig. 2*J*, *M*). Consistently, OA progression was markedly accelerated or delayed by constitutive activation or inhibition of mTORC1 in chondrocytes (Fig. 2*K*, *N*). These findings suggest that articular chondrocyte mTORC1 promotes subchondral angiogenesis and H-type vessel formation during OA progression.

mTORC1 activation promotes articular chondrocyte VEGF-A production during OA development

VEGF-A is an established important proangiogenic factor expressed by hypertrophic chondrocytes in OA.[44] To explore the mechanism by which chondrocyte mTORC1 stimulates subchondral angiogenesis, the potential role of VEGF-A in this process was investigated. As expected, increased VEGF-A-positive cells were detected in OA chondrocytes (Fig. 3*A*, *B*). Upon IL-1β stimulation, secretion of VEGF-A was enhanced in primary cultured chondrocytes (Fig. 3*C*). The supernatant of OA chondrocyte culture promoted HUVEC in vitro tube formation (Fig. 3*D*, *E*). Moreover, other potential triggers, such as transforming growth factor (TGF)-β, were investigated. We also observed that TGF-β enhanced VEGF-A secretion in cultured articular cartilage explants. However, the effect of TGF-β1 on VEGF-A expression was not rapamycin dependent. The mechanisms through which TGF-β1 regulates VEGF-A need to be further investigated (Supporting Fig. 5). Strong VEGF-A immunostaining and marked increase of VEGF-A-positive articular chondrocytes were observed in *Tsc1* CKO mice but not in their littermate controls or rapamycin-treated *Tsc1* CKO mice (Fig. 3*F*, *H*). Cultured chondrocytes from *Tsc1* CKO mice secreted more VEGF-A than the control and rapamycin-treated *Tsc1* CKO mice did (Fig. 3*I*). Accordingly, in vitro tube formation stimulated by the supernatant of *Tsc1* CKO chondrocyte culture was significantly enhanced as compared with that of control and rapamycin-treated mice (Fig. 3*G*, *J*). Consistent with the increased H-type vessels in subchondral bone of aged mice (Fig. 1*E–H*), mTORC1 activity (p-S6) and VEGF-A expression in articular chondrocytes were enhanced in aged mice (Supporting Fig. 6). A similar result can be observed in cartilage explant (Supporting Fig. 5). All these results suggest that mTORC1 activation induces production of VEGF-A in OA articular chondrocytes.

Chondrocyte mTORC1 activation promotes OA partially through VEGF-A–stimulated subchondral H-type vessel formation

We next investigated the role of VEGF-A in chondrocyte mTORC1-stimulated subchondral angiogenesis and OA development. Four-week-old *Tsc1* CKO mice were intraarticularly injected with bevacizumab; a recombinant monoclonal anti-VEGF-A antibody that blocks angiogenesis.[44] Four-week treatment with bevacizumab not only markedly reduced subchondral H-type vessel formation (Fig. 4*A*, *C*; Supporting Fig. 4), but also prevented cartilage proteoglycan loss and cartilage degeneration and reduced OARSI score in *Tsc1* CKO mice (Fig. 4*B*, *D*). Similarly, VEGF-A antibody significantly decreased subchondral H-type vessels and delayed OA development in *Tsc1* CKO^{ER} mice (Fig. 4*E–H*). These findings clearly demonstrate that VEGF-A–stimulated subchondral H-type vessel formation is essential for chondrocyte mTORC1 activation-induced OA.

Enhanced H-type vessel formation in subchondral bone contributes to chondrocyte mTORC1 activation and OA development

Although it is well established that chondrocyte mTORC1 activation plays a vital role in the pathogenesis of OA, little is known about the mechanism through which articular chondrocyte mTROC1 is activated during this process. Bevacizumab not only blocked subchondral H-type vessel formation (Fig. 5*A*, *B*), but also reduced chondrocyte mTORC1 activation in DMM OA mice (Fig. 5*C–E*). Serum or nutrients (amino acids) stimulated mTORC1 in culture chondrocyte cell line ATDC5 that were subjected to serum or nutrient starvation (Fig. 5*F*, *G*). Similarly, serum or amino acids strongly activated mTORC1 in primary cultured chondrocytes and cartilage explants (Fig. 5*H–K*). We further examined the effect of cyclic stretch on the capability of chondrocytes to promote vessel formation. We found that stretch significantly enhanced mTORC1 activity and the ability of chondrocytes to promote tube formation (Supporting Fig. 7). These findings imply that chondrocyte mTORC1 activation enhances nutrients and factors supplementation by increasing angiogenesis during OA development in mice.

Previous studies have shown that mTORC1 activation induces articular chondrocyte hypertrophic differentiation,[35] autophagy deficiency and apoptosis to initiate and promote OA.[34] Blockade of angiogenesis by bevacizumab reduced chondrocyte mTORC1 activity in OA mice (Fig. 5*C–E*); therefore, we examined the effect of bevacizumab on these chondrocyte phenotypes related to mTORC1 in OA. As expected, chondrocyte hypertrophic differentiation was prevented by blockade of angiogenesis, manifested by reduced matrix metallopeptidase (MMP)13, collagen X, and runt-related transcription factor (Runx)2 expression in bevacizumab-treated OA mice (Fig. 6*A–F*). Furthermore, autophagy marker light chain 3 (LC3B)-positive chondrocytes were augmented while TUNEL-positive cells were reduced by bevacizumab (Fig. 6*G–J*), indicating that OA chondrocyte autophagy was recovered and cell death was decreased. These results showed that enhanced angiogenesis may activate chondrocyte mTORC1 and promote OA in mice.

Discussion

Accumulating evidence demonstrates that OA is a systemic disease, not just of cartilage or bone. Pathological changes in cartilage and subchondral bone and crosstalk between cartilage and subchondral play a vital role in this process.[45] Angiogenesis and biochemical interplay between subchondral bone and articular cartilage are critical in the pathogenesis of OA. Here, we demonstrate that during OA development, activation of mTORC1 in articular chondrocytes is essential for subchondral H-type vessel formation via production of VEGF. Moreover, subchondral angiogenesis activates nutrient-sensing mTORC1 signaling in articular chondrocytes. These data establish a novel positive-feedback regulation of subchondral vascular formation by articular chondrocyte nutrient-sensing signaling, which is essential for the pathogenesis of OA (Supporting Fig. 8).

H-type vessels are a specific subtype that couples angiogenesis with osteogenesis.[41,42] A recent study has shown that H-type vessels are increased in subchondral bone (osteoid islet) of mouse models of anterior cruciate ligament transection.[46] However, how H-type vessel formation is induced during OA is unknown. We found that H-type vessels were increased in the subchondral bone of DMM mice. Importantly, activation of

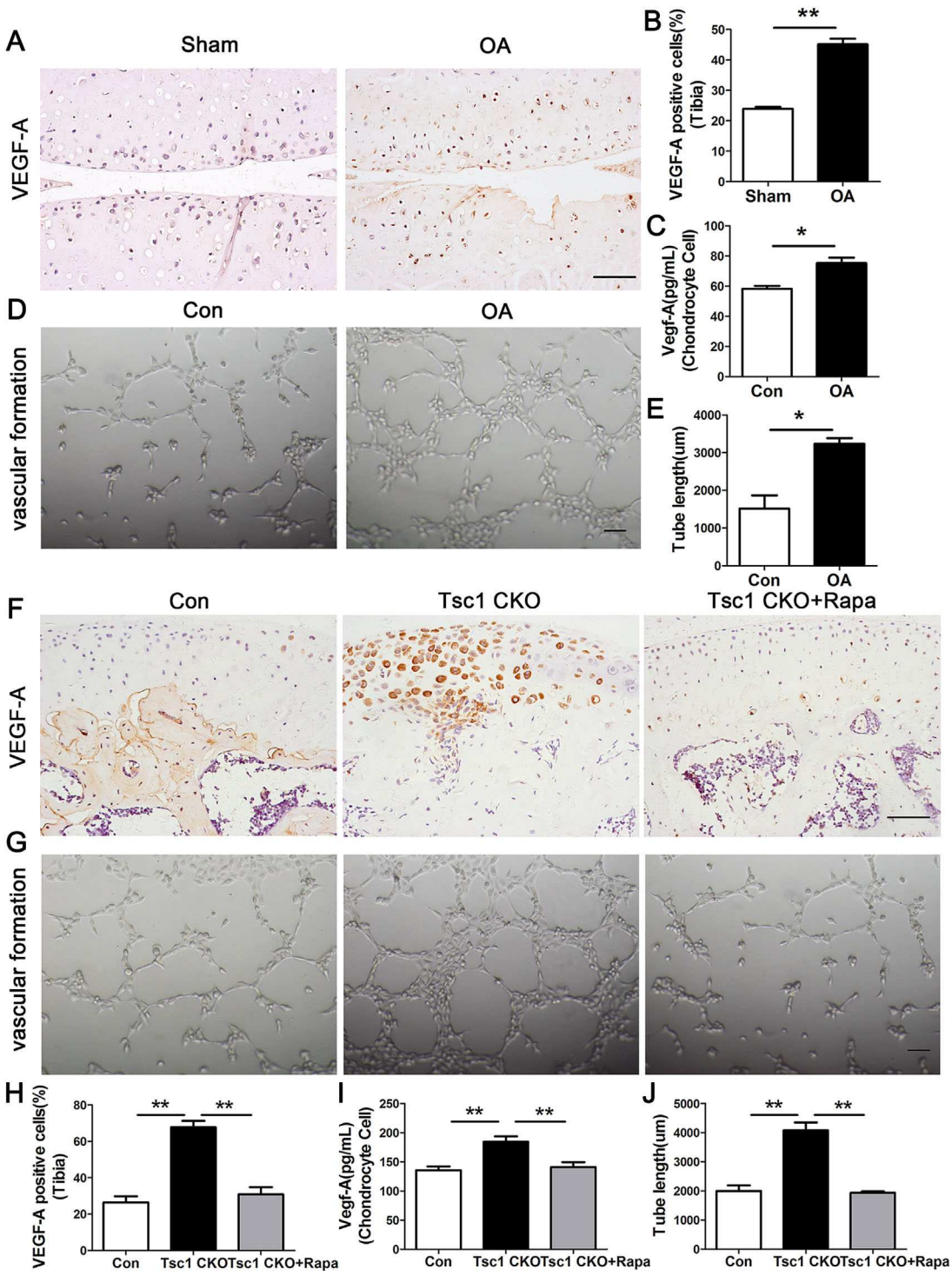

Fig. 3. mTORC1 activation promotes VEGF-A secretion in chondrocytes in vitro and in vivo. (*A*, *B*, *F*, *H*) Immunostaining (*A*, *F*) and quantitative analysis of VEGF-A–positive cells (*B*, *H*). Scale bars = 50 μm. (*C*, *I*) VEGF-A levels in supernatant of primary chondrocytes were determined by ELISA. (*D*, *G*) HUVECs were cocultured with supernatant of primary chondrocytes, and tube formation was measured by tube formation assay. (*E*, *J*) HUVEC tube length was evaluated. ($n \geq 4$), $^{*}p < 0.05$, $^{**}p < 0.01$.

chondrocyte mTORC1 is sufficient to stimulate H-type vessel formation and OA, while inhibition of chondrocyte mTORC1 prevents subchondral H-type vessel formation and attenuates OA development. These findings suggest that articular chondrocyte mTORC1 is essential for H-type vessel formation, which may couple subchondral angiogenesis with osteogenesis and contribute to the sclerosis and the formation of cysts and osteophytes in OA. However, the implications of increased vessel formation may be much more complex and not necessarily serum derived. Biomechanics may also contribute

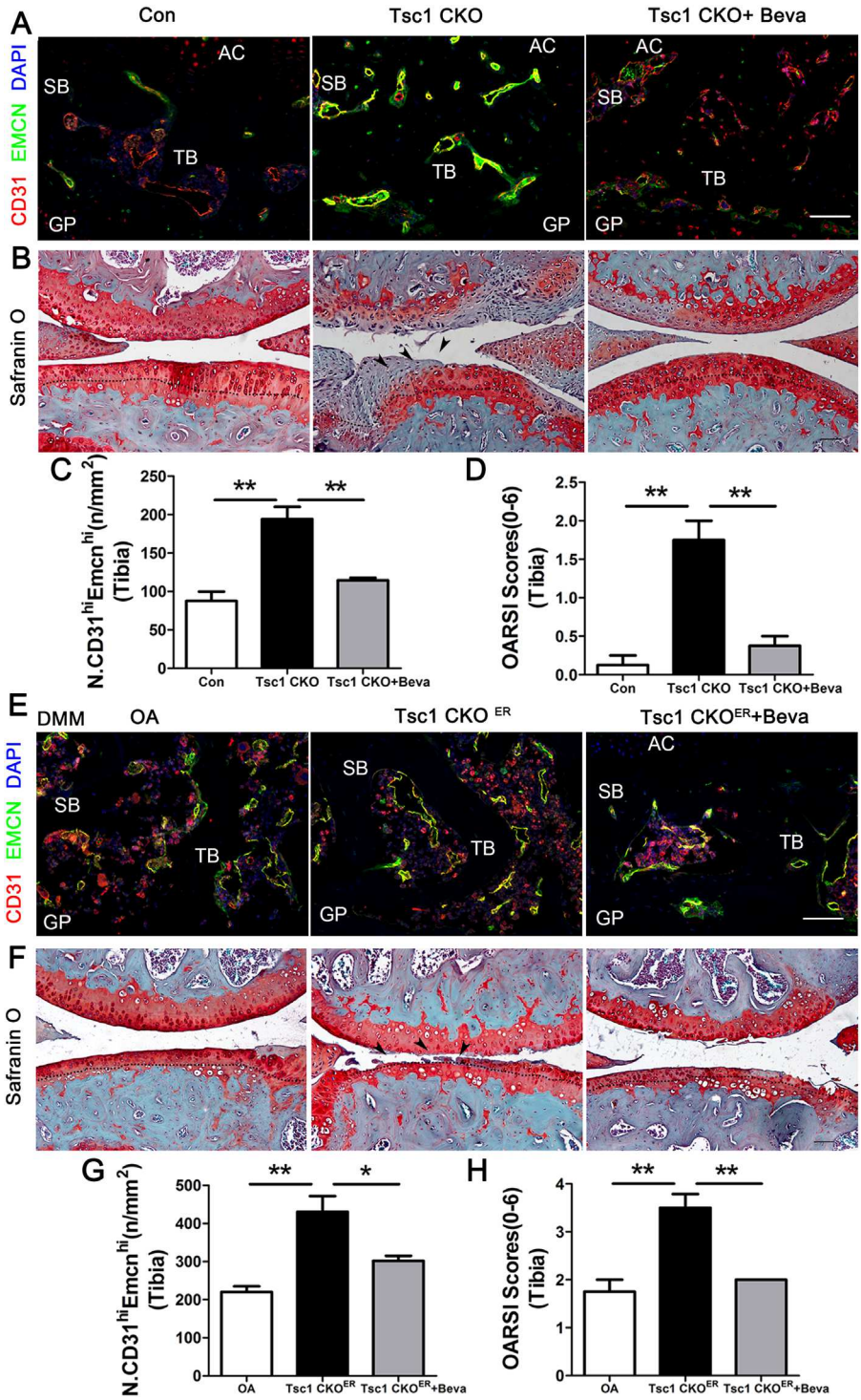

Fig. 4. Bevacizumab inhibits subchondral H-type vessel formation and delays OA development in Tsc1CKO and Tsc1CKO^{ER} mice. (*A*, *C*, *E*, *G*) Representative immunofluorescence double staining (*A*, *E*) and quantification (*C*, *G*) of cells positive for CD31 (green) and endomucin (red) in Tsc1CKO mice with or without bevacizumab treatment and in Tsc1CKO^{ER} mice 5 weeks after DMM surgery treated with or without bevacizumab. Scale bars = 50 μm. (*B*, *F*) Safranin O and fast green staining. Solid arrows indicate the regions had evident fibrosis and cartilage degeneration. Scale bars = 100 μm. (*D*, *H*) OARSI score in control, Tsc1CKO, and bevacizumab-treated Tsc1CKO mice (*D*) and in OA, Tsc1CKO^{ER}, and bevacizumab-treated Tsc1CKO^{ER} mice (*H*). ($n \geq 4$), $^{*}p < 0.05$, $^{**}p < 0.01$. AC = articular cartilage; TB = trabecular bone; SB = subchondral bone; GP = growth plate; BM = bone marrow cavity; Sham = sham surgery; Beva = bevacizumab.

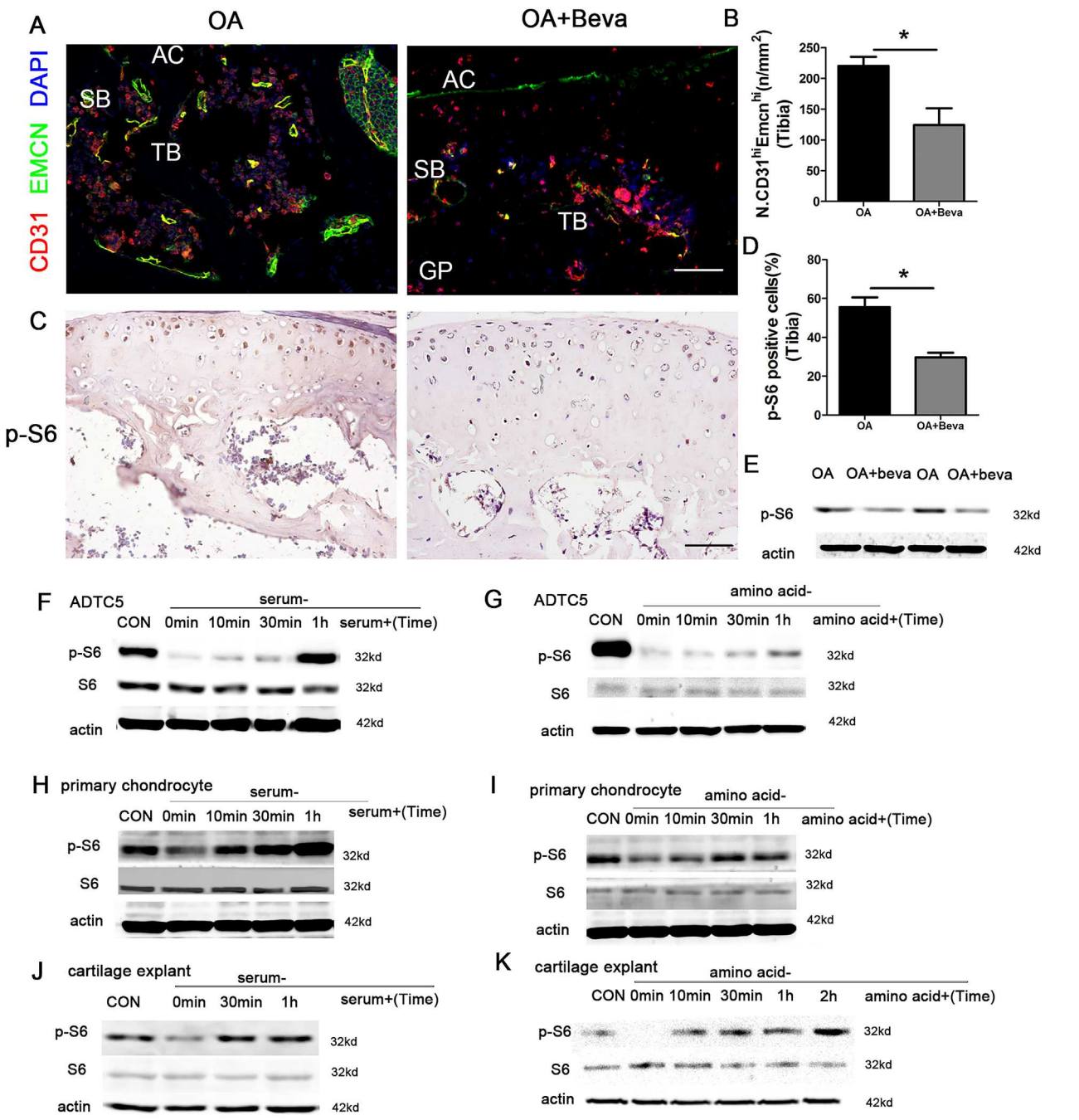

Fig. 5. Subchondral vascular formation is essential for articular chondrocyte mTORC1 activation during OA development. (*A*, *B*) Immunostaining and quantitative analysis of cells positive for CD31 (green) and endomucin (red) in OA and bevacizumab-treated OA mice. (*C*–*E*) p-S6 (S235/236) was examined by immunohistochemistry (*C*) and Western blotting (*E*) in articular cartilage of OA mice with or without bevacizumab. Quantitative analysis of p-S6 (S235/236) in articular cartilage (*D*). Scale bars = 50 μm. (*F*–*K*) Expression of p-S6 (S235/236) was analyzed by Western blotting. Upon serum deprivation for 16 hours or amino-acid starvation for 30 min, ATDC5 cells (*F*, *G*), primary cultured chondrocytes (*H*, *I*) and cartilage explants (*J*, *K*) were stimulated with DMEM/F12 (with or without FBS). ($n \geq 4$), $^{*}p < 0.05$.

to vessel formation. We observed that stretch significantly enhanced mTORC1 activity and the ability of chondrocyte to promote tube formation. Targeting mTORC1 may attenuate OA progression partially by prevention of pathological angiogenesis and osteophytes formation.

It has been shown that H-type vessels are markedly reduced in long bones of aging mice, accompanied by reduced osteoprogenitors, osteogenesis, and bone mass.[41,47] We observed pronounced reduction of H-type vessels in metaphyseal bone marrow cavities, but increased vessels in subchondral bone of aged mice. We speculate that the inconsistency may have resulted from the distinct biochemical and biomechanical microenvironment between subchondral bone and long bone marrow cavities, because activated

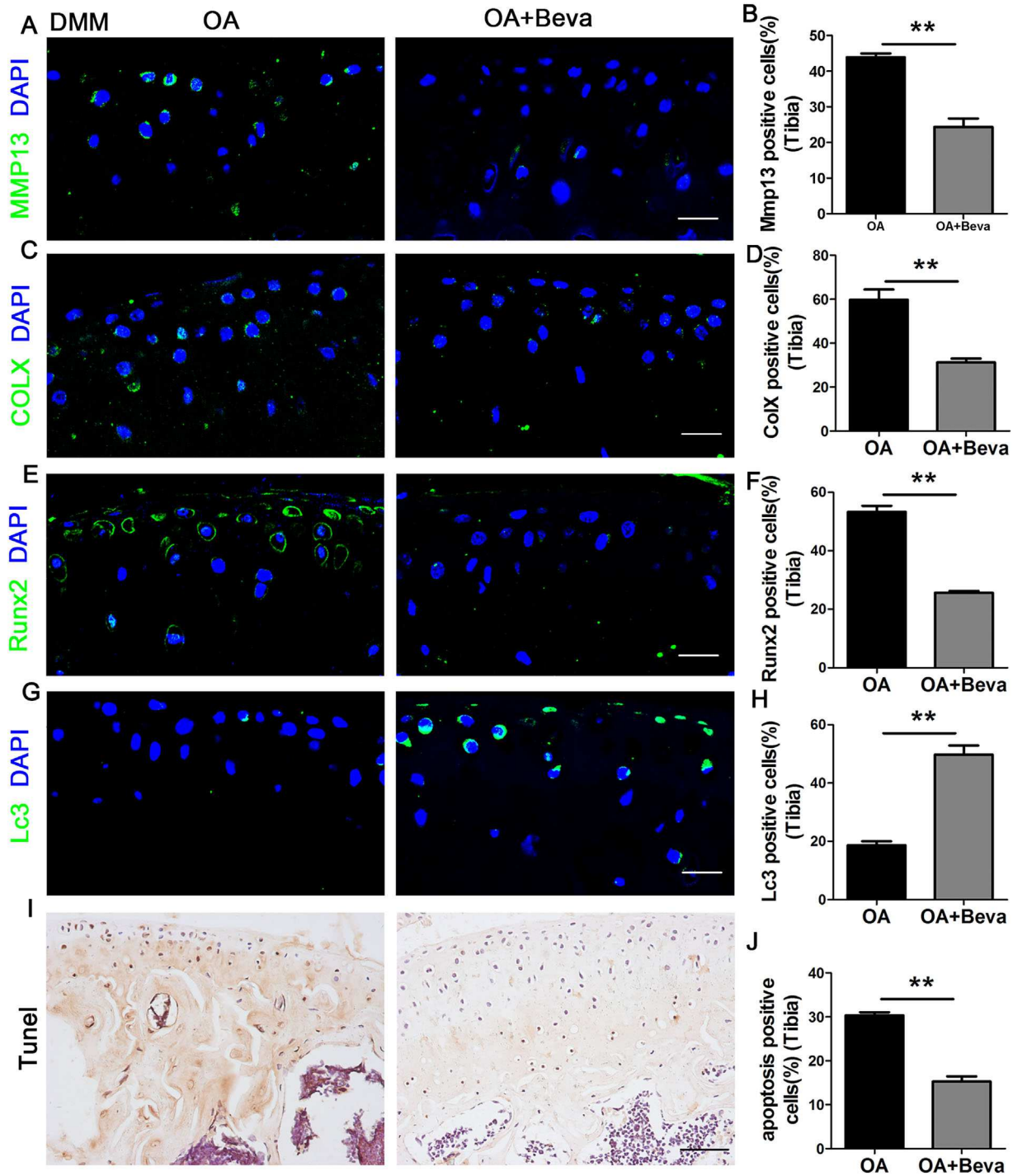

Fig. 6. Bevacizumab prevents articular cartilage degeneration during OA development. (*A–H*) Immunostaining and quantitative analysis of cells positive for MMP13 (*A*, *B*), ColX (*C*, *D*), Runx2 (*E*, *F*), and LC3 (*G*, *H*) in OA mice treated with or without bevacizumab. Scale bars = 10 μm. (*I*, *J*) TUNEL staining and quantitative analysis of apoptotic cells. Scale bars = 50 μm. ($n \geq 4$), $^{**}p < 0.01$.

articular chondrocytes may release many cytokines and factors such as VEGF to subchondral bone during pathogenesis of OA.[25] Subchondral vessels may contribute to chondrocytes and osteoblasts, while metaphyseal vessels may contribute to osteoblasts. Bone vasculature is modulated by numerous biochemical factors through different signaling pathways. Halofuginone can reduce vessel formation through inhibiting MMP2 and collagen type α1 expression.[48] Furthermore, other studies have shown that TGF-β signaling can regulate H-type vessels[46] and we also observed that TGF-β enhanced VEGF-A secretion in cultured articular cartilage explants. However, the mechanisms through which TGF-β1 regulates VEGF-A need to be further investigated. Similarly, we have recently shown that activation of mTORC1 in osteoblast prevents H-type vessel

formation in long bone,[40] while activation of mTORC1 in chondrocytes stimulated vessel formation in subchondral bone in the current study. We revealed that mTORC1 activation stimulates the production of VEGF and chemokine XCX ligand (CXCL)9 in osteoblasts. However, CXCL9 can interact with VEGF and prevent its binding to endothelial cells, thus abrogating angiogenesis in bone. Moreover, CXCL9 is constitutively expressed by osteoblasts residing in bone,[40] but was undetectable in chondrocytes (Supporting Fig. 9). Although blocking subchondral vascular formation by anti-VEGF-A antibody bevacizumab ameliorates OA in mice according to previous reports[44] and the results from this study, systemic targeting of H-type vessels for OA prevention and treatment should be undertaken cautiously because of the increased risk for osteoporosis.

Emerging evidence highlights the vital role of mTORC1 in the pathogenesis and progression of OA.[30–36] mTORC1 is activated in articular chondrocytes in experimental OA, age-related OA, and RA mice. Both cartilage-specific deletion of mTOR[33] and pharmacological inhibition of mTORC1 by rapamycin have been shown to decrease the severity of experimental OA in mouse models.[32,34] We have found that endogenously produced n-3 polyunsaturated fatty acids (PUFAs) inhibit mTORC1 signaling and attenuate OA progression in mice.[49] Mice with chondrocyte-specific activation of mTORC1 develop a severe OA phenotype.[35] However, the mechanism underlying mTORC1 activation in OA chondrocytes is not known. mTORC1 is the major intracellular nutrient sensing signaling that is activated by nutrients such as amino acids and glucose to control cell growth and metabolism.[29,50] We found that blocking subchondral angiogenesis reduced mTORC1 activation in OA articular chondrocytes. We suppose that normal cartilage is avascular with low levels of nutrients, and the articular chondrocytes are maintained in a quiescent state with low mTORC1 activity. During development of OA, cartilage is vascularized and more nutrients are carried to activate chondrocyte mTORC1. This suggests that subchondral vascular formation is important for sustained cartilage chondrocyte mTORC1 activation during OA development.

Clinically, characteristics of OA include osteophyte formation, subchondral bone sclerosis, disruption of tidemark accompanied by angiogenesis at the osteochondral junction, synovitis, and degeneration and loss of articular cartilage.[1,3] Subchondral bone cysts do not necessarily lead to OA, but they can suggest abnormal interaction in subchondral bone.[51] Early osteophyte formation may provide a mechanism for repairing damage during mechanical loading.[52] Some osteophytes without joint space narrowing can be a sign of compensation and restoration of homeostasis. Therapy that is able to target the multiple pathological changes in joints is desirable. Evidence from this study supports that the mTORC1 pathway is one of the critical signaling pathways that activate cellular and molecular processes in crosstalk among joint compartments. Previous studies have established that activation of mTORC1 inhibits autophagy to promote articular chondrocyte apoptosis during OA development.[32,34] We have shown that mTORC1 activation induces chondrocyte aberrant proliferation, differentiation, and activation to initiate cartilage degeneration and OA development.[35] The current study shows that mTORC1 activation stimulates angiogenesis in subchondral bone and synovial membrane and establishes links between cartilage, subchondral bone, and synovial membranes via an mTORC1-mediated positive feedback mechanism. In this model it is hard to explain whether cartilage or bone is a trigger, but it may show that multiple OA pathological joint compartments benefit from targeting of mTORC1.

Disclosure

All authors state that they have no conflicts of interest.

Acknowledgments

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Authors' roles: XB and DC conceived the study, and analyzed and interpreted the data. HF, CZ, DX, and CZ provided samples. JL, PL, DL, YS, YJ, and JOY performed histological review. JL and HZ performed statistical analysis. JL, DL, YS, BH, JY, and JOY acquired the data. JL, HZ, and DC have the same contribution in this article. All authors meet the International Committee of Medical Journal Editors recommendations and have critically reviewed the manuscript.

References

1. Glyn-Jones S, Palmer AJR, Agricola R, et al. Osteoarthritis. Lancet. 2015;386(9991):376–87.
2. Nefla M, Holzinger D, Berenbaum F, Jacques C. The danger from within: alarmins in arthritis. Nat Rev Rheumatol. 2016;12(11):669–83.
3. Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: an update with relevance for clinical practice. Lancet. 2011;377(9783):2115–26.
4. Mobasheri A, Rayman MP, Gualillo O, Sellam J, van der Kraan P, Fearon U. The role of metabolism in the pathogenesis of osteoarthritis. Nat Rev Rheumatol. 2017;13(5):302–11.
5. Lane NE, Schnitzer TJ, Birbara CA, et al. Tanezumab for the treatment of pain from osteoarthritis of the knee. N Engl J Med. 2010;363(16):1521–31.
6. Edwards JJ, Khanna M, Jordan KP, Jordan JL, Bedson J, Dziedzic KS. Quality indicators for the primary care of osteoarthritis: a systematic review. Ann Rheum Dis. 2015;74(3):490–8.
7. Pitsillides AA, Beier F. Cartilage biology in osteoarthritis—lessons from developmental biology. Nat Rev Rheumatol. 2011;7(11):654–63.
8. van der Kraan PM, van den Berg WB. Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? Osteoarthritis Cartilage. 2012;20(3):223–32.
9. Yahara Y, Takemori H, Okada M, et al. Pterosin B prevents chondrocyte hypertrophy and osteoarthritis in mice by inhibiting Sik3. Nat Commun. 2016;7:10959.
10. Pap T, Korb-Pap A. Cartilage damage in osteoarthritis and rheumatoid arthritis—two unequal siblings. Nat Rev Rheumatol. 2015;11(10):606–15.
11. Poole AR, Kobayashi M, Yasuda T, et al. Type II collagen degradation and its regulation in articular cartilage in osteoarthritis. Ann Rheum Dis. 2002; 61 Suppl 2:ii78–ii81.
12. Zhen G, Wen C, Jia X, et al. Inhibition of TGF-beta signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis. Nat Med. 2013;19(6):704–12.
13. Funck-Brentano T, Cohen-Solal M. Subchondral bone and osteoarthritis. Curr Opin Rheumatol. 2015;27(4):420–6.
14. Raynauld JP, Martel-Pelletier J, Berthiaume MJ, et al. Correlation between bone lesion changes and cartilage volume loss in patients with osteoarthritis of the knee as assessed by quantitative magnetic

resonance imaging over a 24-month period. Ann Rheum Dis. 2008;67(5):683–8.

15. Krasnokutsky S, Belitskaya-Lévy I, Bencardino J, et al. Quantitative magnetic resonance imaging evidence of synovial proliferation is associated with radiographic severity of knee osteoarthritis. Arthritis Rheum. 2011;63(10):2983–91.
16. Goldring SR, Goldring MB. Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. Nat Rev Rheumatol. 2016;12(11):632–44.
17. Karsdal MA, Bay-Jensen AC, Lories RJ, et al. The coupling of bone and cartilage turnover in osteoarthritis: opportunities for bone antiresorptives and anabolics as potential treatments? Ann Rheum Dis. 2014;73(2):336–48.
18. Pouran B, Arbabi V, Bleys RL, René Van Weeren P, Zadpoor AA, Weinans H. Solute transport at the interface of cartilage and subchondral bone plate: effect of micro-architecture. J Biomech. 2017;52:148–54.
19. Prasadam I, Crawford R, Xiao Y. Aggravation of ADAMTS and matrix metalloproteinase production and role of ERK1/2 pathway in the interaction of osteoarthritic subchondral bone osteoblasts and articular cartilage chondrocytes—possible pathogenic role in osteoarthritis. J Rheumatol. 2012;39(3):621–34.
20. Conaghan PG, Vanharanta H, Dieppe PA. Is progressive osteoarthritis an atheromatous vascular disease? Ann Rheum Dis. 2005;64(11): 1539–41.
21. Dyke JP, Aaron RK. Noninvasive methods of measuring bone blood perfusion. Ann N Y Acad Sci. 2010;1192(1):95–102.
22. Imhof H, Breitenseher M, Kainberger F, Trattnig S. Degenerative joint disease: cartilage or vascular disease? Skelet Radiol. 1997;26(7):398–403.
23. Imhof H, Breitenseher M, Kainberger F, Rand T, Trattnig S. Importance of subchondral bone to articular cartilage in health and disease. Top Magn Reson Imaging. 1999;10(3):180–92.
24. Aaron RK, Dyke JP, Ciombor DM, et al. Perfusion abnormalities in subchondral bone associated with marrow edema, osteoarthritis, and avascular necrosis. Ann N Y Acad Sci. 2007;1117(1):124–37.
25. Pesesse L, Sanchez C, Delcour JP, et al. Consequences of chondrocyte hypertrophy on osteoarthritic cartilage: potential effect on angiogenesis. Osteoarthritis Cartilage. 2013;21(12):1913–23.
26. Sanchez C, Deberg M, Msika P, Baudoin C, Henrotin Y. Study of in vitro conditions promoting hypertrophic differentiation of osteoarthritic articular chondrocytes [Abstract]. Osteoarthritis Cartilage. 2007; 15 Suppl C: C109–10.
27. Goldring MB, Goldring SR. Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. Ann N Y Acad Sci. 2010;1192(1):230–7.
28. Pan J, Wang B, Li W, Zhou X, Scherr T, Yang Y, Price C, Wang L 2012 Elevated cross-talk between subchondral bone and cartilage in osteoarthritic joints. Bone 51(2):212–217.
29. Zoncu R, Efeyan A, Sabatini DM 2011 mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol 12(1):21–35.
30. Perl A 2016 Activation of mTOR (mechanistic target of rapamycin) in rheumatic diseases. Nat Rev Rheumatol 12(3):169–82.
31. Pal B, Endisha H, Zhang Y, Kapoor M. mTOR: a potential therapeutic target in osteoarthritis? Drugs R D. 2015;15(1):27–36.
32. Matsuzaki T, Matsushita T, Tabata Y, et al. Intra-articular administration of gelatin hydrogels incorporating rapamycin-micelles reduces the development of experimental osteoarthritis in a murine model. Biomaterials. 2014;35(37):9904–11.
33. Zhang Y, Vasheghani F, Li YH, et al. Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis. Ann Rheum Dis. 2015;74(7):1432–40.
34. Carames B, Hasegawa A, Taniguchi N, Miyaki S, Blanco FJ, Lotz M. Autophagy activation by rapamycin reduces severity of experimental osteoarthritis. Ann Rheum Dis. 2012;71(4):575–81.
35. Zhang H, Wang H, Zeng C, et al. mTORC1 activation downregulates FGFR3 and PTH/PTHrP receptor in articular chondrocytes to initiate osteoarthritis. Osteoarthritis Cartilage. 2017;25(6):952–63.
36. Yan B, Zhang Z, Jin D, et al. mTORC1 regulates PTHrP to coordinate chondrocyte growth, proliferation and differentiation. Nat Commun. 2016;7:11151.
37. Schwabe K, Garcia M, Ubieta K, et al. Inhibition of osteoarthritis by adipose-derived stromal cells overexpressing Fra-1 in mice. Arthritis Rheumatol. 2016;68(1):138–51.
38. Zhen G, Cao X. Targeting TGFβ signaling in subchondral bone and articular cartilage homeostasis. Trends Pharmacol Sci. 2014;35(5): 227–36.
39. Talbot J, Bianchini FJ, Nascimento DC, et al. CCR2 expression in neutrophils plays a critical role in their migration into the joints in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(7):1751–9.
40. Huang B, Wang W, Li Q, et al. Osteoblasts secrete Cxcl9 to regulate angiogenesis in bone. Nat Commun. 2016;7:13885.
41. Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature. 2014;507(7492):323–8.
42. Xie H, Cui Z, Wang L, et al. PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis. Nat Med. 2014;20(11):1270–8.
43. Mapp PI, Walsh DA. Mechanisms and targets of angiogenesis and nerve growth in osteoarthritis. Nat Rev Rheumatol. 2012;8(7):390–8.
44. Hamilton JL, Nagao M, Levine BR, Chen D, Olsen BR, Im HJ. Targeting VEGF and its receptors for the treatment of osteoarthritis and associated pain. J Bone Miner Res. 2016;31(5):911–24.
45. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum. 2012;64(6): 1697–707.
46. Cui Z, Crane J, Xie H, et al. Halofuginone attenuates osteoarthritis by inhibition of TGF-beta activity and H-type vessel formation in subchondral bone. Ann Rheum Dis. 2016;75(9):1714–21.
47. Ramasamy SK, Kusumbe AP, Wang L, Adams RH. Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. Nature. 2014;507(7492):376–80.
48. Elkin M, Miao HQ, Nagler A, et al. Halofuginone: a potent inhibitor of critical steps in angiogenesis progression. FASEB.J. 2000;14(15): 2477–85.
49. Huang MJ, Wang L, Jin DD, et al. Enhancement of the synthesis of n-3 PUFAs in fat-1 transgenic mice inhibits mTORC1 signalling and delays surgically induced osteoarthritis in comparison with wild-type mice. Ann Rheum Dis. 2014;73(9):1719–27.
50. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. Cell. 2017;169(2):361–71.
51. Chen Y, Wang T, Guan M, et al. Bone turnover and articular cartilage differences localized to subchondral cysts in knees with advanced osteoarthritis. Osteoarthritis Cartilage. 2015;23(12):2174–83.
52. Dieppe PA, L ohmander LS. Pathogenesis and management of pain in osteoarthritis. Lancet. 2005;365(9463):965–73.