Low-intensity pulsed ultrasound inhibits VEGFA expression in chondrocytes and protects against cartilage degeneration in experimental osteoarthritis

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Osteoarthritis (OA) is the most common joint disease, which affects approximately 40% of the world population aged > 70 years [1]. OA is pathologically characterised by articular cartilage degeneration, synovial inflammation, subchondral bone remodelling and osteophyte formation [2]. The main clinical manifestations of OA patients are joint swelling and pain, which markedly affect the patients’ quality of life [3]. However, due to the complicated

Low-intensity pulsed ultrasound (LIPUS), a noninvasive physical therapy, was recently demonstrated to be an effective treatment for osteoarthritis (OA). Vascular endothelium growth factor A (VEGFA) has been found to be upregulated in the articular cartilage, synovium and subchondral bone of OA patients, leading to cartilage degeneration, synovitis and osteophyte formation. However, the functions and mechanisms of LIPUS in regulating chondrocyte-derived VEGFA expression are still unclear. In this study, we investigated whether LIPUS attenuated OA progression by (a) decreasing the percentage of VEGFA-positive cells in mouse articular cartilage destabilised through medial meniscus surgery and (b) relieving interleukin-1β-induced VEGFA expression in mouse primary chondrocytes. However, this function was negated by a p38 mitogen-activated protein kinase (p38 MAPK) inhibitor. In addition, we found that LIPUS ameliorated VEGFA-mediated disorders in cartilage extracellular matrix metabolism and chondrocyte hypertrophy during OA development. In conclusion, our data indicate a novel effect of LIPUS in regulating the expression of osteoarthritic chondrocyte-derived VEGFA through the suppression of p38 MAPK activity.

Abbreviations
DMM, destabilisation of the medial meniscus; ECM, extracellular matrix; IL-1β, interleukin-1β; LIPUS, low-intensity pulsed ultrasound; MMP-13, metalloproteinase-13; OA, osteoarthritis; p38 MAPK, p38 mitogen-activated protein kinase; VEGFA, vascular endothelium growth factor A.

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pathogenesis of OA, the current therapies focus on pain control or total knee replacement [4]. Therefore, developing noninvasive methods of OA treatment would be more ideal for patients in the future.

Low-intensity pulsed ultrasound (LIPUS) is a noninvasive and safe physical therapy [5], which has been widely used in promoting the healing of fresh bone fracture and nonunited fracture [6–8]. Recent clinical trials and basic researches initially confirmed that LIPUS can alleviate OA progression while reducing joint cartilage damage and relieving joint pain [9–12]. A further study demonstrated that LIPUS reduces cartilage damage by promoting chondrocyte proliferation and cartilage matrix secretion [12,13]. However, the comprehensive functions of LIPUS in OA are largely unknown. LIPUS has been clinically used as a supplemental therapy for promoting the healing of bone fracture and wound [14]. Moreover, a related study has reported that LIPUS increases the vascular endothelium growth factor A (VEGFA) level of periosteal cells in a mouse femur fracture model, facilitating local angiogenesis and promoting fracture healing [15]. However, articular cartilage is a nonvascular tissue, which is composed of extracellular matrix (ECM) and chondrocytes [16]. VEGFA is barely expressed by healthy cartilage; however, markedly increased VEGFA expression is related to the severity of OA [17]. In addition, injecting VEGFA into both keen and temporomandibular joint induced OA phenotype in mice [18,19]. Correspondingly, another study reported that either deleting VEGFA in Col II-Cre lineage cells or intra-articular injecting anti-VEGFA antibody attenuated progression of surgically induced OA in mice [20]. These findings suggest that controlling VEGFA secretion may help prevent OA escalation and OA-associated joint deterioration. However, the regulation of VEGFA expression in OA articular chondrocytes by LIPUS is still not explicated.

To address this issue, we investigated whether LIPUS attenuated OA progression by decreasing the percentage of VEGFA-positive cells in mouse articular cartilage with destabilisation of the medial meniscus (DMM) surgery and relieving interleukin-1β (IL-1β)-induced VEGFA expression in mouse primary chondrocytes.

Materials and methods

Isolation and culture of mouse primary chondrocytes

Primary chondrocytes were isolated from the knee articular cartilage of 5-day-old mice. Knee joints were first digested by 0.25% trypsinase (Gibco/Life Technologies, Carlsbad, CA, USA) at 37 °C for 15 min, and adjacent muscles, ligaments and bone tissues were removed using a stereomicroscope (Olympus BX51, Tokyo, Japan). Chondrocytes were isolated from the cartilage by additional digestion with 0.1% collagenase II (Gibco/Life Technologies) overnight at 37 °C in a 5% CO2 incubator [21]. Cells were seeded in 35-mm-diameter dishes and cultured in Dulbecco’s modified Eagle’s medium/F12 (1 : 1) supplemented with 1% penicillin/streptomycin (HyClone, Logan, UT, USA) and 10% FBS (HyClone) and incubated in a humidified atmosphere of 5% CO2 at 37 °C. The culture medium was changed every 2 days.

Cells treated with LIPUS

We treated chondrocytes cultured in 35-mm-diameter dishes with LIPUS (Exogen 4000; Smith & Nephew, Jericho, NY, USA) at an average intensity of 30 mW·cm−2, frequency of 1.5 MHz, pulse repetition rate of 1 kHz and the on–off ratio of 20% for 20 min (Fig. 1A,B). The stimulations were conducted in a sterile environment at room temperature. The acoustic gel (< 1 mm thick) was used as a coupler between the transducer and cell plate to ensure optimal ultrasound exposure [22].

Real-time polymerase chain reaction

Total RNA was isolated from chondrocytes using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and used to generate cDNA template for real-time PCR, which was carried out on the Mx3000P system (Exscript, Takara, Japan) using the SYBR Green RT-PCR Kit (Takara Bio, Shiga, Japan). All samples were measured in triplicate, and the cyclophilin was amplified as an internal control. The primer sequences for RT–PCR are shown in Table 1.

ELISA

The chondrocytes’ culture supernatant was collected and operated according to the instructions of the Mouse VEGFA ELISA Kit (Beyotime, Shanghai, China). The absorbance at 450-nm wavelength was measured with a microplate reader, and the expression of VEGFA of chondrocytes was calculated according to the standard curve.

Western blotting

Mice chondrocyte cultures were extracted with RIPA lysis buffer containing protease inhibitors (Roche, West Sussex, UK). Proteins were resolved by 12% SDS/PAGE electrophoresis and transferred to a poly(vinylidene difluoride) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% nonfat milk in TBST buffer, probed with primary antibodies specific for phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK, 1 : 1000;
Ten-week-old male C57BL/6J mice were purchased from HuaFukang Biotechnology Company (Beijing, China). DMM surgery was performed on the right knee joints of the 10-week-old male mice according to the procedure described in a previous study [23]. The mice were first anaesthetised (1% pentobarbital sodium), the joint capsule was incised, and then the medial meniscotibial ligament was sectioned by using microsurgical scissors. As a control, sham operation was performed on the left knee joint with medial capsulotomy only, and the joints were categorised into the Sham group. Animals were randomly divided into the DMM and DMM + LIPUS groups (n = 7, each group), maintained in the animal facility (specific pathogen-free) of Daping Hospital and allowed to move freely in the cages. All animal protocols were approved by the Institutional Animal Care and Use Committee of Daping Hospital (Chongqing, China, No: SYXK-PLA-2017-0058).

**Animals treated with LIPUS**

In order to avoid wound infection, we began to treat the animals with LIPUS on the third day after the DMM surgery. Mice were anaesthetised, the hair around the knee joint was shaved, and then a layer of coupling agent was applied between the LIPUS probe and mice joint (Fig. 1C, D). Mice underwent treatment with LIPUS at an average intensity of 30 mW/cm², frequency of 1.5 MHz, pulse repetition rate of 1 kHz and the on–off ratio of 20% for 20 min per day, as well as therapeutic parameters in clinic, once daily for 6 days as one cycle, which was continued for two cycles. We affixed the LIPUS device to the DMM group without electricity at the same time [11].

**Specimen preparation**

Mice were sacrificed by CO₂ inhalation at 8 and 12 weeks after the DMM surgery, knee joints were fixed in 4% paraformaldehyde, decalcified in 20% formic acid and embedded in paraffin.
Serial sagittal sections were obtained across the entire joint by collecting 5-µm sections. Sections were stained with Safranin O–Fast Green for histological analysis. The intervening sections were used for the immunohistochemical analysis.

**Immunohistochemical analysis**

Knee joint sections were deparaffinised with xylene, and endogenous peroxidase activity was quenched by 3% H2O2, followed by antigen retrieval with 0.1% trypsinisation. Sections were then blocked with goat serum and incubated at 4 °C overnight with primary antibody followed by the appropriate biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin–biotin staining. Immunoreactivity was visualised with a 3,3′-diaminobenzidine tetrahydrochloride kit (ZSGB-BIO) followed by counterstaining with methyl green. Primary antibodies against the following proteins were used: VEGFA (1 : 200; Abcam, Cambridge, MA, USA), collagen II (1 : 200; Chondrex, Redmond, WA, USA), aggrecan (1 : 100; Abcam) and metalloproteinase-13 (MMP-13, 1 : 200; Abcam). The number of immunoreactive cells in the sections was counted using IM‐AGE‐PRO PLUS 6.0 (Media Cybernetics, Rockville, MD, USA).

**Histologic assessment of articular cartilage degeneration**

Histological changes in the medial tibial plateau (MTP) and medial femoral condyle (MFC) of the knee joints were scored on a scale of 0–6 according to the recommendations of the Osteoarthritis Research Society International (OARSI) [24]. The maximum scores (the highest score in all slides) and summed scores (sum of the four highest scores in all slides) for the medial femora and medial tibiae were calculated separately to evaluate the severity of cartilage destruction. Scoring was carried out by three independent investigators, and all investigators were blinded to the allocation during experiments.

**Statistical analysis**

Statistical analysis was performed using GRAPHPAD PRISM v.6.01 software (GraphPad Inc, La Jolla, CA, USA). Results were presented as mean ± SEM. Mean differences between two groups were analysed with the Student’s t‐test. Statistical analysis for multicomparisons was performed by one‐way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001).

**Results**

**LIPUS directly reduces the expression of VEGFA and catabolic genes in IL-1β-treated mouse primary chondrocytes**

To clarify whether LIPUS directly regulates the expression of VEGFA in chondrocytes, we used mouse primary chondrocytes stimulated by IL-1β to establish the OA model in vitro. IL-1β has been well known to play a key role in the degradation of articular cartilage by inhibiting ECM synthesis and accelerating cartilage breakdown [25]. Real‐time PCR and the ELISA results showed that LIPUS significantly alleviated the IL-1β‐induced VEGFA expression at both mRNA (Fig. 2A) and protein (Fig. 2B) levels. However, there was no significant difference in normal chondrocytes with or without LIPUS treatment (Fig. 2A,B). Previous studies have demonstrated that VEGFA accelerates OA progression by promoting cartilage matrix degradation and chondrocyte hypertrophy [18]. In mouse primary chondrocytes treated with IL-1β, LIPUS significantly downregulated the levels of MMP-13 and collagen X, as well as increased the expression of collagen II (Fig. 2C–E). These results demonstrated that LIPUS directly reduced the expression of VEGFA and inhibited catabolic events of cartilage matrix in IL-1β-treated chondrocytes.

**LIPUS inhibits IL-1β-induced VEGFA expression by decreasing the phosphorylation of p38 MAPK**

Previous researches have shown that the abnormal activation of p38 MAPK and JNK signalling pathways is associated with increased VEGFA expression in osteoarthritic chondrocytes [26,27]. Therefore, through western blotting, we analysed phosphorylated and total protein levels of p38 MAPK and JNK in the total cell lysates of mouse primary chondrocytes cultured in the absence or presence of IL-1β and LIPUS. We found that IL-1β significantly increased the expression of p-p38 MAPK and phosphorylated JNK (p-JNK) compared with the control. Moreover, LIPUS attenuated IL-1β-upregulated p-p38 MAPK protein levels of the primary chondrocytes (Fig. 3A), but it demonstrated no significant regulation of p-JNK expression (Fig. 3A). These results indicated that LIPUS inhibits IL-1β-induced abnormal activation of the p38 MAPK signalling pathway.

To investigate whether pharmacological inhibition of p38 MAPK signalling could attenuate the downregulated VEGFA caused by LIPUS in IL-1β‐treated chondrocytes, we pretreated chondrocytes with SB203580, a p38 MAPK inhibitor, which suppresses it by inhibiting the activity of phosphoinositide‐dependent kinase-1. The qPCR results displayed that the presence of SB203580 negated the effects of LIPUS on VEGFA expression (Fig. 3B). This demonstrated that LIPUS abrogated IL-1β‐induced VEGFA expression partially by regulating the p38 MAPK pathway in the articular chondrocytes.
LIPUS decreases the expression of VEGFA and cartilage matrix loss in vivo

To investigate whether LIPUS modulated the expression of VEGFA in vivo, we performed DMM surgery on the right knee joint of 10-week-old male C57BL/6J mice, which is relevant to the actual state of human OA. After the DMM surgery, the mice received LIPUS treatment for 2 weeks and then were sacrificed at 8 weeks after DMM. We performed IHC staining to examine the expression of VEGFA, MMP13,
aggrecan and type II collagen (collagen II). The results showed that LIPUS treatment significantly decreased the percentage of VEGFA-positive cells in the articular cartilage (Fig. 4A,E). Furthermore, the IHC results showed that the LIPUS relieved loss of aggrecan and collagen II as well as reduced the expression of MMP-13, which is an essential cartilage matrix-degrading enzyme (Fig. 4B–D,F). Collectively, these results suggested that LIPUS reduced the expression of VEGFA in the articular cartilage and alleviated the loss of cartilage matrix in the DMM-induced OA model.

LIPUS attenuates the cartilage degeneration in the DMM model

We further examined the therapeutic role of LIPUS in the development of OA. In the DMM model, Safranin O–Fast Green staining showed a significant reduction in hypertrophic chondrocytes, proteoglycan loss and articular cartilage degeneration in mice treated with LIPUS compared with control mice at 12 weeks after surgery (Fig. 5A). The OARSI histologic scoring system was applied to quantitatively analyse the cartilage degeneration in each group. The summed and maximal OARSI scores of the femurs and tibiae demonstrated that LIPUS-treated mice had a significantly lower score than the DMM mice at 12 weeks after the surgery (Fig. 5B,C). These results suggested that LIPUS can significantly delay the progression of DMM-induced knee OA in mice.

Discussion

In this study, we explored a previously unrecognised effect of LIPUS on the inhibition of chondrocyte-derived VEGFA in OA. We proved that LIPUS directly reduces the expression of VEGFA in IL-1β-treated mouse primary chondrocytes and downregulates VEGFA-positive cells in the articular cartilage of the DMM mice model.

LIPUS, a noninvasive physical therapy, was recently used in clinical settings to relieve pain and improve the quality of life of patients with knee OA [9]. However, the mechanisms involved in LIPUS-treated OA are largely unknown. A previous study reported that LIPUS facilitated fracture healing by inducing VEGFA expression in periosteal cells and inducing local angiogenesis [15]. Meanwhile, in a steroid-associated osteonecrosis rat model, LIPUS promoted bone repair by increasing BMP2 rather than VEGFA expression in the femoral head [28]. Besides, LIPUS facilitated bone–tendon junction repair through the upregulated VEGFA expression of chondrocytes and osteoblasts in woven bone [29]. However, during rat meniscal

**Fig. 4.** Effects of LIPUS on VEGFA and articular cartilage homeostasis in the mouse after DMM. The knee joints were harvested at 8 weeks after surgery and immunohistochemically stained for VEGFA (A), MMP-13 (B), aggrecan (C) and collagen II (D) expression in the DMM and DMM + LIPUS groups (scale bar: 100 µm). The percentage of VEGFA-positive cells (E) and MMP-13-positive cells (F) of the articular cartilage in each group. Statistical analyses were performed using Student’s t-test. Data are expressed as the mean ± SEM (n = 5 mice per group). **P < 0.01, ***P < 0.001.
healing, LIPUS promotes the migration and the VEGFA expression of meniscus cells in the outer meniscal region, but in the inner region with avascular tissue as articular cartilage, the modulation of VEGFA expression by LIPUS is still unknown [30]. Therefore, LIPUS may regulate VEGFA expression in a cell-intrinsic manner under different pathophysiological situations.

To date, numerous studies have suggested that VEGFA plays an important role in cartilage development and OA progression [17,20,31–33]. Articular cartilage is a special and avascular tissue, which is composed of chondrocytes and ECM [34]. VEGFA is barely expressed by healthy articular cartilage, whereas it acts as a promotive factor in the chain of events leading to OA [17]. In knee OA patients, the expression of VEGFA in the articular cartilage, synovial fluid and synovium has been found to be significantly correlated with the grade of OA severity and the degree of pain [31]. Moreover, intra-articular administration of the anti-VEGFA antibody or reduction in collagen II lineage cell-derived VEGFA attenuated the progression of surgically induced OA in rabbit or mice, respectively [20,35]. The above-mentioned studies combined with our findings revealed that the therapeutic effects of LIPUS on OA may partially be mediated by downregulation of the expression of VEGFA in the articular cartilage.

Previous researches have shown that intra-articular injection of VEGFA in the knee and temporo-mandibular joints of mice induced cartilage degeneration, which is correlated with subchondral bone sclerosis and increased expression of metalloproteinases and loss of cartilage matrix [18,19]. We have confirmed that LIPUS downregulated MMP-13 expression and relieved loss of collagen II in both the articular cartilage and synovium of OA mice. This finding suggests that LIPUS may have direct effects on the articular cartilage and synovium, which may contribute to the therapeutic effects of LIPUS on OA.
Moreover, we also noticed that LIPUS inhibited the IL-1β-induced expression of collagen X, which is considered as the standard marker of chondrocyte hypertrophy. Previously, it was shown that VEGFA inhibition attenuated the expression of collagen X in chondrocytes both in a surgery-induced rat OA model and in TNF-α-induced hypertrophic chondrocyte model [38]. Therefore, we speculated that LIPUS ameliorated VEGFA-mediated disorder in cartilage ECM metabolism and chondrocyte hypertrophy during OA development.

Evidences demonstrated that abnormal activation of p38 MAPK signalling pathway in OA chondrocytes was directly involved in the upregulation of VEGFA [26,27]. However, we found that LIPUS significantly inhibited the activation of p38 MAPK signalling in chondrocytes treated with IL-1β. A previous study has suggested that the p38 MAPK is a major signalling molecule in LIPUS-induced cartilage matrix maintenance in the rabbit OA model [39]. We also showed that SB203580, a p38 MAPK inhibitor, negated the effects of LIPUS on VEGFA expression in IL-1β-treated chondrocytes. Our findings suggested that LIPUS reduced VEGFA expression in OA chondrocytes by inhibiting p38 MAPK signalling to maintain cartilage homeostasis during OA progression (Fig. 5D).

In summary, our findings highlight the novel effect of LIPUS on regulating the expression of osteoarthritic chondrocyte-derived VEGFA. We found that LIPUS directly protects articular cartilage from degeneration by inhibiting VEGFA expression, and this effect is mainly exerted through the inhibition of p38 MAPK signalling. However, the exact mechanisms underlying this phenomenon warrant further study.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

DQB, XLD, MTG and YZ conceived and designed the project; MTG, YZ, BL, BZ and JLH acquired the data; MTG, YZ and HBQ analysed and interpreted the data; MTG, YZ and QYT wrote the paper; all authors were involved in checking the paper and contributed to the preparation of the final manuscript. All authors read and approved the final manuscript.

References

1. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V et al. (2012) Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380, 2163–2196.

2. Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H and Carr AJ (2015) Osteoarthritis. Lancet 386, 376–387.

3. Brown JP and Boulay LJ (2013) Clinical experience with duloxetine in the management of chronic musculoskeletal pain. A focus on osteoarthritis of the knee. Ther Adv Musculoskelet Dis 5, 291–304.

4. Bay-Jensen AC, Thudium CS, Gualillo O and Mobasheri A (2018) Biochemical marker discovery, testing and evaluation for facilitating OA drug discovery and development. Drug Discov Today 23, 349–358.

5. Claes L and Willie B (2007) The enhancement of bone regeneration by ultrasound. Prog Biophys Mol Biol 93, 384–398.

6. Malizos KN, Hantes ME, Protopappas V and Papachristos A (2006) Low-intensity pulsed ultrasound for bone healing: an overview. Injury 37, S56–S62.

7. Fung CH, Cheung WH, Pounder NM, de Ana FJ, Harrison A and Leung KS (2014) Investigation of rat bone fracture healing using pulsed 1.5 MHz, 30 mW/cm² burst ultrasound–axial distance dependency. Ultrasonomics 54, 850–859.

8. Manaka S, Tanabe N, Kariya T, Naito M, Takayama T, Nagao M, Liu D, Ito K, Maeno M, Suzuki N et al. (2015) Low-intensity pulsed ultrasound-induced ATP increases bone formation via the P2X7 receptor in osteoblast-like MC3T3-E1 cells. FEBS Lett 589, 310–318.

9. Jia L, Wang Y, Chen J and Chen W (2016) Efficacy of focused low-intensity pulsed ultrasound therapy for the management of knee osteoarthritis: a randomized, double blind, placebo-controlled trial. Sci Rep 6, 35453.

10. Sekino J, Nagao M, Kato S, Sakai M, Abe K, Nakayama E, Sato M, Nagashima Y, Hino H, Tanabe N (2018) Low-intensity pulsed ultrasound induces cartilage matrix synthesis and reduced MMP13
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expression in chondrocytes. Biochem Biophys Res Commun 506, 290–297.

11 Zhang B, Chen H, Ouyang J, Xie Y, Chen L, Tan Q, Du X, Su N, Ni Z and Chen L (2019) SQSTM1-dependent autophagic degradation of PKM2 inhibits the production of mature IL1B/IL-1β and contributes to LIPUS-mediated anti-inflammatory effect. Autophagy 1–17.

12 Nishida T, Kubota S, Aoyama E, Yamanaka N, Lyons KM and Takigawa M (2017) Low-intensity pulsed ultrasound (LIPUS) treatment of cultured chondrocytes stimulates production of CCN family protein 2 (CCN2), a protein involved in the regeneration of articular cartilage: mechanism underlying this stimulation. Osteoarthritis Cartilage 25, 759–769.

13 Rothenberg JB, Jayaram P, Naqvi U, Gober J and Nishida T, Kubota S, Aoyama E, Yamanaka N, Lyons KM and Takigawa M (2017) The role of low-intensity pulsed ultrasound on cartilage healing in knee osteoarthritis: a review. Phys Med Rehabil 9, 1268–1277.

14 Harrison A, Lin S, Pounder N and Mikuni-Takagaki Y (2016) Mode & mechanism of low intensity pulsed ultrasound (LIPUS) in fracture repair. Ultrasonics 70, 45–52.

15 Uchida K, Urake K, Naruse K, Mikuni-Takagaki Y, Inoue G and Takaso M (2016) 5. Accelerated fracture healing targeting periosteal cells: possibility of combined therapy of low-intensity pulsed ultrasound (lipus), bone graft, and growth factor (bFGF). J Orthop Trauma 30, S3.

16 Sophia FAJ, Bedi A and Rdeo SA (2009) The basic science of articular cartilage: structure, composition, and function. Sports Health 1, 461–468.

17 Saito T, Fukai A, Mabuchi A, Ikeda T, Yano F, Obha S et al. (2010) Transcriptional regulation of endochondral ossification by HIF-2alpha during skeletal growth and osteoarthritis development. Nat Med 16, 678–686.

18 Ludin A, Sela JJ, Schroeder A, Samuni Y, Nitzan DW and Amir G (2013) Injection of vascular endothelial growth factor into knee joints induces osteoarthritis in mice. Osteoarthritis Cartilage 21, 491–497.

19 Shen P, Jiao Z, Zheng JS, Xu WF, Zhang SY, Qin A and Yang C (2015) Injecting vascular endothelial growth factor into the temporomandibular joint induces osteoarthritis in mice. Sci Rep 5, 16244.

20 Nagao M, Hamilton JL, Ke R, Berendsen AD, Duan X, Cheong CW Li X, Im H-J and Olsen BR (2017) Vascular endothelial growth factor in cartilage development and osteoarthritis. Sci Rep 7, 13027.

21 Gosset M, Berenbaum F, Thirion S and Jacques C (2008) Primary culture and phenotyping of murine chondrocytes. Nat Protoc 3, 1253–1260.

22 Uddin SM and Qin YX (2013) Enhancement of osteogenic differentiation and proliferation in human mesenchymal stem cells by a modified low intensity ultrasound stimulation under simulated microgravity. PLoS ONE 8, e73914.

23 Glasson SS, Blanchet TJ and Morris EA (2007) The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. Osteoarthritis Cartilage 15, 1061–1069.

24 Glasson SS, Chambers MG, Van Den Berg WB and Little CB (2010) The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis Cartilage 18, S17–S23.

25 Joos H, Wildner A, Hogrefe C, Reichel H and Brenner RE (2013) Interleukin-1 beta and tumor necrosis factor alpha inhibit migration activity of chondrogenic progenitor cells from non-fibrillated osteoarthritic cartilage. Arthritis Res Ther 15, R119.

26 Murata M, Yudoh K, Nakamura H, Kato T, Inoue K, Chiba J, Nishioka K and Masuko-Hongo K (2006) Distinct signaling pathways are involved in hypoxia- and IL-1-induced VEGF expression in human articular chondrocytes. J Orthop Res 24, 1544–1554.

27 Nakashima M, Sakai T, Hiraiva H, Hamada T, Omachi T, Ono Y, Inukai N, Ishizuka S, Matsukawa T, Oda T et al. (2012) Role of S100A12 in the pathogenesis of osteoarthritis. Biochem Biophys Res Commun 422, 508–514.

28 Zhu H, Cai X, Lin T, Shi Z and Yan S (2015) Low-intensity pulsed ultrasound enhances bone repair in a rabbit model of steroid-associated osteonecrosis. Clin Orthop Relat Res 473, 1830–1839.

29 Lu H, Qin L, Cheung W, Lee K, Wong W and Leung K (2008) Low-intensity pulsed ultrasound accelerated bone-tendon junction healing through regulation of vascular endothelial growth factor expression and cartilage formation. Ultrasound Med Biol 34, 1248–1260.

30 Kamatsuki Y, Aoyama E, Furumatsu T, Miyazawa S, Maehara A, Yamanaka N, Nishida T, Kubota S, Ozaki T and Takigawa M (2018) Possible reparative effect of low-intensity pulsed ultrasound (LIPUS) on injured meniscus. J Cell Commun Signal 13, 193–207.

31 Hamilton JL, Nagao M, Levine BR, Chen D, Olsen BR and Im HJ (2016) Targeting VEGF and its receptors for the treatment of osteoarthritis and associated pain. J Bone Miner Res 31, 911–924.

32 Maes C (2017) Signaling pathways effecting crosstalk between cartilage and adjacent tissues: seminars in cell and developmental biology: the biology and pathology of cartilage. Semin Cell Dev Biol 62, 16–33.

33 Pfander D, Körtje D, Zimmermann R, Wesołog H, Kirsch T, Gesslein M, Cramer T and Swoboda B (2001) Vascular endothelial growth factor in articular cartilage of healthy and osteoarthritic human knee joints. Ann Rheum Dis 60, 1070–1073.

34 Jiang Y and Tuan RS (2015) Origin and function of cartilage stem/progenitor cells in osteoarthritis. Nat Rev Rheumatol 11, 206–212.
35 Li W, Jiang CY, Wang ZW and Xiao DM (2016) [Intraarticular injection of bevacizumab in treatment of osteoarthritis: a laboratory research on a rabbit model]. Beijing Da Xue Xue Bao Yi Xue Ban 48, 203–209.

36 Li X, Li J, Cheng K, Lin Q, Wang D, Zhang H, An H, Gao M and Chen A (2011) Effect of low-intensity pulsed ultrasound on MMP-13 and MAPKs signaling pathway in rabbit knee osteoarthritis. Cell Biochem Biophys 61, 427–434.

37 Naito K, Watari T, Muta T, Furuhata A, Iwase H, Igarashi M, Kurosawa H, Nagaoka I and Kaneko K (2010) Low-intensity pulsed ultrasound (LIPUS) increases the articular cartilage type II collagen in a rat osteoarthritis model. J Orthop Res 28, 361–369.

38 Zhang X, Crawford R and Xiao Y (2016) Inhibition of vascular endothelial growth factor with shRNA in chondrocytes ameliorates osteoarthritis. J Mol Med 94, 787–798.

39 Xia P, Ren S, Lin Q, Cheng K, Shen S, Gao M and Li X (2015) Low-intensity pulsed ultrasound affects chondrocyte extracellular matrix production via an integrin-mediated p38 MAPK signaling pathway. Ultrasound Med Biol 41, 1690–1700.