Matrix-targeted Nanoparticles for MMP13 RNA

Interference Blocks Post-Traumatic Osteoarthritis.

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Abstract

Osteoarthritis (OA) is a debilitating and prevalent chronic disease, but there are no approved disease modifying OA drugs (DMOADs), only pharmaceuticals for pain management. OA progression, particularly for post-traumatic osteoarthritis (PTOA), is associated with inflammation and enzymatic degradation of the extracellular matrix. In particular, Matrix Metalloproteinase 13 (MMP13) breaks down collagen type 2 (CII), a key structural component of cartilage extracellular matrix, and consequently, matrix degradation fragments perpetuate inflammation and a degenerative cycle that leads to progressive joint pathology. Here, we tested targeted delivery of endosome-escaping, MMP13 RNA interference (RNAi) nanoparticles (NPs) as a DMOAD. The new targeting approach pursued here deviates from the convention of targeting specific cell types (*e.g.*, through cell surface receptors) and instead leverages a monoclonal antibody (mAbCII) that targets extracellular CII that becomes uniquely accessible at early OA focal defects. Targeted mAbCII-siNPs create an *in situ* NP depot for retention and potent activity within OA joints. The mAbCII-siNPs loaded with MMP13 siRNA (mAbCII-siNP/siMMP13) potently suppressed MMP13 expression (95% silencing) in TNF α -stimulated chondrocytes *in vitro*, and the targeted mAbCII-siNPs had higher binding to trypsin-damaged porcine cartilage than untargeted control NPs. In an acute mechanical injury mouse model of PTOA, mAbCII-siNP/siMMP13 achieved 80% reduction in MMP13 expression ($p = 0.00231$), whereas a non-targeted control achieved only 55% silencing. In a more severe, PTOA model, weekly mAbCII-siNP/siMMP13 long-term treatment provided significant protection of cartilage integrity (0.45 ± 0.3 vs 1.6 ± 0.5 on the OARSI scale; $p=0.0166$), and overall joint structure (1.3 ± 0.6 vs 2.8 ± 0.2 on the Degenerative Joint Disease scale; $p<0.05$). Intra-articular mAbCII-siNPs better protected articular cartilage (OARSI score) relative to either single or weekly treatment with the clinical gold

stand steroid treatment methylprednisolone. Finally, multiplexed gene expression analysis of 254 inflammation-related genes showed that MMP13 inhibition suppressed clusters of genes associated with tissue restructuring, angiogenesis (associated with synovial inflammation and thickening), innate immune response, and proteolysis. This work establishes the new concept of targeting unique local extracellular matrix signatures to sustain retention and increase delivery efficacy of biologics with intracellular activity and also validates the promise of MMP13 RNAi as a DMOAD in a clinically-relevant therapeutic context.

Abstract Figure: PTOA targeted delivery of MMP13 siRNA to block disease progression.

The top left schematic illustrates the progression (left to right) from healthy knee joint, through inflammation induction following traumatic injury, to cartilage loss and degenerative joint disease (including synovial response). Degradation of cartilage enhances inflammation, inducing a degenerative cycle (middle right). The bottom of the graphic illustrates the concept of the matrix targeted nanocarriers for enhanced retention and activity of MMP13 siRNA at sites of cartilage injury.

Keywords:

Osteoarthritis, non-viral delivery, RNA interference, MMP13, antibody targeted drug delivery, matrix targeted drug delivery.

Introduction

Osteoarthritis (OA) is a chronic degenerative disease affecting joints and their surrounding tissues. Initial symptoms include pain and loss of mobility, resulting in significant loss in quality of life. The burden of OA on the United States healthcare system is estimated to be over \$44 billion annually in direct costs and up to double that in indirect economic burden (1). More than 25% of the American population over the age of 45 is afflicted with OA, and its prevalence is anticipated to increase over the next twenty years (2).

OA is a disease of the entire joint that involves a complex interplay between mechanical and biochemical factors (3). Some well-established risk factors include poor joint alignment or injury (4), obesity (5), genetic disposition (6), and aging (7). Multiple signaling molecules are known to be central to OA pathogenesis such as interleukin (IL-1 β), Wnt, c-Jun N-terminal kinase (JNK), and reactive oxygen species (ROS) (3, 8, 9). All of these signaling pathways independently

converge toward increased production of matrix metalloproteinases (MMPs), a step of critical importance in cartilage degradation and progression of OA symptoms (3).

Post-traumatic osteoarthritis (PTOA) is a form of OA induced by a mechanical joint injury. Common injuries include ligament and meniscal tears, cartilage damage, bone fractures from high impact landings, and dislocations. These injuries are particularly common among young athletes and military personnel and result in an accelerated pathology, requiring surgical intervention 7-9 years earlier on average than standard OA (10). Approximately 12% of all OA cases are PTOA in the United States, and PTOA tends to have greater cost relative to normal OA both in annual expenditure (~\$3 billion) and in quality adjusted life years (QALYs) due to its younger age of onset and accelerated progression (11). Following a PTOA-initiating injury, the mechanical disturbance of the extracellular matrix (ECM) stimulates synoviocytes and chondrocytes to produce inflammatory cytokines and MMPs (12). MMP13 is an enzyme able to catalytically degrade collagen II (CII), a key cartilage structural component. Degradation of CII disrupts chondrocytes by destroying the ECM in their surroundings and also releases soluble ECM degradation biproducts which have inflammatory signaling properties that trigger the OA degenerative cycle (3, 13). Inflammatory activation of the full joint and surrounding tissues (synovium and subchondral bone) perpetuates this cycle until cartilage destruction is complete. Because PTOA commonly has a defined and predictable initiating event, there is potential for early therapeutic intervention to block disease onset or progression at an early stage.

Current pharmaceutical management of OA is solely palliative, and no disease modifying OA drugs (DMOADs) are clinically approved. There are five FDA-approved corticosteroids for intra-articular OA therapy, but these therapies provide only temporary pain relief. Steroids do not

target the underlying cause of disease and are not recommended for long-term management(14), as they have been shown to actually cause cartilage volume loss (when given 4 times per year for 2 years)(15) and have associations with chondrotoxicity (16). MMP13 is a key proteolytic driver of cartilage loss in OA as indicated by reduced OA progression in surgically induced OA in MMP13 knockout mice and in wild type mice treated with broad MMP inhibitors (17). Unfortunately, clinical trials on MMP small molecules inhibitors (tested mostly for cancer treatment) have been suspended due to pain associated with musculoskeletal syndrome (MSS). Patient MSS is believed to be linked to systemic delivery of small molecules that non-selectively inhibit multiple MMPs, some of which (MMP2, 3, 4, 7 and 9) are involved in normal tissue homeostasis (18-20). Production of selective small molecule inhibitors is complicated by shared domains of the collagenases and the homology of the catalytic site (21). One tested MMP13 “specific” inhibitor PF152 reduced lesion severity in a canine PTOA model (22) but unfortunately caused nephrotoxicity believed to be caused by off-target effects on the human organic anion transporter 3 (which can be circumvented with our proposed RNAi due to sequence specificity and not structural specificity) (23). For these reasons, we hypothesize that selectively targeting MMP13 (which has not been associated with MSS) through delivery of a locally-retained therapy could be an effective and safe approach for blocking the degenerative PTOA process following joint injury.

Local, intraarticular injections are clinically-utilized in OA, yet face unique drug delivery challenges. One of the major barriers is that synovial fluid is continuously exchanged in the joint, causing most drugs to be rapidly cleared into the lymphatic system (24, 25). The synovial vasculature clears small molecules, while the lymphatics drain away macromolecules (26, 27), resulting in joint half-lives ranging 1-4 hours for commonly used steroids (25). These challenges leave an unmet need for OA therapies that are targeted and/or are better retained within the joint

after local injection. Targeting of nanoscale particles is one promising approach that has traditionally relied on using chondroitin sulfate, CII-binding peptides, and bisphosphonates (28-30). Chondroitin sulfate and CII-binding peptides target the cartilage matrix to tether/embed the particles and reduce convective transport through synovial fluid flow, while the bisphosphonates are focused on advanced cases of OA and target exposed subchondral bone. Extracellular substrate targeting has been minimally utilized for delivery of small molecules and has not been investigated, to our knowledge, for local retention of biologics with intracellular targets (31).

Here, we sought to target and retain delivery of RNAi therapy against MMP13 to sites of early cartilage damage in OA and to confirm that matrix targeting can provide functional benefit for delivery of intracellular-acting biologics. RNAi is critical in this application because siRNA can be designed to have selective complementarity with MMP13, obviating the enzyme selectivity concerns associated with small molecule inhibitors. The clinical utility of siRNA medicines has been validated by the recent clinical trial success and FDA approval of both Alnylam's ONPATRO™ (patisiran) for treatment of hereditary transthyretin-mediated amyloidosis and GIVLAARI™ (givosiran) for acute hepatic porphyria (32, 33). Here, we extended polymeric siRNA nanopolyplexes (siNPs) recently innovated by our research group (34-37) to develop the first targeted form of this carrier that binds to sites of early OA cartilage damage using a collagen type 2 monoclonal antibody (mAbCII). Cartilage CII is more exposed and accessible for binding after injury (38), and the mAbCII antibody has previously been used as a targeted nano-diagnostic for intravital measuring severity of OA (39). In the current report, we formulated and therapeutically tested mAbCII-targeted siNPs (mAbCII-siNPs) as a locally injectable system that creates an *in situ* depot of MMP13 RNAi nanomedicine in PTOA-afflicted joints. Matrix-targeted delivery of an intracellular-acting biologic such as siRNA represents a significant departure from

the convention of targeting internalizing cellular receptors. Herein, we validate the utility of this approach and prove it therapeutically significant as a DMOAD in a model of mechanical PTOA.

Results and discussion

Synthesis and conjugation of polymers

The mAbCII-siNPs were synthesized comprising an endosome-escaping, RNA-condensing core and a passivating, colloiddally-stabilizing poly (ethylene glycol) (PEG) surface amenable to antibody conjugation (**Figure 1A**). The diblock copolymer that is assembled to generate this system was synthesized through reversible addition-fragmentation chain transfer (RAFT) polymerization of a random copolymer of 50 mol% 2-(dimethylamino)ethyl methacrylate (DMAEMA) and 50 mol% butyl methacrylate (BMA) from a carboxy-PEG-ECT (4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid) macro-chain transfer agent (macro-CTA) and verified by NMR (**Figure S1, S2**). The poly(DMAEMA-co-BMA) (DB) random copolymer block has a balance of hydrophobic BMA and cationic DMAEMA monomers that has been finely tuned to drive NP self-assembly and stabilization (BMA), enable electrostatic siRNA packaging (DMAEMA), and have an appropriate pK_a and level of hydrophobicity that drives pH-dependent membrane disruptive function in the early endosomal pH range (4.0-4.3). The collagen II targeting mAbCII was conjugated to COOH-PEG-ECT by N-hydroxysulfosuccinimide; 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide (sNHS/EDC) chemistry. Successful conjugation between PEG-*bl*-DB and mAb-CII to form mAbCII-siNPs was validated by size exclusion chromatography of the conjugate created at a 1:1 molar ratio of antibody to polymer (**Figure 1B**). The resultant polymers were formulated into siNPs by complexation with siRNA at pH 4 followed by raising to physiologic pH (**Figure 1C**). The control groups included bare siNPs and siNPs functionalized

with an off-target antibody (mAbCtrl siNPs). “Dual hydrophobization” was also employed in all siNP formulations. This approach combines the hydrophobicity of BMA in the core of the siNP with C16 modification of the siRNA through conjugation to palmitic acid in order to improve siNP stability and gene silencing longevity of action (36, 44).

A Synthesis

B Confirmation

C Formulation

Figure 1: Synthesis of mAbCII-siNPs – (A) Polymer synthesis and mAbCII conjugation scheme; (B) Size exclusion chromatography confirming mAbCII conjugation to polymer; (C) Formulation schematic for siRNA loading and assembly of mAbCII-siNPs.

Chemicophysical and In Vitro Characterization of mAbCII-siNPs

The siNP hydrodynamic diameter, siRNA encapsulation efficiency, pH-dependent membrane disruptive behavior (as an indirect indicator of endosome escape), and cell viability were assayed for mAbCII-siNPs compared to non-targeted siNPs. The mAbCII-siNPs were statistically equivalent to the non-functionalized siNPs in all these assays (**Figure 2A-D**). The mAbCII-siNPs, prepared at a 1:40 antibody:polymer ratio for optimized targeting (see below), had an average hydrodynamic diameter of 124 nm with a PDI of 1.1 as determined by dynamic light scattering. Encapsulation of siRNA was found to be efficient (~80%) at N⁺:P⁻ ratios (ratio of positive nitrogen groups in polymer side chains to negative phosphodiester groups in the siRNA backbone) of 10 or above. The hemolysis assay demonstrated significant membrane lysis at the early endosome pH (6.8) and below and negligible activity at extracellular pH (7.4). Cell viability was approximately 80% or greater for doses of 150 nM or less.

Silencing of MMP13 was also tested in cultured, chondrogenic ATDC5 cells stimulated with TNF α . The cells were pretreated for 24 h with the siRNA formulations, stimulated with 20 ng mL⁻¹ TNF α for 24 h, and then assayed for MMP13 gene expression using TaqMan PCR. Information on screens that identified the leading MMP13 siRNA sequence are found in **Table S1** and **Figure S3**. The best candidate (siMMP13) demonstrated greater than 80% knockdown with a 50 nM dose delivered by the mAbCII-siNPs when compared with a nontargeting siRNA sequence (siNEG) (**Figure 2E**). No substantial change is observed in siNP bioactivity following

decoration with a targeting antibody and that the mAbCII-siNPs can achieve potent MMP13 silencing in cells under pro-inflammatory conditions.

***Ex vivo* CII targeting and substrate-mediated RNAi in ATDC5 cells**

The mAbCII-siNPs were assessed for binding to trypsin-damaged porcine cartilage explants. The mAbCII-siNPs were prepared with a range from 20:1 to 80:1 non-conjugated polymer: antibody modified polymer molar ratios. The polymers used comprised rhodamine acrylate (exc/emm: 548/570 nm) copolymerized at 1 mol% in the poly(DMAEMA-*co*-BMA) block to enable fluorescent measurement of carrier retention on the damaged cartilage plugs. Retention of siNPs on trypsin-damaged cartilage after washing with phosphate buffer saline (PBS) was quantified by IVIS imaging and showed that conjugation of mAbCII to the polymer at 40:1 polymer:mAbCII molar ratio provided the best retention performance (**Figure 2F**). These data confirm that mAbCII conjugation enhances binding of siNPs to exposed CII in damaged cartilage and motivated our focus on the 40:1 conjugation ratio for subsequent studies.

Subsequently, the porcine cartilage binding assay was adapted to confirm whether matrix bound mAbCII-siNPs could achieve effective substrate-mediated siRNA delivery and bioactivity. Following incubation of all siNP groups loaded with anti-luciferase siRNA (siLuciferase) with trypsin-damaged cartilage, a PBS washing step was done to simulate synovial fluid clearance. Murine chondrogenic ATDC5 cells that were lentivirally transduced in-house with a constitutive luciferase reporter were then seeded over the damaged cartilage that had been pre-treated with mAbCII-siNPs, bare-siNPs, mAbCtrl-siNPs, or lipofectamine 2000. In parallel, the same groups were run without the washing step to clearly elucidate the benefit of siNP matrix binding and retention. Significantly higher luciferase silencing was observed with mAbCII-siNPs compared to

bare and mAbCtrl-siNPs when a wash step was used prior to cell seeding (**Figure 2G**). Following the measurement of luciferase expression, cell viability of each group was determined using the Promega CellTiter-Glo luminescent cell viability assay following Promega's standard protocol. Only cells treated with lipofectamine 2000 (100 nM) were found to have significantly lower viability than other treatment groups. These data confirm a potential pharmacokinetic benefit of siNP matrix binding and that substrate-mediated delivery of matrix-targeted siNPs achieves target gene silencing.

Figure 2: In vitro characterization of siNPs, and comparison of mAbCII-functionalized siNPs to bare and control antibody-functionalized siNPs – (A) siNP sizing by dynamic light scattering; (B) siRNA encapsulation by Ribogreen assay; (C) pH-triggered membrane disruption by hemolysis assay; (D) Cytocompatibility of siNPs across a broad dose range; (E) MMP13

silencing by lead siRNA candidate in ATDC5 cells stimulated with TNF α using mAbCII-siNPs for delivery; (F) Retention of siNPs with varied polymer:mAbCII molar ratios on trypsin damaged porcine cartilage explants after thorough washing; (G) Substrate-mediated silencing of MMP13 *in vitro* enhanced by mAbCII-siNP binding and retention on trypsin-damaged porcine cartilage (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

***In vivo* targeting-dependent MMP13 silencing in mice**

An acute PTOA model of noninvasive repetitive joint loading was used by subjecting the left knee joint of 8-week-old C57BL/6 mice to 50 cycles of compressive mechanical loading at 9N (Figure 3A). This procedure was repeated three times per week over a period of two weeks using conditions adapted from previous studies (39, 45). Following loading, mice were treated via intraarticular injection of 0.5 mg/kg per knee of formulated siRNA with mAbCII-siNPs, bare siNPs, or mAbCtrl-siNPs. All forms of siNPs contained a rhodamine acrylate monomer integrated into the poly(DMAEMA-*co*-BMA) block that forms the NP core, enabling IVIS fluorescence imaging to assess pharmacokinetics. Retention in the knees was measured over 72 h, after which knees were excised and the joints were imaged again *ex vivo*. The mAbCII-siNPs had significantly higher retention within the joint compared to mAbCtrl- and bare siNPs (Figure 3B-D). It was also supported that the enhanced joint retention was pathology-driven and due to exposed collagen II associated with cartilage damage (46, 47), as retention was also higher in PTOA-damaged knees relative to non-injured knees (Figure S4). Finally, in this acute PTOA model, mAbCII-siNP/siMMP13 (candidate sequence from screen; Figure S3) treatment more potently silenced

MMP13 expression relative to untargeted siNPs, achieving greater than 90% target gene knockdown in mechanically-loaded PTOA joints 3 days after treatment (**Figure 3E**).

Dose dependent *in vivo* gene silencing activity of mAbCII-siNP/siMMP13 was also measured for siMMP13 (0, 0.125, 0.25, and 0.5 mg/kg per knee injected intraarticularly) or siNEG (0.5 mg/kg) in PTOA knees. Doses were administered after two weeks of cyclic, mechanical loading. At 72 h post-treatment with siNPs, total MMP activity was quantified by IVIS imaging 24 h after intravenous injection with MMPsense (probe activated by MMPs 2, 3, 7, 9, 12, and 13), showing that 0.25 and 0.5 mg/kg siMMP13 doses delivered with mAbCII-siNPs significantly reduced total MMP activity (**Figure 3F-G**). MMP13 and IL-1 β expression were quantified in the same experiment by TaqMan qPCR from joint samples collected at 72 h following treatment (**Figure 3H-I**). While both 0.25 and 0.5 mg/kg injections significantly reduced MMP13 gene expression silencing, reduction in IL-1 β expression in OA knees was only seen for 0.5 mg/kg, suggesting that this dose provided a broader suppression of the OA inflammatory response. Therefore, a 0.5 mg/kg per knee dose was selected for subsequent, longer-term studies. The mAbCII targeting enhances gene knockdown of MMP13 with roughly 40% greater silencing over bare-siNPs/siMMP13 and 70% greater than mAbCtrl-siNPs/siMMP13, motivating testing in a longer term and more challenging OA model to assess mAbCII-siNPs/siMMP13 therapeutic efficacy as a DMOAD.

Figure 3: mAbCII-siNPs are better locally-retained in mechanically loaded knees and potently silence MMP13 in short-term in vivo PTOA studies –(A) Schematic illustrations of the mouse knee mechanical loading apparatus and of the loading regimen used in the short-term PTOA model studies; (B-C) Representative *ex vivo* imaging and quantification of retention of mAbCII-siNPs compared to mAbCtrl- and Bare siNPs in extracted limbs at 3 days; (D) *in vivo* knee retention of mAbCII- and mAbCtrl-siNPs presented as pharmacokinetic area under the curve calculated from intravital imaging over 3 days post-treatment; (E) *In vivo* expression of MMP13 measured by TaqMan qPCR in mouse knees treated with 0.5 mg/kg siRNA dose per knee using mAbCII or control (bare and nonbinding control antibody) siNPs; (F-G) Example images of total MMP activity as visualized with MMPSense at 0.5 mg/kg siRNA dose and total MMP activity quantified; (H-I) Dose dependent effects of mAbCII-siNP delivery of siMMP13 or siNEG (in mg/kg) on expression of MMP13 and IL1 β as quantified by TaqMan qPCR (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

MMP13 silencing in longer-term osteoarthritis mouse model

A 6-week murine study was completed to evaluate the therapeutic effect of weekly doses of MMP13-silencing mAbCII-siNPs in a longer-term and more aggressive PTOA model. Tests for the more aggressive PTOA model were carried out in C56BL/6 mice aged to 6 months and subjected to a more rigorous cyclic mechanical loading protocol of 9N, 500 cycles, 5 times per week, for 6 weeks (**Figure 4A**) (48). Doses of 0.5 mg/kg siRNA were administered into each knee weekly, starting concurrently with mechanical loading. MMPSense and Alexafluor-labeled mAbCII antibody were injected intravenously 24 h before sacrifice to gauge total MMP activity

1 and quantify cartilage damage, respectively. Even though the study takedown and gene expression
2 analysis was done a full week following administration of the final dose, MMP13 expression
3 interference achieved with mAbCII-siNPs was consistent with the short-term model, with greater
4 than 80% reduction compared to OA joints treated with siNEG using mAbCII-siNPs (**Figure 4B**).
5 Immunohistochemical (IHC) staining revealed that MMP13 production both in the articular
6 cartilage and the synovial tissue was reduced by mAbCII-siNP/siMMP13 treatment (**Figure 4C,**
7 **D**). The inflamed state associated with PTOA is characterized by detrimental tissue restructuring
8 potentiated by cytokines and growth factors produced in the synovium of the joint (49).
9 Degradation products of degraded CII have signaling properties that contribute to catabolic
10 activity, hypertrophy, and apoptosis (50). The mAbCII-based targeting of siMMP13 in the PTOA
11 joint may have both primary, cartilage-protective effects on MMP13 gene silencing and also
12 secondary effects associated with reduced production of degradation products that amplify
13 downstream inflammation and MMP production.

14 Histological analysis showed that mAbCII-siNP/siMMP13 treatment significantly reduced
15 PTOA-associated joint structural changes. Coronal sections of fixed knee joints were stained with
16 Safranin O and Fast Green to evaluate cartilage histopathology. Sections were then blindly scored
17 by a trained pathologist (**Figure 4E-F**) using criteria detailed in the supplement (**Table S2A, S2B**).
18 Safranin O stains proteoglycans associated with normal cartilage a deep red and is used for
19 histopathological scoring of cartilage as recommended by the Osteoarthritis Research Society
20 International (OARSI) (51). The reduced Safranin O staining and surface discontinuity observed
21 in untreated OA and siNEG-treated OA is indicative of proteoglycan loss and cartilage erosion
22 and was significantly prevented in siMMP13-treated joints.

1 Total MMP activity was also significantly reduced in the mAbCII-siNP treated animals
2 compared to controls that were either untreated or treated with mAbCII-siNPs loaded with siNEG
3 (**Figure 4G**). Relative binding of Alexafluor-labeled mAbCII antibody to pathologically exposed
4 CII is a biomarker for degree of cartilage damage (39, 52, 53) and was thus used here as an
5 intravital readout. Significantly greater binding of mAbCII was observed in mAbCII-siNP/siNEG
6 treated and untreated mice compared to mAbCII-siNP/siMMP13 treated mice, indicating that
7 targeted MMP13 silencing protected the cartilage architecture (**Figure 4H, I**). Levels of
8 Alexafluor680-mAbCII binding in the PTOA joint were similar between untreated mice and mAb-
9 CII-siNP/siNEG treated mice, indicating that treatment with mAbCII-functionalized siNPs one
10 week prior did not in itself interfere with the subsequent mAbCII-based cartilage damage
11 measurement.

Figure 4: Long-term MMP13 silencing reduces MMP13 protein levels in cartilage and synovium and protects mechanically-loaded joints from OA progression – (A) Loading and treatment regimen used in the long-term mouse model; (B) MMP13 expression at the end of week 6; (C-D) IHC for MMP13 showing that targeted siMMP13 treatment reduced MMP13 protein levels both in the articular surface of the tibia and in the synovial tissue; (E-F) Representative Safranin O staining of the articular surface of the femur and quantification of cartilage damage by the OARSI osteoarthritis cartilage histopathology assessment system. (G) Total MMP activity at 6 weeks measured by MMPsense. (H-I) Representative images and quantification of the binding of fluorescently labeled mAbCII in the joint at 6 weeks as a marker for relative cartilage damage and resultant superficial exposure of Collagen II (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Hematoxylin and eosin (H&E) staining was used to evaluate overall joint status, including synovial response (**Figure 5A**). The loading protocol utilized induced robust synovial thickening and ectopic mineralization. Whole joint histology was blindly scored by a pathologist (**Figure 5B**) based on the degenerative joint disease criteria outlined in the supplemental information (**Table S2**). While cartilage structure was fully protected by mAbCII-siNP/siMMP13 treatment (treated mice had statistically equivalent OARSI score to normal mice with no load-induced PTOA), there was also a significant protection of other joint tissues, including less synovial hyperplasia compared to control-treated animals. The H&E joint sections for siNEG and untreated groups also appeared to contain higher ectopic calcification in the synovial membrane compared to the mAbCII-siNP/siMMP13 group. To more globally visualize and quantitatively assess ectopic calcification of the synovium, we utilized microCT, revealing that mAbCII-siNP/siMMP13 treatment protected against synovial mineralization (**Figure 5C-D**). These data support that

1 MMP13 silencing does have secondary benefits on the joint beyond directly reducing articular
2 cartilage loss.

3 When evaluating OA joints clinically, the presence of osteophytes and ossified nodules
4 within the synovium is especially important, as these characteristics are used to gauge OA
5 progression and appear in the most advanced stages of the disease (54). These rigid calcium
6 deposits are associated with synovial macrophage activation in experimental OA (55) where they
7 concentrate local mechanical stress. Further, presence of calcium phosphate can exacerbate
8 synovitis by activating inflammasomes and consequently triggering production of the OA driver
9 IL-1 β (56). While inflammation affects patient comfort, complications and acute pain from
10 osteophytes and calcium deposits are often cited as primary reasons for advanced OA patients to
11 cease exercise altogether and to resort to total knee replacement (57). Inhibiting pathological
12 ossification would be anticipated to enable maintenance of an active lifestyle and to delay total
13 knee replacement.

14 To look more broadly at the joint and surrounding tissues, subchondral trabecular bone was
15 also characterized using microCT. Interestingly, mAbCII-siNP/siMMP13 treatment significantly
16 reduced loss of subchondral trabecular bone volume associated with PTOA (**Figure 5E**). TRAP
17 (tartrate-resistant acid phosphatase) staining was also performed to assess osteoclast activity as a
18 snapshot of bone resorption (**Figure 5F**). Activated osteoclasts were increased in untreated,
19 mechanically loaded knees compared to those receiving mAbCII-siNP/siMMP13 treatment. These
20 data reinforce the complexity of OA as a full joint disease, while also indicating that MMP13
21 RNAi has beneficial, global impacts within the joint by also impacting cartilage crosstalk with
22 surrounding tissue. There is strong clinical precedent for observing multiple, concomitant types

1 of aberrant mineral homeostasis within the OA joint. Clinically, calcium “crystals” often manifest
2 in the synovial tissue and fluid. At the same time, pathological vascularization and thickening of
3 the subchondral bone plate can cause loss of subchondral trabecular bone (56). Animal models and
4 human samples suggest an influence of MMP13 and cathepsin K, specifically, in driving
5 pathological subchondral bone resorption in late-stage OA in human tissues (58). In accordance
6 with these studies, we find that subchondral bone resorption and pathologic bone restructuring
7 within the whole joint are reduced with MMP13 inhibition.

Figure 5: mAbCII-siNP/siMMP13 silencing decreases joint destruction by reducing synovial response and subchondral bone loss. – (A-B) H&E staining of joints, focusing on the synovial regions, and average Degenerative Joint Disease Score by treatment group. Blinded histological scoring was completed by a trained histopathologist; (C) 3-dimensional renderings of synovial ossification in the joint space from microCT scans; (D) Quantified degree of ectopic synovial ossification in quantified hydroxyapatite (HA); (E) microCT-derived bone volume fraction of the femoral and tibial subchondral bone; (F) TRAP staining of PTOA groups highlighting osteoclast activity in the subchondral bone (Arrows highlight active osteoclasts; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Benchmark comparisons to clinical gold standard steroid treatment

The proposed therapy is positioned as an early intervention for PTOA. The most prevalent clinical intervention beyond the use of oral NSAIDS is the intraarticular administration of steroids, which are recommended to be given up to four times per year (59). Of corticosteroids, the most commonly used is methylprednisolone (60). Because the longer-term, 6-week osteoarthritis mouse model used in our therapeutic studies is aggressive, benchmarking was compared against both a single dose of methylprednisolone at the time of first injury (most similar to frequency of dosing used clinically) and a weekly dose, with the latter done to match the protocol used for testing of mAbCII-siNPs/siMMP13 (dosing described in Supplemental Information).

Histological sections on joints extracted at the endpoint of this study were blindly scored by a pathologist following tissue staining with hemotoxalin/eosin and safranin-O (**Figure 6A-D**). Neither single or weekly methylprednisolone injections significantly altered either the OARSI or Degenerative Joint Disease Scores relative to untreated PTOA knees. These findings are in

1 agreement with other studies demonstrating that while steroids temporarily alleviate inflammatory
2 pain, there is no demonstrated protection of cartilage structural integrity (14). Notably, mAbCII-
3 siNPs/siMMP13 treatment significantly reduced both the DJD and the OARSI score relative to
4 controls, while neither single or weekly steroid treatment provided any significant protection by
5 these joint scoring metrics. Similar to the histological scoring outcomes, neither steroid treatment
6 protocol blocked ectopic mineralization in the synovium, unlike mAbCII-siNP/siMMP13 therapy
7 (**Figure 6E**). These distinctions further highlight the therapeutic potential of specific inhibition of
8 MMP13, a molecular driver that broadly underlies several aspects of PTOA joint destruction.

Figure 6: Steroid treatment does not provide DMOAD effects in relation to mAbCII-siNP/siMMP13– (A) Representative whole joint histological sections stained with H&E; (B) Representative safranin-O staining of the articular surface of the tibia and femur; (C) Total joint scores were determined from H&E stained slides by a treatment-blinded histopathologist; (D) Cartilage damage by the OARSI osteoarthritis cartilage histopathology assessment system was also determined by a treatment-blinded histopathologist; Arrows highlight focal cartilage damage and regions of cartilage degeneration; (E) MicroCT imaging of ectopic mineralization in the synovial tissues; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

MMP13 silencing broadly affects expression of OA-associated genes

Targeting MMP13 directly by RNAi successfully silenced MMP13 and was found to have broader effects on overall joint health, including in the synovium and subchondral bone. To further characterize the global impacts of targeted MMP13 inhibition, the extended mechanical loading mouse model was repeated with the same parameters except that samples were harvested at 4 rather than 6 weeks to capture a more intermediate stage of disease (**Figure 7A**). The nanoString nCounter Inflammation panel was used to quantify expression of 254 genes in the knee joint samples (articular cartilage and synovial tissue combined at a consistent 1:1 mass ratio). Unsupervised analysis using nanoString software indicate that the joints treated with mAbCII-siNPs/siMMP13 were closer to normal tissue and more different from untreated OA tissue than mAbCII-siNPs/siNEG and more (**Figure 7B**). Compared to treatment with mAbCII-siNPs/siNEG, treatment with mAbCII-siNPs/siMMP13 significantly suppressed expression levels of several clusters of genes with notable associations with OA progression (**Figure 7C,D**).

1 In PTOA joints, mAbCII-siNPs/siMMP13 treatment significantly reduced clusters of genes
2 associated with tissue restructuring and angiogenesis. Tissue remodeling is an active process in
3 response to injury, in the articular cartilage and especially in the synovium where capsule
4 thickening, vascularization, and hyperplasia occurs. These processes are associated with the
5 upregulation of genes such as MEF2 α (chondrocyte hypertrophy) and PDGF α (a potent synovial
6 fibroblast growth factor) (61, 62), which were suppressed by mAbCII-siNPs/siMMP13. MMP13
7 silencing also suppressed Flt-1, which is a VEGF receptor, predominantly expressed by vascular
8 endothelial cells and involved in angiogenesis. The more active tissue remodeling and thickening
9 processes in control PTOA joints was also indicated by mAbCII-siNP/siMMP13 treatment-
10 associated suppression of gene clusters related to proteolysis and skeletal development. MMP13
11 silencing also led to downregulation of genes associated with apoptosis, such as Jun (which also
12 induces MMP13) and the inflammatory cytokine IL6, which is mechanistically involved in
13 propagation of cellular stress and synovial inflammation (63, 64).

14 Treatment-associated downregulation of genes related to tissue restructuring, apoptosis,
15 angiogenesis, and proteolysis were also complemented by reduction of innate immune activation
16 in joints with targeted MMP13 silencing. OA-relevant PTGS2 (prostaglandin-endoperoxide
17 synthase 2, an inflammation driver that encodes the cyclooxygenase 2 enzyme (COX2) and IL1RN
18 encoding the interleukin 1 receptor antagonist protein (IL1RaP) in which its knock-out correlates
19 with spontaneous arthritis development (65) were, for example, significantly reduced in
20 mechanically loaded joints with mAbCII-siNP delivery of siMMP13 versus siNEG. Coupled with
21 reduction of innate immune response, MMP13 silencing also reduced expression of several serine
22 proteases (C1S, C1RA, and C2) that are components of the complement pathway, which is known
23 to be associated with OA progression (66, 67). The local reduction in these components indicates

1 reduced number or activation of circulating monocytes, macrophages, and monocyte-derived
2 dendritic cells that all express C1s, C1RA, and C2 (68). MMP13 silencing also reduced expression
3 of IL-1 β (MAPK activation cluster), an inflammatory cytokine that drives OA driver by inducing
4 nitric oxide production, increasing synthesis of MMPs and aggrecanases, while suppressing
5 proteoglycan synthesis (69). Interestingly, recent work has highlighted how macrophages
6 embedded in synovial tissue serve to induce a proinflammatory state (70) and, in combination with
7 synovial fibroblasts, appear to be the lead contributors to MMP13 expression within the joint space
8 as observed by MMP13 IHC. Not only does MMP13 inhibition directly reduce cartilage
9 degradation, it has secondary impacts on overall joint health by disrupting the “degenerative cycle”
10 that is perpetuated by degradative products of the cartilage matrix (17).

11

Figure 7: MMP13 silencing globally impacts gene expression patterns within mechanically-loaded PTOA joints – (A) Loading and treatment regimen for the nanoString analysis study; (B) Unsupervised sorting of treatment groups from most to least similar to normal joints as quantified by nanoString at the end of week 4. Gene expression is shown as high- (green) or low-expression (red) sorted vertically by differences between treatment groups; (C-D) Gene clusters were significantly different between siNEG- and siMMP13-treated joints (5 most changed in C, next 5 most changed in D). The plots are arranged such that the clusters' p-values between siMMP13 and siNEG treatment groups is lowest on left and highest on right (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

The global changes to inflammatory gene expression observed in this study, in combination with joint analysis utilizing microCT and histology, all support the therapeutic value and safety of local, MMP13 silencing targeted to cartilage matrix. It is thought that MMP13 activity within the synovial joint drives OA progression (71). By contrast, MMP13 expression by osteocytes is important for normal perilacunar/canalicular remodeling (PLR). Selective genetic ablation of MMP13 expression in osteocytes disrupts subchondral bone homeostasis in a way that exacerbates subchondral bone sclerosis and OA pathology (72). We speculate that a CII-targeted delivery system such as mAbCII-siNPs that is injected directly into the intraarticular space will localize effects to cartilage and possibly adjacent synovial tissues, while any unbound treatment would clear primarily through lymphatic drainage (73), limiting exposure and gene targeting effects on cells embedded within the more remote subchondral bone tissue shielded by mineralized matrices. The concept that mAbCII-siNPs/siMMP13 helped to maintain normal mineralization homeostasis

1 within the joint is supported by the microCT analyses showing that treated animals maintained a
2 more normal subchondral bone volume fraction with reduced abnormal ectopic mineralization
3 versus control treatment. Our matrix-targeted mAbCII-siNPs enable a desirable combination of
4 safe and effective therapeutic MMP13 targeting that may be difficult to achieve with systemic
5 delivery or through delivery of small molecule inhibitors that more readily diffuse into tissues
6 outside of the synovial joint.

7 **Conclusions**

8 RNAi silencing of MMP13 using matrix-targeted nanocarriers to prolong retention within
9 the osteoarthritic joint provides significant therapeutic benefit in blocking PTOA progression.
10 This study validates the unique concept that matrix targeting for local retention of an in situ formed
11 nanoparticle-based depot is a viable strategy to improve potency and longevity of action of
12 intracellular biologics such as siRNAs. While formulation of larger sized (micro-scale) particles
13 may also facilitate retention, it would not be anticipated to be effective for intracellular acting
14 drugs because of endocytosis, endosome escape, and tissue penetration limitations of larger
15 particles (74). The current system, which uses antibody targeting to reduce joint clearance,
16 achieved as high as 90% target gene silencing in vivo with gene silencing remaining potent even
17 1 week after the final treatment in a 6-week study. Furthermore, local retention of the injected dose
18 and specific targeting of MMP13 are anticipated to reduce the toxicity concerns that have become
19 associated with systemically-delivered, non-selective synthetic small molecule MMP inhibitors.
20 The therapeutic relevance of this approach is exemplified in an aggressive, long-term PTOA model
21 which showed a treatment-associated 80% reduction in MMP13 expression ($p = 0.00231$),
22 protection of cartilage integrity ($0.45 \pm .3$ vs $1.6 \pm .5$ on the OARSI scale; $p = 0.0166$),
23 improvement in synovial histopathology ($1.3 \pm .6$ vs $2.8 \pm .2$ on the Degenerative Joint Disease
24 scale; $p < 0.05$), and less destruction of subchondral bone (16% greater bone volume over total

volume; $p < 0.01$). There were also global gene expression changes associated with MMP13 silencing that suggests this treatment produces both primary benefits through cartilage protection and secondary benefits from blocking inflammatory, degradative feedback loops activated by the release of cartilage degradation by-products. In sum, matrix-targeted delivery for potent MMP13 gene silencing is a promising experimental DMOAD that warrants additional preclinical development because of its significant advantages over current treatments.

Experimental section

Experimental details and characterization of all particles, animal procedures, and therapeutic assessment are included in Supporting Information.

Supporting information

Experimental methods are available in the supplementary document.

Acknowledgements

The authors acknowledge the assistance of the Vanderbilt Translational Pathology Shared Resource (TPSR). TPSR is supported by NCI/NIH Cancer Center Support Grant 2P30 CA068485-14. Dynamic light scattering was conducted at the Vanderbilt Institute of Nanoscale Sciences and Engineering. Bone analysis by microCT was supported in part by the NIH (S10RR027631-01). The technical assistance of Carrie B. Wiese, Joshua R Johnson, and Ray Mernaugh is also acknowledged.

We are grateful to the DOD (DOD CDMRP OR130302), NIH (NIH R01 CA224241, NIH R01 EB019409), NIH (NIGMS T32GM007347), the VA Merit Award BX004151, and the

National Science Foundation Graduate Research Fellowship Program (NSF GRF #2016212929) for support.

Experimental section

Materials

Unless otherwise stated, materials and reagents were purchased from Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St. Louis, MO, USA).

Synthesis and conjugation of polymers

N-hydroxysuccinimide-functionalized 4-cyano-4-(ethylsulfanylthiocarbonyl)sulfanylpentanoic acid (NHS-ECT) synthesis was verified by NMR (**Figure S1**), and the product was then conjugated to an amine-carboxy bifunctional 5kD PEG to form carboxy-PEG-conjugated ECT for use as an initial chain transfer agent for RAFT polymerization (40). A co-polymer of DMAEMA (2-(Dimethylamino)ethyl methacrylate) and BMA (butyl methacrylate) was chain extended from the COOH-PEG-ECT with a desired target degree of polymerization of 150 (1:1 molar ratio DMAEMA:BMA) to create PEG-DB which was verified by NMR (**Figure S2**). The reaction was purged with nitrogen for 30 minutes. AIBN was utilized as an initiator (10:1 CTA:Initiator ratio) in 10% w/v dioxane. The reaction was stirred at 65 °C for 24 h before precipitation into ether and vacuum drying for 24 h. Polymer was then dissolved and dialyzed in methanol for 48 hours before transition to dialysis in water for another 48 hours.

1

2

3 **Figure S1.** ¹H-NMR spectrum of NHS-ECT, a functionalized RAFT chain transfer agent in
4 CDCl₃

5

1

2

3 **Figure S2.** $^1\text{H-NMR}$ spectrum of DB-PEG-COOH in CDCl_3 calibrated to the PEG proton

4 content

Subsequently, a two-stage sNHS/EDC conjugation protocol was optimized for activating the polymer and removing excess activating compounds before adding the antibody to avoid antibody crosslinking. Carboxyl terminated PEG-DB polymer was dissolved in ethanol at a 20 mg/mL concentration before addition to 0.05 M MES buffer, pH=6.0 to prepare a final 1 mg/mL solution of COOH-PEG-DB polymer. EDC and sNHS were added at 250 and 500 mM and allowed to react for 15 minutes at room temperature. Excess EDC and sNHS were then eliminated using 10kD MWCO spin filters from Amicon, centrifuging at 3,000 rcf for 13 minutes from an initial volume of 6 mL. Volume lost was replaced with more MES buffer reestablishing initial volume. Antibody at 1 mg/mL in 0.1M PBS, pH=8.0 was added to the activated polymer solution and allowed to react for 18 h at room temperature. Conjugation was verified by size exclusion chromatography as shown above, and fully conjugated mABCII-PEG-DB were mixed with non-functionalized PEG at the appropriate ratio (usually 1:40 conjugated to nonfunctionalized polymer).

Characterization of particles

Antibody conjugation to polymer was verified by size exclusion chromatography, tracking polymer elution by absorbance at 214 nm through Enrich SEC 650 columns at a flow rate of 0.25 mL/min in 10 mM PBS at pH 8.

A sequence of tests was then conducted to verify that the particle functionality was uncompromised by conjugation. The efficiency of siRNA encapsulation $N^+:P^-$ ratios was evaluated using a Quant-iT Ribogreen assay kit (ThermoFisher Scientific, Waltham, MA). siNP size was evaluated using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern, USA). Polymer pH-

dependent membrane disruptive function as a marker for endosome disruption/escape was evaluated using a hemolysis assay, as described previously (75).

Selection of MMP13 siRNA

Seven candidate siRNA sequences targeting different sites of the MMP13 gene were first screened in ATDC5s stimulated with the inflammatory cytokine TNF α (20 ng/mL). Oligonucleotides used in these studies were purchased from Integrated DNA Technologies (Coralville, IA, USA) or Dharmacon, Incorporated (Lafayette, CO, USA). The selected sequence was synthesized with 2' *O*-methyl modification for enhanced *in vivo* activity.

Table S1. Candidate sequences screened for MMP13 knockdown in inflamed ATDC5 cells. Selected sequence is bolded. The palmitic acid-conjugate modification employed thereafter is also described.

Sequence Screening Antisense Sequences

Lead: 5'-UCAAAUGGUCCCAAACGAA-3'

#2: 5'-CUGCGACUCUUGCGGGAU-3'

#3: 5'-AGACUAUGGACAAAGAUUA -3'

#4: 5'-GGCCCAUACAGUUUGAAUA -3'

#5: 5'-GCUAUGCACACUGGUAGAAGAUATT-3'

#6: 5'-UCCCUAGAACACUCAAUGGUCCCA-3'

#7: 5'-UCAAGAGACAGUGUUAUUAACCTG-3'

Palmitic Acid-Conjugated Sequence

Antisense: 5'-UCAAAUGGUCCCAAACGAACUUAACUU -3'

Sense: 5'-GUUAAGUUCGUUUGGGACCAUUUGA 3' Amino Modifier C6 dT -3'

siNEG: 5'-AUACGCGUAUUUAUACGCGAUUAACGA-3'

Figure S3. Candidate sequences screened for MMP13 silencing in TNF α -stimulated ATDC5 cells (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Formation of mAbCII-siNPs

Polyplexes were formed by dissolving polymers in 10 mM citric acid buffer (pH 4) before complexation with siRNA for 30 minutes. Polymer was initially dissolved at 3 mg/mL concentration. The siRNA (or palmitic acid-modified siRNA) were complexed at the N:P ratio of 20. Following complexation, the pH was neutralized to 7.4 with sodium phosphate buffer (10 mM; pH 8; 5:1 v/v ratio).

For *in vivo* experiments, siNPs were formed under the same conditions and concentrated using 50kD MWCO 15 mL Amicon spin filters, washing with PBS, and sterile-filtered before injection.

1 **Cell culture**

2 ATDC5 cells were cultured in DMEM / F-12, GlutaMAX medium with 10% FBS, 1%
3 penicillin/streptomycin at 37 °C in 5% carbon dioxide. Relevant experiments were performed at
4 80% confluency.

5 **Cell viability studies**

6 Cytotoxicity was performed using the CellTiter-Glo assay, in accordance with the
7 manufacturer's protocol.

8 **Luciferase gene silencing assay**

9 For *in vitro* luciferase knockdown assays, ATDC5 cells were lentivirally transduced with
10 constitutively-expressed luciferase gene in a manner previously described (37). Cells were then
11 seeded at 2,000 per well in 96-well black plates, clear-bottom. After allowing cells to adhere for
12 24 hours, siNPs were then introduced into cell media at a concentration of 100 nM siRNA (siLUC
13 or siNEG). Treatments were removed after 24 hours of incubation, and cell bioluminescence was
14 then measured on an IVIS Lumina III imaging system (Caliper Life Sciences, Hopkinton, MA) at
15 24 and 48 hours after treatment by addition of 150 µg/mL luciferin. Luminescence was normalized
16 to that of siNEG NP controls. Finally, cell viability was measured by comparing luminescence of
17 siNEG controls to untreated cells.

18 **Quantitative reverse transcription PCR**

19 Real-time qRT-PCR was performed utilizing TaqMan primers and reagents, and conducted
20 under conditions outlined by the manufacturer. (Thermofisher Scientific, Waltham, MA; GAPDH:

1 Mm99999915_g1, ACTB: Mm02619580_g1, MMP13: Mm00439491_m1, IL6:
2 Mm00446190_m1)

3 **In vitro collagen-targeting, uptake, and viability in ATDC5 cells**

4 In order to test targeting and potency of the mAbCII-siNPs formulation, a reverse
5 transfection assay was employed. First, damaged articular cartilage “model lesions” were created
6 by partial trypsin damage of porcine cartilage with 2.5% trypsin for 15 min at 37 °C. Then, each
7 plug was inserted into a well plate and treated with mAbCIIsiNPs or controls. Damaged tissues
8 were incubated for 1 h with mAbCII/siLuc or control siNPs complexed with luciferase silencing
9 siRNA or negative control siRNA. Following incubation, all explants were washed with PBS, and
10 luciferase-expressing ATDC5 cells (murine, chondrogenic) in DMEM/F12 1:1 media were
11 cultured on top of the treated cartilage for 24 h at 37 °C. Each well was rinsed with PBS before
12 adding luciferin-containing media (150 ug/mL) and evaluating luminescence by IVIS imaging.
13 The reverse transfection assay was used to compare PEG-DB not functionalized with mAbCII,
14 PEG-DB functionalized with mAbCtrl, commercial reagent lipofectamine 2000, and mAbCII-
15 siNPs. Some treated cartilage plugs for each group were left unwashed to verify gene silencing
16 efficacy of the different formulations independent from the cartilage binding / target capacity.
17 Performance was measured against delivery of the same formulations (targeted and control) loaded
18 with siNEG. Following the measurement of luciferase expression, cell viability of each group was
19 determined using the Promega CellTiter-Glo luminescent cell viability assay following Promega’s
20 standard protocol.

***In vivo* short-term mechanical loading OA model**

C57 mice were mechanically loaded, 3 times per week for 2 weeks. The PTOA model of noninvasive repetitive joint loading was induced by subjecting the knee joints of mice (anesthetized with 3% isoflurane) to 250 cycles of compressive mechanical loading at 9 N. This procedure was repeated three times per week over a period of two weeks using conditions adapted from previous studies (39, 45).

In-joint retention of mAbCII-siNPs in PTOA (2-week loading model) versus healthy mice was also assessed. mAbCII-siNPs were shown to selectively retain in the knee. At 24 h following treatment, the amount remaining in the knee was measured by quantifying fluorescence in the rhodamine-labeled siNPs (**Figure S4**; Exc/Emm: 548/570 nm).

Figure S4. In-joint retention of mAbCII-siNPs in healthy and PTOA mice as measured by rhodamine in siNP polymer (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Statistics performed with an unpaired t test.

***In vivo* long-term mechanical loading therapeutic efficacy testing OA model**

Mice were aged to 6 months and then subjected to a more rigorous cyclic mechanical loading protocol of 9 N, 500 cycles, 5 times per week, for 6 weeks (48). Doses of siRNA (0.5 mg/kg) were administered into each knee weekly, starting concurrently with mechanical loading. MMPsense and Alexafluor-labeled mAbCII antibody were injected intravenously 24 h before sacrifice to gauge total MMP activity and quantify cartilage damage, respectively.

A high, effective methylprednisolone dose in rodents of 4 mg/kg was administered by intraarticular injection a single time at the beginning of the 6-week time course, which is the duration of the therapeutic study employed with the other groups and the shortest recommended time a patient should wait before repeating intraarticular steroid injection (76, 77). The dose was prepared by initially dissolving in DMSO, diluting in water, and injecting at a volume of 20 μ L per knee (60). A separate cohort underwent the same mechanical loading with weekly doses of the same dose of methylprednisolone as a control in direct comparison with the weekly administration of siMMP13 that was speculated to better accommodate the aggressive model, even though less clinically relevant.

Histology and Immunohistochemistry

Stifles were fixed in 10% neutral buffered formalin and decalcified in Immunocal (StatLab, McKinney, TX). Tissue handling for histopathology was primarily performed in the Vanderbilt Translational Pathology Shared Resource by certified histotechnicians. Fixed tissues were routinely processed using a standard 8 h processing cycle of graded alcohols, xylenes, and paraffin wax, embedded and sectioned at 5 microns, floated on a water bath, and mounted on positively charged glass slides. Hematoxylin and eosin (H&E) staining was performed on the Gemini

autostainer (Thermo Fisher Scientific, Waltham, MA). Safranin O staining was performed by hand using a kit (StatLab).

Stifle joints were evaluated by H&E and safranin O in at least 2 serial mid-frontal sections. This histopathologic interpretation was conducted by a board-certified veterinary pathologist under blinded conditions (78). OARSI scores (0-6 semiquantitative scale) were provided for the medial tibial plateau and lateral tibial plateau (54). Simultaneously, a generic score (0-4 semiquantitative scale) was assigned based on safranin O staining of the tibial plateau (**Table S2A**) and on H&E features of degenerative joint disease severity, as defined by cartilage degeneration, meniscal metaplasia, subchondral osteosclerosis, synovial hyperplasia and inflammation, and osteophyte formation (**Table S2B**) (79).

Immunohistochemical staining was performed on a Leica Bond-Max autostainer (Leica Biosystems Inc., Buffalo Grove, IL). All steps besides dehydration, clearing, and coverslipping were performed on the Bond-Max where all the slides were deparaffinized. Heat-induced antigen retrieval was performed on the Bond-Max using Epitope Retrieval 1 solution (Leica Biosystems Inc.) for 20 minutes. Slides were incubated with anti-MMP13 (cat# Ab39012, Abcam, Cambridge, MA) for 1 hour at a 1:750 dilution. The Bond Polymer Refine detection system (Leica Biosystems Inc.) was used for visualization. Slides were then dehydrated, cleared, and cover slipped.

Table S2. (A) Description of OARSI scale scoring for Safranin O slides of the tibial plateau cartilage (left); **(B)** criteria for scoring of H&E slides to assess overall joint by the degenerative joint disease scale (right).

Severity Score	OARSI Scale	Degenerative Joint Disease Scale
0	Normal	<u>Within normal limits:</u> mild DJD as a feature of expected age-related change (e.g. attenuation of articular cartilage and proteoglycan loss)
1	Loss of SO staining, articular cartilage thinning; no defects	<u>Moderate:</u> articular cartilage degeneration and beginning of secondary pathology, synovitis, joint capsule fibrosis, meniscal metaplasia
2	1 + fibrillation or pyknotic articular chondrocytes	<u>Marked:</u> metaplasia and/or fragmentation of one meniscus, osteophyte formation, synovitis/hyperplasia
3	2 + loss of articular cartilage <50% (e.g. erosion, flap, or callus)	<u>Severe:</u> metaplasia and/or fragmentation of both menisci, total loss of at least 1 articular surface (eburnation), osteophyte formation, advanced synovitis/hyperplasia
4	3 + fragmentation and fissuring in an area	<u>Extreme:</u> severe changes plus collapse of stifle joint space and epiphyseal osteolysis
5	4 + fragmentation and fissuring in >75% of the cartilage surface	N/A
6	Total loss of normal articular cartilage (end-stage)	N/A

Statistical methods

Data are displayed as mean plus standard error. Statistical tests employed either one-way ANOVA with multiple comparisons test or two-tailed student's T-test between only two groups with $\alpha = 0.05$.

Ethics statement

All animal experiments described herein were carried out according to protocols approved by Vanderbilt University's Institutional Animal Care and Use Committee, and all studies followed the National Institutes of Health's guidelines for the care and use of laboratory animals.

Bibliography

1. Menon J, Mishra P. Health care resource use, health care expenditures and absenteeism costs associated with osteoarthritis in US healthcare system. *Osteoarthritis Cartilage*. 2018;26(4):480-4. Epub 2017/12/18. doi: 10.1016/j.joca.2017.12.007. PubMed PMID: 29269328.
2. Turkiewicz A, Petersson IF, Björk J, Hawker G, Dahlberg LE, Lohmander LS, Englund M. Current and future impact of osteoarthritis on health care: a population-based study with projections to year 2032. *Osteoarthritis and Cartilage*. 22(11):1826-32. doi: 10.1016/j.joca.2014.07.015.
3. Loeser RF. Osteoarthritis year in review 2013: biology. *Osteoarthritis and Cartilage*. 2013;21(10):1436-42. doi: <http://dx.doi.org/10.1016/j.joca.2013.05.020>.
4. Tanamas S, Hanna FS, Cicuttini FM, Wluka AE, Berry P, Urquhart DM. Does knee malalignment increase the risk of development and progression of knee osteoarthritis? A systematic review. *Arthritis Care & Research*. 2009;61(4):459-67. doi: 10.1002/art.24336.
5. Richette P, Poitou C, Garnerio P, Vicaut E, Bouillot J-L, Lacorte J-M, Basdevant A, Clément K, Bardin T, Chevalier X. Benefits of massive weight loss on symptoms, systemic inflammation and cartilage turnover in obese patients with knee osteoarthritis. *Annals of the Rheumatic Diseases*. 2011;70(1):139-44. doi: 10.1136/ard.2010.134015.
6. Valdes AM, Spector TD. Genetic epidemiology of hip and knee osteoarthritis. *Nat Rev Rheumatol*. 2011;7(1):23-32.
7. Issa S, Sharma L. Epidemiology of osteoarthritis: An update. *Curr Rheumatol Rep*. 2006;8(1):7-15. doi: 10.1007/s11926-006-0019-1.
8. Lee AS, Ellman MB, Yan D, Kroin JS, Cole BJ, van Wijnen AJ, Im H-J. A current review of molecular mechanisms regarding osteoarthritis and pain. *Gene*. 2013;527(2):440-7. doi: <http://dx.doi.org/10.1016/j.gene.2013.05.069>.
9. Zhang Y, Jordan JM. Epidemiology of Osteoarthritis. *Clinics in geriatric medicine*. 2010;26(3):355-69. doi: 10.1016/j.cger.2010.03.001. PubMed PMID: PMC2920533.
10. Brophy RH, Gray BL, Nunley RM, Barrack RL, Clohisy JC. Total Knee Arthroplasty After Previous Knee Surgery. *J Bone Joint Surg-Am Vol*. 2014;96A(10):801-5. doi: 10.2106/jbjs.m.00105. PubMed PMID: WOS:000336107700002.
11. Brown TD, Johnston RC, Saltzman CL, Marsh JL, Buckwalter JA. Posttraumatic osteoarthritis: a first estimate of incidence, prevalence, and burden of disease. *Journal of orthopaedic trauma*. 2006;20(10):739-44.
12. Young IC, Chuang ST, Gefen A, Kuo WT, Yang CT, Hsu CH, Lin FH. A novel compressive stress-based osteoarthritis-like chondrocyte system. *Exp Biol Med (Maywood)*. 2017;242(10):1062-71. Epub 2017/03/22. doi: 10.1177/1535370217699534. PubMed PMID: 28492349; PMCID: PMC5444641.
13. Martin J, Buckwalter J. Post-traumatic osteoarthritis: the role of stress induced chondrocyte damage. *Biorheology*. 2006;43(3, 4):517-21.
14. McAlindon TE, Bannuru RR, Sullivan MC, Arden NK, Berenbaum F, Bierma-Zeinstra SM, Hawker GA, Henrotin Y, Hunter DJ, Kawaguchi H, Kwok K, Lohmander S, Rannou F, Roos EM, Underwood M. OARSi guidelines for the non-surgical management of knee osteoarthritis. *Osteoarthritis Cartilage*. 2014;22(3):363-88. Epub 2014/01/24. doi: 10.1016/j.joca.2014.01.003. PubMed PMID: 24462672.
15. McAlindon TE, LaValley MP, Harvey WF, Price LL, Driban JB, Zhang M, Ward RJ. Effect of Intra-articular Triamcinolone vs Saline on Knee Cartilage Volume and Pain in Patients With Knee Osteoarthritis: A Randomized Clinical Trial. *JAMA*. 2017;317(19):1967-75. Epub 2017/05/17. doi: 10.1001/jama.2017.5283. PubMed PMID: 28510679; PMCID: 5815012.

16. Wernecke C, Braun HJ, Dragoo JL. The Effect of Intra-articular Corticosteroids on Articular Cartilage: A Systematic Review. *Orthop J Sports Med.* 2015;3(5):2325967115581163. Epub 2015/12/18. doi: 10.1177/2325967115581163. PubMed PMID: 26674652; PMCID: 4622344.
17. Wang M, Sampson ER, Jin H, Li J, Ke QH, Im HJ, Chen D. MMP13 is a critical target gene during the progression of osteoarthritis. *Arthritis Res Ther.* 2013;15(1):R5. Epub 2013/01/08. doi: 10.1186/ar4133. PubMed PMID: 23298463; PMCID: PMC3672752.
18. Krzeski P, Buckland-Wright C, Bálint G, Cline GA, Stoner K, Lyon R, Beary J, Aronstein WS, Spector TD. Development of musculoskeletal toxicity without clear benefit after administration of PG-116800, a matrix metalloproteinase inhibitor, to patients with knee osteoarthritis: a randomized, 12-month, double-blind, placebo-controlled study. *Arthritis Res Ther.* 2007;9(5):R109. doi: 10.1186/ar2315. PubMed PMID: 17958901; PMCID: PMC2212568.
19. Molina JR, Reid JM, Erlichman C, Sloan JA, Furth A, Safgren SL, Lathia CD, Alberts SR. A phase I and pharmacokinetic study of the selective, non-peptidic inhibitor of matrix metalloproteinase BAY 12-9566 in combination with etoposide and carboplatin. *Anticancer Drugs.* 2005;16(9):997-1002. PubMed PMID: 16162976.
20. Clutterbuck AL, Asplin KE, Harris P, Allaway D, Mobasher A. Targeting matrix metalloproteinases in inflammatory conditions. *Curr Drug Targets.* 2009;10(12):1245-54. Epub 2009/11/17. PubMed PMID: 19909233.
21. Liu J, Khalil RA. Matrix Metalloproteinase Inhibitors as Investigational and Therapeutic Tools in Unrestrained Tissue Remodeling and Pathological Disorders. *Prog Mol Biol Transl Sci.* 2017;148:355-420. Epub 2017/05/10. doi: 10.1016/bs.pmbts.2017.04.003. PubMed PMID: 28662828; PMCID: PMC5548434.
22. Settle S, Vickery L, Nemirovskiy O, Vidmar T, Bendele A, Messing D, Ruminski P, Schnute M, Sunyer T. Cartilage degradation biomarkers predict efficacy of a novel, highly selective matrix metalloproteinase 13 inhibitor in a dog model of osteoarthritis: confirmation by multivariate analysis that modulation of type II collagen and aggrecan degradation peptides parallels pathologic changes. *Arthritis Rheum.* 2010;62(10):3006-15. doi: 10.1002/art.27596. PubMed PMID: 20533541.
23. Cai H, Agrawal AK, Putt DA, Hashim M, Reddy A, Brodfuehrer J, Surendran N, Lash LH. Assessment of the renal toxicity of novel anti-inflammatory compounds using cynomolgus monkey and human kidney cells. *Toxicology.* 2009;258(1):56-63. doi: 10.1016/j.tox.2009.01.006. PubMed PMID: 19378467.
24. Sterner B, Harms M, Wöll S, Weigandt M, Windbergs M, Lehr CM. The effect of polymer size and charge of molecules on permeation through synovial membrane and accumulation in hyaline articular cartilage. *Eur J Pharm Biopharm.* 2016;101:126-36. Epub 2016/02/11. doi: 10.1016/j.ejpb.2016.02.004. PubMed PMID: 26876928.
25. Larsen C, Østergaard J, Larsen SW, Jensen H, Jacobsen S, Lindegaard C, Andersen PH. Intra-articular depot formulation principles: Role in the management of postoperative pain and arthritic disorders. *Journal of pharmaceutical sciences.* 2008;97(11):4622-54.
26. Evans CH, Kraus VB, Setton LA. Progress in intra-articular therapy. *Nature Reviews Rheumatology.* 2013;10:11. doi: 10.1038/nrrheum.2013.159.
27. Simkin PA. Synovial perfusion and synovial fluid solutes. *Ann Rheum Dis.* 1995;54(5):424-8. Epub 1995/05/01. doi: 10.1136/ard.54.5.424. PubMed PMID: 7794054; PMCID: 1005609.
28. Rothenfluh DA, Bermudez H, O'Neil CP, Hubbell JA. Biofunctional polymer nanoparticles for intra-articular targeting and retention in cartilage. *Nat Mater.* 2008;7(3):248-54. doi: http://www.nature.com/nmat/journal/v7/n3/supinfo/nmat2116_S1.html.
29. Zhou F, Zhang X, Cai D, Li J, Mu Q, Zhang W, Zhu S, Jiang Y, Shen W, Zhang S, Ouyang HW. Silk fibroin-chondroitin sulfate scaffold with immuno-inhibition property for articular cartilage repair. *Acta*

- Biomater. 2017;63:64-75. Epub 2017/09/07. doi: 10.1016/j.actbio.2017.09.005. PubMed PMID: 28890259.
30. Hayder M, Poupot M, Baron M, Nigon D, Turrin C-O, Caminade A-M, Majoral J-P, Eisenberg RA, Fournié J-J, Cantagrel A, Poupot R, Davignon J-L. A Phosphorus-Based Dendrimer Targets Inflammation and Osteoclastogenesis in Experimental Arthritis. *Science Translational Medicine*. 2011;3(81):81ra35. doi: 10.1126/scitranslmed.3002212.
 31. Chen K, Chen X. Integrin targeted delivery of chemotherapeutics. *Theranostics*. 2011;1:189-200. Epub 2011/02/17. PubMed PMID: 21547159; PMCID: PMC3086622.
 32. Hoy SM. Patisiran: First Global Approval. *Drugs*. 2018;78(15):1625-31. doi: 10.1007/s40265-018-0983-6. PubMed PMID: 30251172.
 33. de Paula Brandão PR, Titze-de-Almeida SS, Titze-de-Almeida R. Leading RNA Interference Therapeutics Part 2: Silencing Delta-Aminolevulinic Acid Synthase 1, with a Focus on Givosiran. *Molecular Diagnosis & Therapy*. 2019:1-8.
 34. Nelson CE, Kintzing JR, Hanna A, Shannon JM, Gupta MK, Duvall CL. Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time, and bioactivity in vivo. *ACS Nano*. 2013;7(10):8870-80. Epub 2013/09/18. doi: 10.1021/nn403325f. PubMed PMID: 24041122; PMCID: Pmc3857137.
 35. Beavers KR, Nelson CE, Duvall CL. MiRNA inhibition in tissue engineering and regenerative medicine. *Adv Drug Deliv Rev*. 2015;88:123-37. doi: 10.1016/j.addr.2014.12.006. PubMed PMID: 25553957; PMCID: PMC4485980.
 36. Sarett SM, Werfel TA, Chandra I, Jackson MA, Kavanaugh TE, Hattaway ME, Giorgio TD, Duvall CL. Hydrophobic interactions between polymeric carrier and palmitic acid-conjugated siRNA improve PEGylated polyplex stability and enhance in vivo pharmacokinetics and tumor gene silencing. *Biomaterials*. 2016;97:122-32. doi: <http://dx.doi.org/10.1016/j.biomaterials.2016.04.017>.
 37. Jackson MA, Werfel TA, Curvino EJ, Yu F, Kavanaugh TE, Sarett SM, Dockery MD, Kilchrist KV, Jackson AN, Giorgio TD, Duvall CL. Zwitterionic Nanocarrier Surface Chemistry Improves siRNA Tumor Delivery and Silencing Activity Relative to Polyethylene Glycol. *ACS Nano*. 2017. doi: 10.1021/acsnano.7b01110.
 38. Ruan MZ, Erez A, Guse K, Dawson B, Bertin T, Chen Y, Jiang MM, Yustein J, Gannon F, Lee BH. Proteoglycan 4 expression protects against the development of osteoarthritis. *Sci Transl Med*. 2013;5(176):176ra34. doi: 10.1126/scitranslmed.3005409. PubMed PMID: 23486780; PMCID: PMC3804124.
 39. Cho H, Pinkhassik E, David V, Stuart JM, Hasty KA. Detection of early cartilage damage using targeted nanosomes in a post-traumatic osteoarthritis mouse model. *Nanomedicine : nanotechnology, biology, and medicine*. 2015;11(4):939-46. doi: 10.1016/j.nano.2015.01.011. PubMed PMID: 25680539.
 40. Nelson CE, Kintzing JR, Hanna A, Shannon JM, Gupta MK, Duvall CL. Balancing Cationic and Hydrophobic Content of PEGylated siRNA Polyplexes Enhances Endosome Escape, Stability, Blood Circulation Time, and Bioactivity in Vivo. *ACS Nano*. 2013;7(10):8870-80. doi: 10.1021/nn403325f.
 41. Werfel TA, Wang S, Jackson MA, Kavanaugh TE, Joly MM, Lee LH, Hicks DJ, Sanchez V, Ericsson PG, Kilchrist KV, Dimobi SC, Sarett SM, Brantley-Sieders DM, Cook RS, Duvall CL. Selective mTORC2 Inhibitor Therapeutically Blocks Breast Cancer Cell Growth and Survival. *Cancer Res*. 2018;78(7):1845-58. Epub 2018/01/22. doi: 10.1158/0008-5472.CAN-17-2388. PubMed PMID: 29358172; PMCID: PMC5882532.
 42. Werfel TA, Jackson MA, Kavanaugh TE, Kirkbride KC, Miteva M, Giorgio TD, Duvall C. Combinatorial optimization of PEG architecture and hydrophobic content improves ternary siRNA polyplex stability, pharmacokinetics, and potency in vivo. *J Control Release*. 2017;255:12-26. Epub 2017/03/31. doi: 10.1016/j.jconrel.2017.03.389. PubMed PMID: 28366646; PMCID: PMC5484420.

43. Kilchrist KV, Dimobi SC, Jackson MA, Evans BC, Werfel TA, Dailing EA, Bedingfield SK, Kelly IB, Duvall CL. Gal8 Visualization of Endosome Disruption Predicts Carrier-Mediated Biologic Drug Intracellular Bioavailability. *ACS Nano*. 2019;13(2):1136-52. doi: 10.1021/acsnano.8b05482.
44. Jackson MA, Bedingfield SK, Yu F, Stokan ME, Miles RE, Curvino EJ, Hoogenboezem EN, Bonami RH, Patel SS, Kendall PL, Giorgio TD, Duvall CL. Dual carrier-cargo hydrophobization and charge ratio optimization improve the systemic circulation and safety of zwitterionic nano-polyplexes. *Biomaterials*. 2019;192:245-59. Epub 2018/11/10. doi: 10.1016/j.biomaterials.2018.11.010. PubMed PMID: 30458360.
45. Poulet B, Hamilton RW, Shefelbine S, Pitsillides AA. Characterizing a novel and adjustable noninvasive murine joint loading model. *Arthritis and rheumatism*. 2011;63(1):137-47. doi: 10.1002/art.27765. PubMed PMID: 20882669.
46. Noyori K, Koshino T, Takagi T, Okamoto R, Jasin HE. Binding characteristics of antitype II collagen antibody to the surface of diseased human cartilage as a probe for tissue damage. *J Rheumatol*. 1994;21(2):293-6. PubMed PMID: 8182639.
47. Jasin HE, Noyori K, Takagi T, Taurog JD. Characteristics of anti-type II collagen antibody binding to articular cartilage. *Arthritis Rheum*. 1993;36(5):651-9. doi: 10.1002/art.1780360512. PubMed PMID: 8489543.
48. Ko FC, Dragomir C, Plumb DA, Goldring SR, Wright TM, Goldring MB, van der Meulen MC. In vivo cyclic compression causes cartilage degeneration and subchondral bone changes in mouse tibiae. *Arthritis Rheum*. 2013;65(6):1569-78. doi: 10.1002/art.37906. PubMed PMID: 23436303; PMCID: PMC3672344.
49. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. *Bone*. 2012;51(2):249-57. Epub 2012/02/22. doi: 10.1016/j.bone.2012.02.012. PubMed PMID: 22387238; PMCID: PMC3372675.
50. Goldring MB. Articular cartilage degradation in osteoarthritis. *HSS J*. 2012;8(1):7-9. Epub 2012/01/24. doi: 10.1007/s11420-011-9250-z. PubMed PMID: 23372517; PMCID: PMC3295961.
51. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis and Cartilage*. 2010;18:S17-S23. doi: <https://doi.org/10.1016/j.joca.2010.05.025>.
52. Eichaker LR, Cho H, Duvall CL, Werfel TA, Hasty KA. Future nanomedicine for the diagnosis and treatment of osteoarthritis. *Nanomedicine*. 2014;9(14):2203-15. doi: 10.2217/nnm.14.138. PubMed PMID: WOS:000345285700012.
53. O'Grady KP, Kavanaugh TE, Cho H, Ye H, Gupta MK, Madonna MC, Lee J, O'Brien CM, Skala MC, Hasty KA, Duvall CL. Drug-Free ROS Sponge Polymeric Microspheres Reduce Tissue Damage from Ischemic and Mechanical Injury. *ACS Biomaterials Science & Engineering*. 2017. doi: 10.1021/acsbomaterials.6b00804.
54. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage*. 2010;18 Suppl 3:S17-23. doi: 10.1016/j.joca.2010.05.025. PubMed PMID: 20864019.
55. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, van den Berg WB. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. *Arthritis Rheum*. 2007;56(1):147-57. doi: 10.1002/art.22337. PubMed PMID: 17195217.
56. Hügler T, Geurts J. What drives osteoarthritis?—Synovial versus subchondral bone pathology. *Rheumatology*. 2016;56(9):1461-71.
57. Goldring SR. Alterations in periarticular bone and cross talk between subchondral bone and articular cartilage in osteoarthritis. *Ther Adv Musculoskelet Dis*. 2012;4(4):249-58. doi: 10.1177/1759720X12437353. PubMed PMID: 22859924; PMCID: PMC3403248.

58. Boileau C, Tat SK, Pelletier JP, Cheng S, Martel-Pelletier J. Diacerein inhibits the synthesis of resorptive enzymes and reduces osteoclastic differentiation/survival in osteoarthritic subchondral bone: a possible mechanism for a protective effect against subchondral bone remodelling. *Arthritis Res Ther*. 2008;10(3):R71. Epub 2008/06/25. doi: 10.1186/ar2444. PubMed PMID: 18578867; PMCID: PMC2483463.
59. Garg N, Perry L, Deodhar A. Intra-articular and soft tissue injections, a systematic review of relative efficacy of various corticosteroids. *Clinical rheumatology*. 2014;33(12):1695-706.
60. Cho H, Walker A, Williams J, Hasty KA. Study of osteoarthritis treatment with anti-inflammatory drugs: cyclooxygenase-2 inhibitor and steroids. *Biomed Res Int*. 2015;2015:595273. doi: 10.1155/2015/595273. PubMed PMID: 26000299; PMCID: PMC4427003.
61. Almasry SM, Soliman HM, El-Tarhouny SA, Algaidi SA, Ragab EM. Platelet rich plasma enhances the immunohistochemical expression of platelet derived growth factor and vascular endothelial growth factor in the synovium of the meniscectomized rat models of osteoarthritis. *Ann Anat*. 2015;197:38-49. Epub 2014/11/04. doi: 10.1016/j.aanat.2014.10.006. PubMed PMID: 25466931.
62. Solomon LA, Bérubé NG, Beier F. Transcriptional regulators of chondrocyte hypertrophy. *Birth Defects Res C Embryo Today*. 2008;84(2):123-30. doi: 10.1002/bdrc.20124. PubMed PMID: 18546336.
63. Zhai G, Doré J, Rahman P. TGF- β signal transduction pathways and osteoarthritis. *Rheumatol Int*. 2015;35(8):1283-92. Epub 2015/03/15. doi: 10.1007/s00296-015-3251-z. PubMed PMID: 25773660.
64. Shen J, Li S, Chen D. TGF- β signaling and the development of osteoarthritis. *Bone Res*. 2014;2. doi: 10.1038/boneres.2014.2. PubMed PMID: 25541594; PMCID: PMC4274935.
65. Lee YH, Wu HC, Yeh CW, Kuan CH, Liao HT, Hsu HC, Tsai JC, Sun JS, Wang TW. Enzyme-crosslinked gene-activated matrix for the induction of mesenchymal stem cells in osteochondral tissue regeneration. *Acta Biomater*. 2017;63:210-26. Epub 2017/09/09. doi: 10.1016/j.actbio.2017.09.008. PubMed PMID: 28899816.
66. John T, Stahel PF, Morgan SJ, Schulze-Tanzil G. Impact of the complement cascade on posttraumatic cartilage inflammation and degradation. *Histol Histopathol*. 2007;22(7):781-90. doi: 10.14670/HH-22.781. PubMed PMID: 17455152.
67. Silawal S, Triebel J, Bertsch T, Schulze-Tanzil G. Osteoarthritis and the Complement Cascade. *Clin Med Insights Arthritis Musculoskelet Disord*. 2018;11:1179544117751430. Epub 2018/01/03. doi: 10.1177/1179544117751430. PubMed PMID: 29434479; PMCID: PMC5805003.
68. Lubbers R, van Essen MF, van Kooten C, Trouw LA. Production of complement components by cells of the immune system. *Clin Exp Immunol*. 2017;188(2):183-94. Epub 2017/03/24. doi: 10.1111/cei.12952. PubMed PMID: 28249350; PMCID: PMC5383442.
69. Takahashi N, Rieneck K, van der Kraan PM, van Beuningen HM, Vitters EL, Bendtzen K, van den Berg WB. Elucidation of IL-1/TGF-beta interactions in mouse chondrocyte cell line by genome-wide gene expression. *Osteoarthritis Cartilage*. 2005;13(5):426-38. doi: 10.1016/j.joca.2004.12.010. PubMed PMID: 15882566.
70. Klein-Wieringa IR, de Lange-Brokaar BJ, Yusuf E, Andersen SN, Kwekkeboom JC, Kroon HM, van Osch GJ, Zuurmond AM, Stojanovic-Susulic V, Nelissen RG, Toes RE, Kloppenburg M, Ioan-Facsinay A. Inflammatory Cells in Patients with Endstage Knee Osteoarthritis: A Comparison between the Synovium and the Infrapatellar Fat Pad. *J Rheumatol*. 2016;43(4):771-8. Epub 2016/03/15. doi: 10.3899/jrheum.151068. PubMed PMID: 26980579.
71. Wu CL, Harasymowicz NS, Klimak MA, Collins KH, Guilak F. The Role of Macrophages in Osteoarthritis and Cartilage Repair. *Osteoarthritis Cartilage*. 2020. Epub 2020/01/08. doi: 10.1016/j.joca.2019.12.007. PubMed PMID: 31926267.
72. Mazur CM, Woo JJ, Yee CS, Fields AJ, Acevedo C, Bailey KN, Kaya S, Fowler TW, Lotz JC, Dang A, Kuo AC, Vail TP, Alliston T. Osteocyte dysfunction promotes osteoarthritis through MMP13-dependent

- suppression of subchondral bone homeostasis. *Bone Res.* 2019;7:34. Epub 2019/11/05. doi: 10.1038/s41413-019-0070-y. PubMed PMID: 31700695; PMCID: PMC6828661.
73. Goldring SR, Goldring MB. Biology of the normal joint. *Kelley's textbook of rheumatology*: Elsevier; 2017. p. 1-19. e6.
 74. Hoshyar N, Gray S, Han H, Bao G. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine (Lond)*. 2016;11(6):673-92. Epub 2016/03/22. doi: 10.2217/nnm.16.5. PubMed PMID: 27003448; PMCID: PMC5561790.
 75. Evans BC, Nelson CE, Yu SS, Beavers KR, Kim AJ, Li H, Nelson HM, Giorgio TD, Duvall CL. Ex Vivo Red Blood Cell Hemolysis Assay for the Evaluation of pH-responsive Endosomolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs. *Journal of Visualized Experiments : JoVE*. 2013(73):50166. doi: 10.3791/50166. PubMed PMID: PMC3626231.
 76. Polderman JA, Farhang-Razi V, Van Dieren S, Kranke P, DeVries JH, Hollmann MW, Preckel B, Hermanides J. Adverse side effects of dexamethasone in surgical patients. *Cochrane Database Syst Rev*. 2018;11:CD011940. Epub 2018/11/23. doi: 10.1002/14651858.CD011940.pub3. PubMed PMID: 30480776.
 77. Orak MM, Ak D, Midi A, Lacin B, Purisa S, Bulut G. Comparison of the effects of chronic intra-articular administration of tenoxicam, diclofenac, and methylprednisolone in healthy rats. *Acta Orthop Traumatol Turc*. 2015;49(4):438-46.
 78. Bolon B, Stolina M, King C, Middleton S, Gasser J, Zack D, Feige U. Rodent preclinical models for developing novel antiarthritic molecules: comparative biology and preferred methods for evaluating efficacy. *J Biomed Biotechnol*. 2011;2011:569068. Epub 2010/12/28. doi: 10.1155/2011/569068. PubMed PMID: 21253435; PMCID: PMC3022224.
 79. Aigner T, Söder S. Histopathologische Begutachtung der Gelenkdegeneration. *Der Pathologe*. 2006;27(6):431-8. doi: 10.1007/s00292-006-0864-8.