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Expression of semaphorin-3A in the joint and role in osteoarthritis

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Abstract

Osteoarthritis (OA) is characterised by the deterioration of cartilage in the joints and pain. We hypothesise that semaphorin-3A (sema-3A), a chemorepellent for sensory nerves, plays a role in joint degradation and pain. We used the mechanical joint loading (MJL) model of OA to investigate sema-3A expression in the joint and examine its association with the development of OA and pain. We also analyse its effect on chondrocyte differentiation using the ATDC5 cell line. We demonstrate that sema-3A is present in most tissues in the healthy joint and its expression increases in highly innervated tissues, such as cruciate ligaments, synovial lining and subchondral bone, in loaded compared to nonloaded control joints. In contrast, sema-3A expression in cartilage was decreased in the severe OA induced by the application of high loads. There was a significant increase in circulating sema-3A, 6 weeks after MJL compared to the nonloaded mice. mRNA for sema-3A and its receptor Plexin A1 were upregulated in the dorsal root ganglia of mice submitted to MJL. These increases were supressed by zoledronate, an inhibitor of bone pain. Sema-3A was expressed at all stages of Chondrocyte maturation and, when added exogenously, stimulated expression of markers of chondrocyte differentiation. This indicates that sema-3A could affect joint tissues distinctively during the development of OA. In highly innervated joint tissues, sema-3A could control innervation and/or induce pain-associated neuronal changes. In cartilage, sema-3A could favour its degeneration by modifying chondrocyte differentiation.

KEYWORDS
chondrocyte, innervation, joint, osteoarthritis, semaphorin-3A

1 | INTRODUCTION

Osteoarthritis (OA) is the most common joint disorder, with an estimated 28% of the older population (over 60 years) affected.[1,2] It is the most common cause of disability in older adults and poses a massive health and social care system burden.[3] OA is characterised by the deterioration of cartilage in the joints associated with pathologies in multiple joint tissues. The main symptoms are chronic joint pain and stiffness associated with progressive changes in the joint tissues.[4,5] There are no disease-modifying treatments for OA rendering the management of

this disease difficult. In addition, current OA pain treatments are limited and largely consist of weak pain killers such as paracetamol, NSAIDS that modulate inflammation and opioids if these are unsuccessful. Bisphosphonates were suggested to preserve the structural integrity of subchondral bone in knee OA[6] but there is a lack of evidence of their effectiveness as a disease-modifying drug in OA. They may however have a therapeutic potential for OA-associated pain.[7]

There is a need for basic and translational research to better understand joint pain to allow treatment that does not hasten the progression of the disease. The origin of pain lies in the joint where pain signals are detected by nociceptors present locally and carried to the spinal cord and brain.[8] Cartilage is aneural but innervation is abundant in the ligaments, menisci and periosteum.[9] Exposure of the subchondral bone allows the vascularization of cartilage and ingrowth of nerve fibres as OA pathologies progress. The nerves that innervate the joint can undergo a notable reorganisation, changing their morphology, increasing density and sprouting in areas which are normally poorly or noninnervated. This nerve sprouting has been observed in several skeletal pathologies including OA and is often associated with the generation of pain.[10–12] Its cause is mainly unknown but could involve contributions from factors released by cartilage cells during its degradation, and/or inflammatory and immune cells.

Semaphorins are a large and highly conserved family of proteins, both secreted and membrane bound, that guide neuronal axons during the development of the nervous system.[13] They include many members and can be divided into 8 classes. Among them, class 3 semaphorins are secreted family members that have autocrine and paracrine functions via the plexin/neuropilin complex.[14–16] Semaphorin-3A (sema-3A) axonal guidance protein has been the most studied. In the nervous system, it inhibits axonal growth in a concentration-dependent manner via a receptor complex that consists of neuropilin 1 (Nrp-1) and plexin-A1 (PlxA).[17] It has been shown to serve several other functions, including in rheumatic diseases by controlling immune cells, angiogenesis, bone remodelling, cell migration and apoptosis.[18] Several studies have examined the expressions of sema-3A and its receptors in joint tissues and their changes with degeneration of intervertebral disk,[19,20] bone remodelling[21,22] and rheumatic diseases.[18,23] Most studies showed that sema-3A expression is reduced in serum and synovial tissues of patients with rheumatoid arthritis (RA) and in systemic Lupus Erythematosus,[18,23] although some results are contradictory as sema-3A was also shown to be significantly higher in patients with RA and to correlate with inflammatory factors.[24] The role of sema-3A in chondrocyte function and changes of its expression in the joint with the development of OA have been less explored and this is the goal of this study.

Animal models of OA, mainly in rodents, have been extensively used to address questions regarding the pathological development of OA and the associated pain. They include those that spontaneously develop OA such as the STR/ort mouse,[25] those in which OA is surgically induced (such as anterior cruciate ligament resection and medial meniscectomy) and those that involve the administration of irritants in the joint, such as the monoiodo acetate (MIA) and Freund's Complete Adjuvant (FCA) which are well characterised.[26] Mechanical loading of the knee joint is a novel, noninvasive murine model of OA. It is characterised by OA lesions and a reproducible pain phenotype that can be reversed using known analgesics.[27,28] The mechanical joint loading (MJL) model is now well-established to study the pathogenesis of OA, as the knee joint remains intact and it takes into account mechanical factors that are important for the initiation and progression of OA.[25,28]

This novel model of OA pain was used for the first time to study the role of sema-3A in OA. The MJL model presents the advantage that the joint can be mechanically loaded with different intensities and durations accounting for different OA severity. We had two aims: (1) to analyse sema-3A expression in the different joint tissues and determine whether sema-3a expression in the joint changes with OA severity. Since the potent bisphosphonate Zoledronate has been suggested to help reduce bone pain in OA, some mice were also treated with Zoledronate to investigate if this affects sema-3A expression in the dorsal root ganglia (DRGs); (2) to examine the role of sema-3A in chondrocyte function. We investigated whether exogenous sema-3A affects the different stages of chondrocyte differentiation using the chondrogenic ATDC5 cell line.

Significance statement

Semaphorin-3A (sema-3A) is an axon guidance molecule previously shown to play a role in neural ingrowth and vascularisation during degeneration of tissues. We investigated its expression in tissues of the mouse joint and examined changes in expression with the development of osteoarthritis (OA). We show that sema-3A expression is increased in highly innervated joint tissues with OA and may control innervation in these tissues. In contrast, sema-3A expression in cartilage decreases with the severity of OA. We also demonstrate using a chondrocytic cell line that sema-3A stimulates expression of markers of chondrocyte differentiation and may play a role in cartilage degeneration.

2 | MATERIALS AND METHODS

2.1 | Animals

All mice were housed in groups of four in individually ventilated cages and fed a standard RM1 maintenance diet ad libitum. Each experiment was carried out in compliance with the Animals (Scientific Procedures) Act (1986) and approved by the Royal Veterinary College's Ethics and Welfare Committee and UK Home Office.

2.2 | Induction of osteoarthritis

We used the MJL model of OA. The right knees of naïve, male 12-week-old C57bl/6 mice (Charles River) were loaded using a servo-hydraulic materials testing machine (Model HC10, Dartec Ltd.), as previously described.[27,28] Osteoarthritis was induced in the right knees

by a 2-week loading regimen of 40 cycles with peak force of 9N or 11N (three times/week). We previously showed that loading at both 9N and 11N induced OA lesions compared to nonloaded controls 6 weeks after loading and these lesions were more severe in the joints of mice loaded at 11N compared to 9N.[28] Briefly, the tibia was positioned vertically between two custom-made cups to fixate the knee and ankle joint in deep flexion. Axial compressive loads were applied to the knee joint via the upper loading cup controlled by software delivered by the loading system (WinTest7 Bose). One loading cycle consisted of 9.9 s holding time with a load magnitude of 2N after which a peak load of 9N or 11N was applied for 0.05 s. This 10 s trapezoidal wave loading cycle was repeated 40 times within each loading episode. Loading episodes were applied three times per week, performed on alternating days, for 2 consecutive weeks. The left hindlimb was left unloaded.

Two experiments were performed using the MJL model. One was aimed at examining the changes in sema-3A expression in joints after MJL of different severity. In this experiment, mice were therefore loaded at both 9N and 11N (6 mice/group). In the second MJL experiment, the effect of Zoledronate treatment on sema-3A expression in the DRGs was performed on mice loaded at 9N only. We had three groups: Unloaded mice treated with PBS ($n = 6$), loaded mice treated with PBS ($n = 6$) and loaded mice treated with Zoledronate ($n = 6$). Zoledronic acid (Sigma-Aldrich, SML0223-10MG) was administered weekly (IP) at a concentration of 100 µg/kg for 6 weeks from 3 days after the last loading episode.

2.3 | Tissue collection

The knee joints, DRGs and blood samples of mice subjected to MJL were collected 6 weeks after the last loading episode and processed immediately after euthanasia.

Blood was allowed to clot at room temperature, centrifuged at 2000 g for 10 min and serum collected and frozen at −80°C.

Knee joints were fixed in 10% neutral buffered formalin for 1 day and subjected to 10 days of decalcification in Immunocal (StarLab). Joints were then embedded in paraffin and sectioned at 6 µm coronally across the whole joint using Microm HM325 manual rotary microtome.

The spinal cord was dissected out and the lumbar DRGs (L3–L6) were isolated. They were flash frozen at −80°C for future RNA extraction.

2.4 | Measurement of sema-3A in serum

Quantification of sema-3A in mouse serum was performed using a specific solid Phase Sandwich ELISA (Mouse Sema-3A ELISA kit, CUSABIO) according to the manufacturer's recommendations.

2.5 | Grading of OA lesions

The entire knee was sectioned and collected on slides. We selected one in every four slides of the entire joint to grade OA lesions. These coronal sections of the knee joint were stained with toluidine blue to visualise the cartilage and bone. Each knee will get an average OA score and a maximum OA score for further statistical analysis. Cartilage integrity was scored using the Osteoarthritis Research Society International grading system (range: 0–6). Grade 0: Normal AC surface; Grade 0.5: loss of toluidine Blue staining but no lesions; Grade 1: lesions in the superficial zone of the AC; Grade 2: lesions down to the intermediate zone; Grade 3: lesions down to the tidemark with or with or out possible loss of AC up to 20% of the surface of the condyle; Grade 4: loss of AC tissue from 20% to 50% of the condyle surface; Grade 5: loss of AC tissue from 50% to 80% of the condyle surface; Grade 6: more than 80% loss of surface and/or exposed subchondral bone.

2.6 | Immunohistochemistry

Knee joint sections were dewaxed and rehydrated before antigen retrieval process (0.02 M HCL incubation for 15 min at 37°C). Sections were treated with pepsin for 45 min at 37°C (3 mg Pepsin in 1 mL 0.02 M HCL) and exogenous peroxidase activity was blocked by 0.3% Hydrogen Peroxide for 15 min at 37°C. Sections were then incubated in blocking buffer (10% normal goat serum [sigma G3023] in 0.1% PBS-Bovine Serum Albumin) for 1 h at room temperature, then incubated with rabbit polyclonal anti-sema-3A antibody (1/200 dilution) (Abcam) or with polyclonal rabbit IgG (as a control, R&D Systems) overnight at 4°C in the dark. On the following day, slides were rinsed with PBS (three times for 10 min), then incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit, 1:200) (P0160, Dako). Sections were washed three times in PBS for 10 min each time, followed by DAB staining. Sections were then dehydrated and mounted with DPX. Images were taken using DM4000 upright bright field microscope.

2.7 | Culture of the mouse chondrogenic cell line ATDC5

ATDC5 cells were cultured in growth medium (DMEM/F-12 medium [Gibco] containing 1% [vol/vol] antibiotic-antimycotic [Gibco], 5% FBS [Gibco], 10 µg/mL human transferrin [Sigma] and 3×10^{-8} M sodium selenite [Sigma]). 2×10^5 cells were used for each micro-mass culture. From Days 1 to 14, cells were cultured with chondrogenic medium which contains 1% (vol/vol) antibiotic-antimycotic, 5% FBS, supplemented with an ITS premix containing 10 µg/mL insulin, 5 µg/mL human transferrin, 3×10^{-8} M sodium selenite (Gibco), and 5 µg/mL human transferrin (Sigma) in DMEM/F-12 medium. Mineralization medium was used from Days 14 to 21 containing 5% FBS (Gibco), 10 µg/mL human transferrin (Sigma) and 3×10^{-8} M sodium selenite (Sigma) and 7 mM β-glycerophosphate (Sigma) in alpha-MEM medium (Gibco). RNA from cells was collected at Day 0 before the change of medium for cell differentiation in micro-mass and at Days 7, 14 and 21.

2.8 | Treatment of cells with sema-3A

Micro-mass cultures were treated with either mouse recombinant (*r*) sema-3A or infected with sema-3A lentivirus. Recombinant sema-3A (20 ng/mL) treatment started from Day 1 of culture and added every 2 days during medium change. Sema-3A lentivirus was added during the micro-mass formation at multiplicity of infection (MOI) of 20.

2.9 | Quantitative PCR (qPCR) analysis

qPCR was used to analyse sema-3A mRNA expression levels during differentiation of the mouse ATDC5 chondrogenic cell line and DRGs. We followed as much as possible the MIQE guidelines.[29]

We had two different RTqPCR methods, one for the ATDC5 chondrogenic cell line and one for DRGs.

For the RT-qPCR performed in the DRGs of loaded mice treated with Zoledronate, purified RNA from DRGs was isolated employing the RNeasy Mini Kit (QIAGEN). The 260/280 absorbance ratio for RNA in the NanoDrop (Thermo Scientific) was between 1.8 and 2.0 for every sample. Intron-spanning primers were designed with the Primer3 Input software (version 0.4.0). Selected primers with similar melting temperatures were then assessed via blast to avoid within-species off-target amplification. With an input of 5 µg, RNA was reverse-transcribed together with cDNA amplification by qPCRs in duplicates using the PCRBIO 1-Step Go RT-PCR Kit (PCR Biosystems) and specific intron-spanning primers (Integrated DNA Technologies [IDT] in a CFX96 Touch Real-Time PCR Detection System [Biorad]). Gene expression was normalized to the expression of housekeeping gene (*Actin*), which remained stable with an approximate Ct between 19 and 22 cycles for all samples. Relative gene expression was calculated by using the formula $2^{-\Delta Ct} \times 1000$. As per MIQE guidelines, the sequence of primers used were the following: Actin: Primer forward (F) 5′ AGGTCATCACTATTGGCAACG 3′, Reverse (R) Primer 5′ CACTTCATGATGGAATTGAATGTAGTT 3′; Nrp1: F 5′ GGAGCTA CTGGGCTGTGAAG 3′, R 5′ ACCGTATGTCGGGAACTCTG 3′; Nrp2 F 5′ ATTCAGAAAGCTGGGGGTTT 3′, R 5′ GAGCCTCAAATCAGCC AAAG 3′; PlexnA1: F 5′ CTTCTGGACTGGGCTCTGAC 3′, R 5′ TAG AGGGTGGCTCTGAGCAT 3′; Sema-3A: F 5′ CCTCCCAAAACCTCA AACAA 3′, R 5′ TGATCTCTGTCAAGCGTTGG 3′.

For the RT-qPCRs performed in ATDC5 cell line, samples were lysed using Nucleospin RNA isolation kit (Macherey-Nagel) and quantified using a nanodrop (Thermo Scientific) according to manufacturer's instructions. Complementary DNA (cDNA) was generated using Thermofisher Maxima H Minus First Strand cDNA Synthesis according to manufacturer's instructions. Primers were designed using the Ensembl genome browser, Roche website and according to the literature. Levels of actin mRNA expression were used for cDNA normalization. In line with MIQE recommendation, we use two reference genes (beta-actin and Hprt). All gene expression data was normalised to either actin or Hprt, both known to be stable within this chondrocytic cell line.[30] The following primers were used: mouse sema-3A; forward (F), GCCTGCAGAAGAAGGATTCA; mouse sema-3a reverse (R), TCAGGTTGGGGTGGTTAATG; mouse (MUS) actin F, GAT CTG GCA CCA CAC CTT CT; Mus actin R, GGG GTG TTG AAG GTC TCA AA; Mus Sox9 F, GCT GGA AGT CGG AGA GCC GAG A; Mus Sox9 R, AGA GAA CGA AAC CGG GGC CAC; Mus aggrecan F, GAA ATG ACA ACC CCA AGC AC; Mus aggrecan R, CTG ATG GCA ACA TTC ACC TC; Mus Hprt F, CTG GTG AAA AGG ACC TCT CGA A; Mus Hprt R, CTG AAG TAC TCA TTA TAG TCA AGG GCA T; Mus col2a1 F, CTG CCA GTG GAA AAT TAG GG; Mus col2a1 R, TTC TCC CTT GTC ACC ACG AT; col10a1 F, CCT GGT TCA TGG GAT GTT TT; col10a1, CAG GAA TGC CTT GTT CTC CT.

2.10 | Statistical analysis

Data are presented as mean ± SD. Multiple comparisons were performed using two-way analysis of variance and Tukey's multiple comparison post-hoc test where appropriate.

3 | RESULTS

3.1 | Sema-3A is expressed in several joint tissues

To investigate the role of sema-3A in joint pathology, we first examined its expression in a normal mouse knee joint (not submitted to MJL) using immunohistochemistry. Our results show that sema-3A is expressed in several tissues in the healthy joint (Figure 1). It is mainly expressed in articular cartilage and in the meniscus (Figure 1B), in cruciate ligaments (Figure 1D), in the synovial lining (Figure 1A) and in subchondral bone. While sema-3A is present in chondrocytes in articular cartilage, chondrocytes in the growth plate seem to be devoid of any expression (Figure 1C). Use of a normal pre-immune IgG serum on serial sections as a negative control confirmed the specificity of the labelling with anti-sema-3A antibody (Figure 1E).

3.2 | Sema-3A expression in most tissues but not in the articular cartilage of the joint increases with the progression of osteoarthritis

We next determined if there were changes in this relatively restricted pattern of sema-3A expression between healthy and OA joints using the MJL OA model. Immunolabelling demonstrated that the overall expression of sema-3A in joint tissues was increased with OA severity (Figure 2A–C). We found an increase in sema-3A expression in cruciate ligaments, in the synovial lining and in subchondral bone in loaded compared to nonloaded control joints (Figure 2C). There was also more staining in the ligaments in the 11N-loaded knees compared to the 9N-loaded ones (Figure 2C). Sema-3A was expressed in articular cartilage (Figure 2D–F), but in contrast to other joint tissues, expression was not increased in chondrocytes in 9N-loaded cartilage compared to nonloaded cartilage (Figure 2H,I). In fact, sema-3A levels exhibited a reduced expression in the severe OA

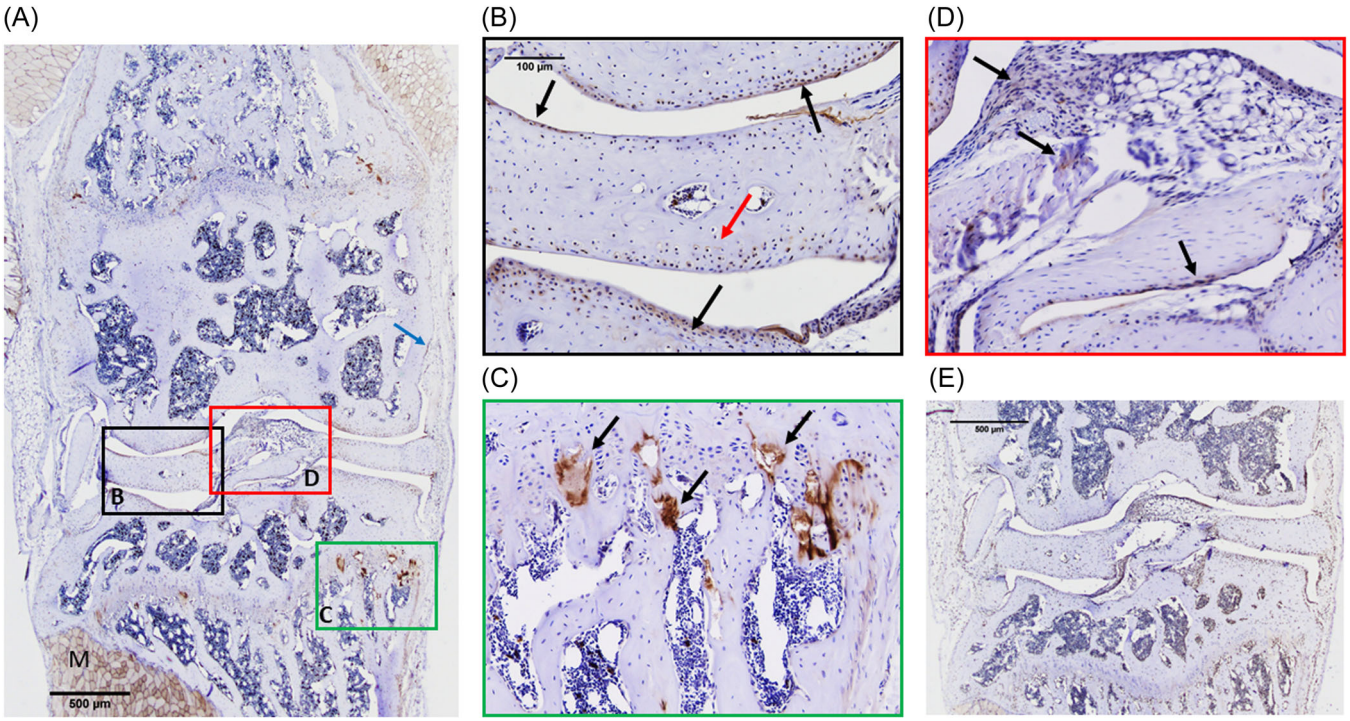

FIGURE 1 Immunolabelling for semaphorin-3A (sema-3A) in the mouse knee joint. Paraffin sections were stained with either sema-3A antibody (A–D, Abcam) or normal IgG control antibody (E). Sema-3A is expressed in articular cartilage (B, black arrows), meniscus (B, red arrow), cruciate ligaments (D, black arrows) and in the synovial lining (A, blue arrow). Nonspecific binding is shown in skeletal muscle (M). Immunolabelling for sema-3A is found in the growth plate (black arrows, C) but not in chondrocytes (C). Squares in (A) indicate joint areas with higher magnification images shown as B–D.

induced by the application of loads at 11N (Figure 2F,J). Use of a normal pre-immune IgG serum on serial sections as a negative control confirmed the specificity of the labelling with anti-sema-3A antibody (Figure 2G,K). Loaded knees showed signs of cartilage damage as previously shown[9] (Figure 2J) and the lesions increased in the 11N-loaded joints compared to the 9N-loaded ones (Figure 3).

3.3 | Sema-3A concentration increases in the serum of mice after MJL

As sema-3A circulates, we collected serum at one and 6 weeks after MJL at 9N and quantified sema-3A concentrations using a specific ELISA. We showed that there was a significant increase in sema-3A concentration (+25%) in mouse serum 6 weeks after MJL at 9N compared to the nonloaded mice (Figure 4). This increase in sema-3A concentration was not observed 1 week after loading. This may suggest that it is not an early consequence of MJL or that the changes in the joint are too early to be detected.

3.4 | Expression of sema-3A in the DRGs of mice increases with MJL

Since sema-3A has been shown to regulate sensory innervation and could contribute to pain in OA, its expression in the lumbar L3-L6 DRG that innervates the hind limb was examined. We show that sema-3A mRNA (Figure 5A) and its receptor Plexin A1 (Plx-A1) (Figure 5B) were upregulated in the DRGs of mice that have been submitted to MJL at 9N. The increase in sema-3A gene expression after MJL was however not significant. Neuropilin-1 (NP1) expression was not significantly changed by MJL (Figure 5C). Interestingly, when mice were treated with Zoledronate, an inhibitor of bone resorption and bone pain, the MJL-induced increases in sema-3A and Plexin-A1 expression were supressed (Figure 5A,B).

3.5 | Sema-3A is expressed at all stages of chondrocyte differentiation and chondrocyte markers are affected by treatment with recombinant sema-3A or transfection of sema-3A

We used the ATDC5 chondrocytic cell line to also study the expression of sema-3A during chondrocytic differentiation. Sema-3A was expressed at all stages of ATDC5 chondrocyte differentiation, with expression peaking during chondrocyte maturation before a decline in expression during hypertrophic differentiation when chondrocytes show raised expression of type X collagen (Figure 6).[31]

Since sema-3A is involved in cartilage OA pathophysiology, we analyse its influence on chondrocyte differentiation. For this, we analysed the expression of chondrocyte markers in the ATDC5 cell

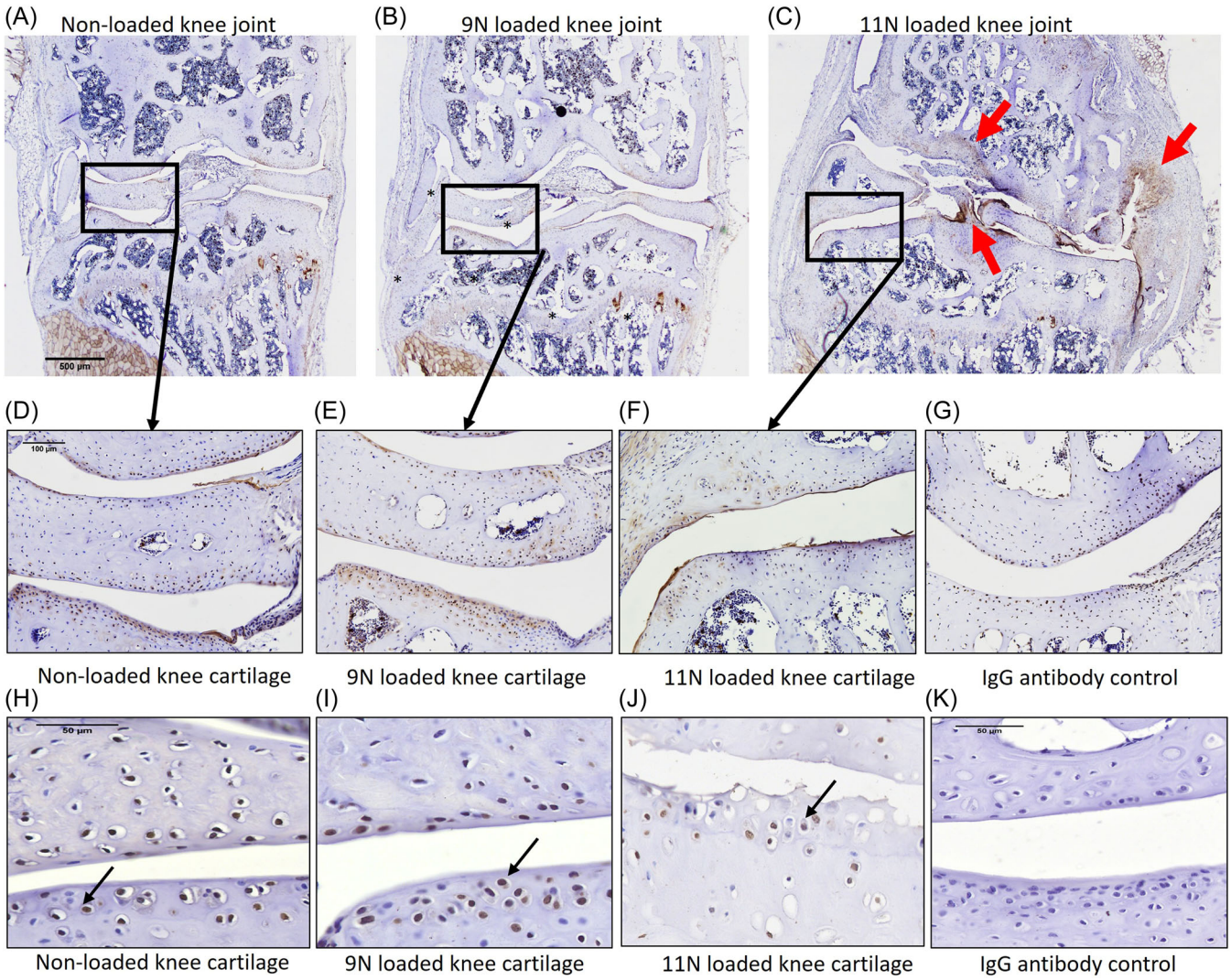

FIGURE 2 Changes in semaphorin-3A (sema-3A) expression in OA mouse knee joints. Osteoarthritis was induced by mechanical joint loading (MJL) at 9N (B) and 11N (C). Loading at 9N (B) and at 11N (C) increases the expression of sema-3A in cruciate ligaments, in the synovial lining and in subchondral bone compared to nonloaded controls. In 11N-loaded joints, OA was severe and the high expression of sema-3A is indicated by red arrows (C). Sema-3A was also expressed in articular cartilage (D–F) but the intensity of labelling in chondrocytes did not increase in 9N-loaded cartilage (E, I) compared to nonloaded cartilage (D, H). In contrast, it was reduced in 11N-loaded joints (F, J). Black squares in (A–C) indicate cartilage areas with higher magnification images shown as D–F. Black arrows in H–J indicate positive chondrocytes for sema-3A. Paraffin sections were stained with either sema-3A antibody (A–F) or normal IgG control antibody for serial sections of knees loaded at 9N (G, K).

line when cells were treated with either recombinant sema-3A (r sema-3A) or a lentivirus overexpressing sema-3A (L sema-3A), added during all stages of ATDC5 differentiation from Day 0 to 21. Both r sema-3A and L sema-3A stimulated the mRNA expression of type 2 collagen by ATDC5 chondrocytes, although the effect occurred at all time points for r sema-3A while only from Week 3 for L sema-3A (Figure 7A,B). mRNA expression of collagen type X was also enhanced with r and L sema-3A (Figure 7C,D). While r sema-3A had no effect on aggrecan expression, L sema-3A increased its expression at Weeks 2 and 3 (Figure 7F). They were no consistent effects of r and L sema-3A on Sox 9 expression (Figure 7G,H). r sema-3A also increased MMP13 expression at Week 3 (Figure 7I).

4 | DISCUSSION

Using the MJL model of OA, our results show that sema-3A is expressed in several joint tissues, highly present in the cruciate ligaments, the synovial lining and the subchondral bone, tissues that are the most innervated in the joint.[9,32] Immunocytochemistry in our study was qualitative, meaning that we just wanted to evaluate in which tissues in the joint sema-3A was present. Sema-3A is needed for normal patterning and growth of nerves[33] and was shown to play a role in bone innervation.[21,34] It was recently shown that sema-3A is associated with sensory nerves and type H vessels during in situ osteogenesis,[35] confirming the close interaction between sema-3A

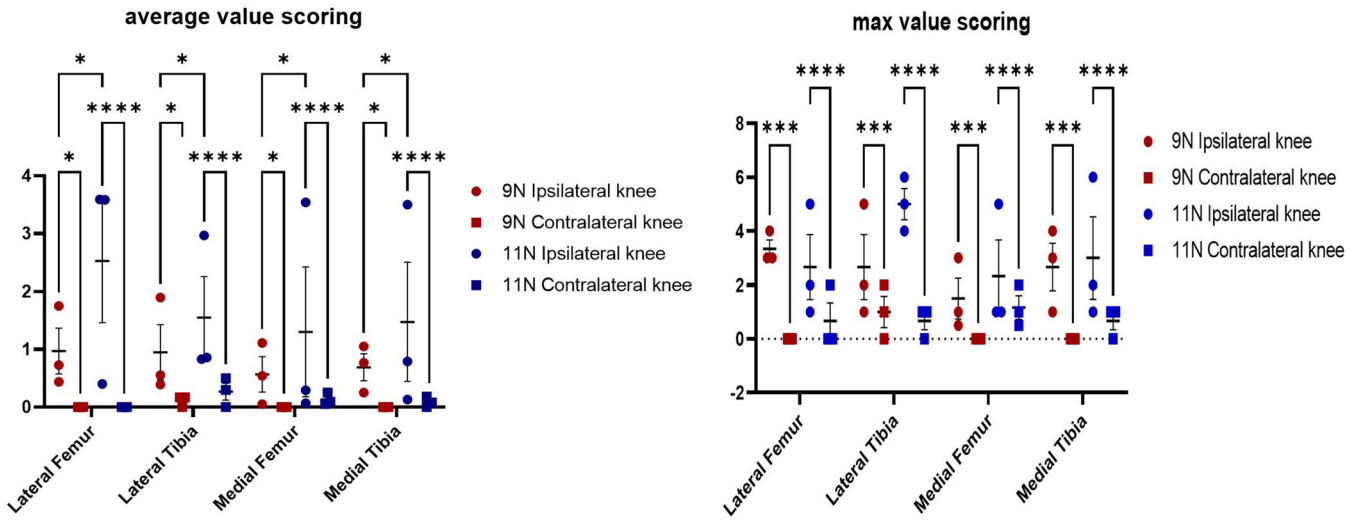

FIGURE 3 Severity of osteoarthritis (OA) cartilage lesions following mechanical joint loading (MJL). Severity of OA lesions on each joint section following MJL was scored as either low (grade 0–2), mild (grade 3–4) or severe (grade 5–6). For each sample, average value score and maximum scores, determined as the lesions with the highest severity are given. Differences in the severity of OA lesions between groups (ipsilateral vs. contralateral and 11N vs. 9N) are indicated with a $^{*}p < .05$, $^{***}p < .001$, $^{****}p < .0001$.

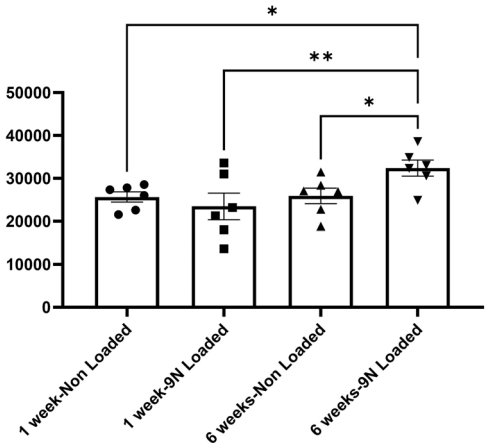

FIGURE 4 Quantification of semaphorin-3A (sema-3A) levels in serum of mice with osteoarthritis (OA). Serum sema-3A levels were measured in C57BL/6 male mice (12-week-old), 1 or 6 weeks after application of mechanical joint loading at 9N. Nonloaded mice served as controls. Bars represent mean ± SEM of $n = 6$ mice/group. $^{*}p < .05$, versus nonloaded controls; $^{**}p < .01$, versus 1 week after loading.

and sensory nerves. Our data show that sema-3A expression is upregulated during OA in those highly innervated joint tissues. These results are novel and suggest that sema-3A may play a role in the changes in peripheral innervation occurring during joint degeneration. Innervation is closely linked to vascularisation and nerve growth together with angiogenesis may contribute to skeletal pain in several pathologies including bone metastases.[36,37] Sema-3A usually inhibits angiogenesis and nerve growth[38] and this implies that sema-3A serves to protect against joint degeneration. This may be the case in articular cartilage which is avascular and aneural. We show that sema-3A is present in chondrocytes in articular cartilage and its expression in these cells only decreases in 11N-loaded mice corresponding to intense OA severity, suggesting that sema-3A function in cartilage could be to protect against its degradation by limiting nerve and blood vessels ingrowth. With OA severity, a decrease in sema-3A expression in chondrocytes could favour vascular invasion and nerve growth in this tissue. This possibility has been supported by studies in the human vertebral disc where sema-3A was proposed to act as a barrier to neuronal ingrowth with the healthy disc.[20] We did not examine however if there was a reduction in chondrocytes numbers or an increased chondrocyte apoptosis with OA severity in the MJL model of OA. In contrast, class 3 semaphorins were shown to be upregulated in the degenerate human intervertebral disc where they were associated with the presence of nerve and blood vessels.[19] Sema-3A may also play other roles during joint degradation including in the inflammatory process and the immune response.[18,24] Interestingly, while sema-3A was highly expressed in articular cartilage chondrocytes, it was poorly expressed by chondrocytes of the growth plate being mainly present in blood vessels associated with chondrocyte hypertrophy.

We used the MJL model of OA to investigate the changes of sema-3A expression with joint degeneration. We previously demonstrated that there is a pain phenotype in this model which correlates with cartilage damage.[9] This is the first demonstration of sema-3A expression in this model. There is evidence that sema-3A attenuates hyperalgesia, mainly by preventing the sprouting of unmyelinated sensory nerve endings although other mechanisms have been suggested.[38] This was shown for low back pain where sema-3A reduced nerve ingrowth and vascular proliferation as well as the expression of matrix metalloproteinases (MMPs).[39] Our demonstration that sema-3A expression is upregulated in innervated joint tissues during the development of OA suggests that it may play a protective role in skeletal pain by modulating innervation or

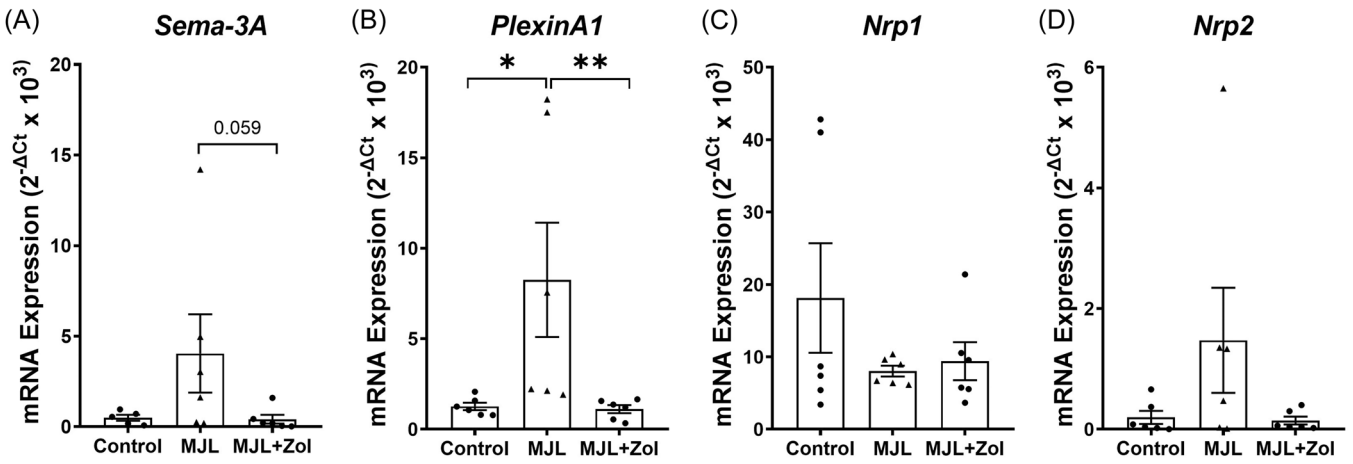

FIGURE 5 Changes in semaphorin-3A (sema-3A) and receptors mRNA levels in dorsal root ganglia (DRGs) of mice with osteoarthritis (OA). Sema-3A (A), Plexin A-1 (PlxA1) (B), Neuropilin 1 (Nrp1, C) and Nrp2 (D) were quantified in L3–L6 DRGs isolated from C57BL/6 male mice (12-weeks-old), which were submitted to joint mechanical loading at 9N with or without Zoledronate (Zol) treatment. DRGs were isolated 6 weeks after loading. Nonloaded mice served as controls. Data are expressed as mean ± SEM of relative gene expression, calculated using the formula $2^{-\Delta Ct} \times 1000$ (ΔCT values normalised to Actin, $n = 6$ per group). *$p < .05$ versus nonloaded controls; **$p < .01$ versus no zoledronate treatment.

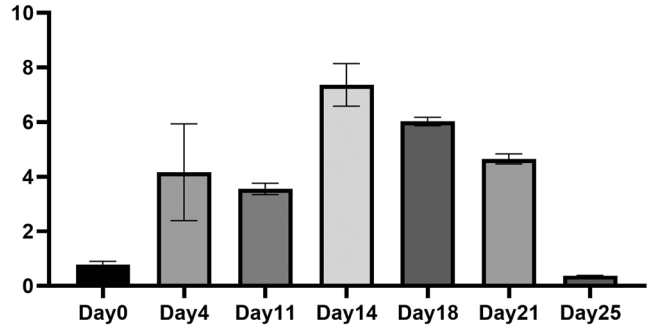

FIGURE 6 Expression of semaphorin-3A (sema-3A) mRNA during chondrocyte differentiation. Mouse chondrogenic ATDC5 cells were cultured in chondrogenic medium for 25 days and mRNA levels of sema-3A in chondrocytes were assessed at Days 0, 4, 11, 14, 18, 21, and 25 of chondrocyte differentiation using real-time quantitative PCR. Results are expressed as mean ± SEM of fold change over actin. One experiment only was performed, in triplicate.

inflammation. However, we didn't examine joint innervation in this study, but our previous work showed no alteration of joint innervation over time following MJL.[9] This would require further examination as it is possible that our immunocytochemistry protocol to quantify nerves is not adequate or sensitive enough to detect nerve growth, that the MJL model doesn't cause enough inflammation to induce nerve sprouting and/or that other neuronal changes contribute to skeletal pain in this model.

Sema-3A is expressed in joint tissues but is also a secreted semaphorin. There is however limited information regarding the role of circulating sema-3A in skeletal diseases. Sema-3A in serum has been shown to be either significantly elevated or decreased in patients with RA.[24,40] In addition, sema-3A levels in serum are decreased in patients with auto-immune diseases such as multiple sclerosis and systemic lupus erythematosus.[41] To our knowledge no study has examined the changes in sema-3A concentration in the serum of OA patients compared to healthy patients. Our study shows a significant increase of serum sema-3A levels 6 weeks after loading in the MJL model. The significance of this increase has yet to be determined and may reflect the increased expression of sema-3A in the joint induced by mechanical loading. It may also reflect changes in deformation and dynamic fluid shifts in the bone induced by repetitive loading cycles. The sema-3A cell surface receptor comprises a complex of two different transmembrane receptors, Nrp-1 and PlxA. Their expression was not analysed in the joint in this study and it is possible that there is increased concentration of sema-3A in serum in compensation to a decreased expression of its receptors. Our group has previously performed microarray analyses comparing mRNA transcripts expression in articular cartilage from OA-prone STR/ort mice to non-OA prone CBA controls at different ages to identify genes involved in OA induction or progression.[42] Interrogating these published data further, we found that STR/ort mice express more sema-3A in articular cartilage than CBA mice but that the expressions levels in both strains of mice decrease with age and with the development of OA. Interestingly, Nrp-1 expression levels in articular cartilage dramatically decreased with age in the STR/ort mice while unchanged in CBA mice. Sema-3A, NP1 isoform 1, NP2, Plx A1 isoform 2, Plx A2 and A3 were also decreased in STR/ort mice at 40 weeks of age compared to 18 weeks (see Figure SX), further suggesting that sema-3A signalling could play a role in the development of OA. The identification of the components of the receptor complexes for sema-3A in joint is important as the different expression of particular receptors and co-receptors complexes may induce differential signalling pathways.

Primary sensory neurons located in the DRGs transmit pain information from the joint to the brain. Sema-3A and Nrp-1 are expressed in DRGs neurons where they may play a role in nerve regeneration after dorsal root injury.[43] Our study is the first one to

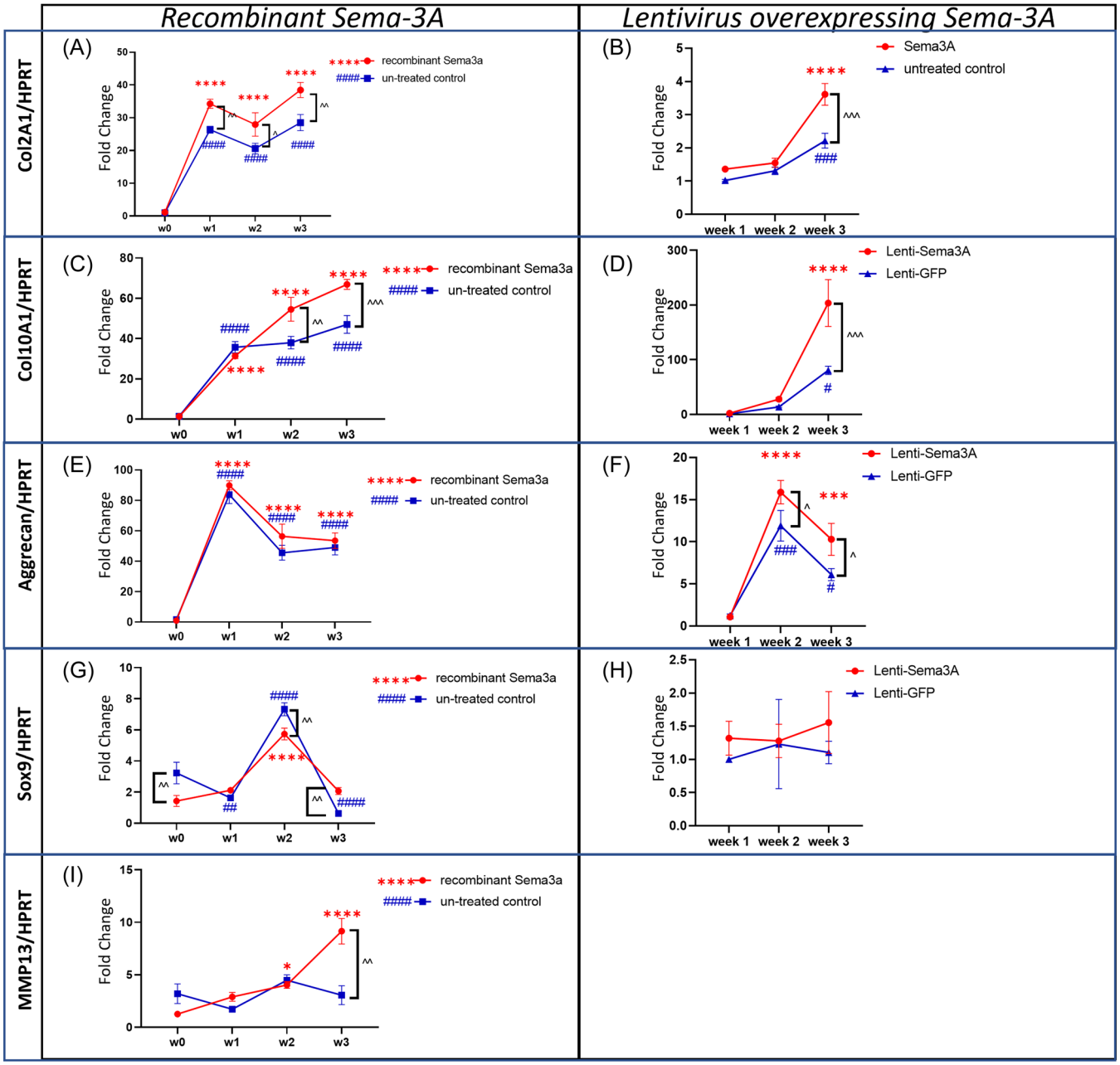

FIGURE 7 Effect of semaphorin-3A (sema-3A) treatment on chondrocyte differentiation. Mouse chondrogenic ATDC5 cells were cultured in chondrogenic medium for 21 days in the presence or absence of sema-3A added either as a recombinant protein (left panels) or overexpressed in a lentivirus (right panels). RNA was collected from chondrocytes at time zero when differentiation was initiated and weekly for 21 days. mRNA levels for chondrocytes markers were quantified by qPCR. The following markers were assessed: type 2 collagen (A, B), type 10 collagen (C, D), Aggrecan (E, F), Sox9 (G, H), MMP13 (I). Expression level of each gene was normalized by HPRT, and results are expressed as mean ± SEM of fold change over Day 0 or Week 1. Two experiments were performed for recombinant sema-3A and one for lentivirus overexpressing sema-3A, each done in triplicate. Both experiments performed with recombinant sema-3A show the same results but we chose to show only one representative experiment rather than pooling the data as there were some variations in the protocol including no week zero for one experiment. ^Represents the significant difference between treatment and control groups within the same week. *Represents the significant difference in sema-3A treatment group to Day 0 or Week 1. #Represents the significant difference in control group to Day 0 or Week 1. ^, * or $^{\#}p < .05$; ^^, ** or $^{\#\#}p < .01$; ^^^, *** or $^{\#\#\#}p < .001$; ^^^^, **** or $^{\#\#\#\#}p < .0001$.

examine sema-3A and its receptors expression in the DRGs during OA. We used Reverse transcription polymerase chain reaction (RT-PCR) to analyse sema-3A, NP1 and Plx-A1 expression in the DRGs (L1–L6) after MJL. We found that sema-3A and Plx-A1 were upregulated in the DRGs 6 weeks after mechanical loading, while not NP-1. Curiously NP2 was upregulated by MJL, suggesting that other semaphorins may be modulated by joint loading in the DRGs. Previous work has shown that pain behaviours in mouse models of OA are associated with molecular and cellular changes in the DRGs innervating the joints,[44] but the role of sema-3A signalling in the

DRGs in these models was not examined before. This upregulation of sema-3A in the DRGs of mice submitted to mechanical loading also supports a role for sema-3A in joint pain in this model. Treatment of mice with the bisphosphonate Zoledronate supressed the increases in sema-3A, Plx-A1 and NP2 expression in the DRGs induced by MJL. These results are novel and interesting as bisphosphonates are widely used to treat osteoporosis by inhibiting bone resorption,[45] but their protective effects on OA are less clear.[46,47] They however reduce knee pain and bone marrow lesions in clinics.[48] Subchondral bone osteoclasts were shown to induce sensory innervation and OA pain, which was reversed by alendronate, another bisphosphonate.[49] Sema-3A affects bone remodelling and we cannot exclude that it could modulate joint innervation and OA pain indirectly by affecting subchondral bone remodelling.

Although sema-3A, Nrp-1 and Plx-A1 are expressed in chondrocytes,[50] the role of sema-3A signalling in their function remains unknown. Interestingly, our data indicates that expression of sema-3A is high in articular cartilage chondrocytes but very low in hypertrophic chondrocytes of the growth plate, suggesting that sema-3A may have distinct roles in chondrocytes depending on the stage of differentiation and/or the cartilage location. We used the chondrogenic ATDC5 cell line, which has been fully characterised in terms of markers of chondrogenic differentiation,[31] to investigate the role of sema-3A in the regulation of chondrocyte differentiation. We showed that sema-3A is expressed at all stages of ATDC5 differentiation with a peak expression during maturation and a decline during hypertrophy when chondrocytes express type X collagen, indicating that it is not a late marker of chondrogenic differentiation. Sema-3A in articular cartilage could play a role during chondrocyte production of the extracellular matrix rather than during its late mineralisation. This role could be different from that of sema-3A expressed in hypertrophic chondrocytes during endochondral ossification where it could play a role in angiogenesis and innervation during bone development.[34]

Recombinant sema-3A has previously been used in ATDC5 cells to examine its role in inflammation in chondrocytes under excessive mechanical stress[51] and upon treatment with lipopolysaccharide (LPS),[52] and it was shown that it reduced LPS-induced inflammatory changes as well as gene expression of inflammatory cytokines. Here we demonstrate that r sema-3A increased aggrecan and type II collagen mRNA expression during all stages of chondrocyte differentiation while it stimulated type X collagen mRNA expression only in mature chondrocytes. This suggests that sema-3A induces the differentiation potential of chondrocytes; making them more mature. *MMP13* gene expression was also increased by r sema-3A at late stages of chondrocyte differentiation. It is an essential enzyme targeting cartilage for degradation making it a critical target gene during the progression of OA.[53] Recent data showing that sema-3A-NP1 Signalling modulates MMP13 expression in human OA chondrocytes confirms this data.[54] Sema-3A overexpression could contribute to the development of OA by stimulating cartilage degeneration. The effects of sema-3A on markers of chondrocyte differentiation were confirmed by the use of lentivirus overexpressing sema-3A. A previous study in the same ATDC5 chondrocytic cell line has shown that overexpression of sema-3A using a viral vector promotes chondrocyte apoptosis,[55] further suggesting that sema-3A may contribute to cartilage degradation during OA by promoting chondrocyte apoptosis. The use of this cell line is however a limitation of those studies and this data needs to be confirmed with primary chondrocytes.

Overall, we have demonstrated that sema-3A is expressed in several joint tissues in which it may serve diverse functions during the development of OA. In highly innervated joint tissues such as bone, synovium and ligaments, sema-3A may regulate peripheral innervation by acting on axonal growth or induce neuronal changes that contribute to pain. In cartilage which is not innervated, sema-3A could favour matrix degeneration by modifying chondrocyte differentiation and survival. Although work remains to be done to understand the mechanisms and signalling pathways involved in the regulation of the different joint tissues by sema-3A, our findings suggest it could have a role in OA development and associated pain.

AUTHOR CONTRIBUTIONS

Xiang Li contributed to the data collection, all animal experiments of mechanical joint loading, RT-PCR analysis, in vitro experiments, immunocytochemistry, data analysis and interpretation. Sara Martinez-Ramos contributed to data collection, RT-PCR analysis and data interpretation. Freija T. Heedge contributed to data collection, immunocytochemistry, data analysis and interpretation. Andrew Pitsillides and George Bou-Gharios contributed to data analysis and interpretation and the drafting and revision of the manuscript. Blandine Poulet contributed to data collection, microarray analyses in STR/ort mice and CBA controls and data interpretation. Chantal Chenu contributed to experimental design, data analysis and interpretation and the drafting and revision of the manuscript. The authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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