Protease-activated receptor-2: a novel pathogenic pathway in a murine model of osteoarthritis

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Abstract

Objective: Osteoarthritis (OA) is a global clinical challenge for which no effective disease modifying agents currently exist. Herein we identify protease-activated receptor-2 (PAR-2) as a novel pathogenic mechanism and potential therapeutic target in OA.

Methods: Experimental OA was induced in wild-type and PAR-2 deficient mice by sectioning the medial menisco-tibial ligament (MMTL), leading to development of a mild arthropathy. Cartilage degradation and increased subchondral bone formation were assessed as indicators of OA pathology.

Results: Four weeks following MMTL section, cartilage erosion and increased subchondral bone formation was evident in wild type mice but substantially reduced in PAR-2 deficient mice. Crucially, therapeutic inhibition of PAR-2 in wild type mice, using either a PAR-2 antagonist or a monoclonal antibody targeting the protease cleavage site of PAR-2, was also equally effective at reducing OA progression *in vivo*. PAR-2 was upregulated in chondrocytes of wild-type but not sham-operated mice. Wild type mice showed further joint degradation eight weeks following induction of OA, but PAR-2 deficient mice were still protected.

Conclusions: The substantial protection from pathology afforded by PAR-2 deficiency following induction of OA provides proof of concept that PAR-2 has a key role in OA and suggests this receptor as a potential therapeutic target.

Osteoarthritis (OA) is a chronic disabling condition currently affecting millions globally [1] with radiological evidence of OA in approximately 80% of the population aged over 65. [2] OA is characterised by cartilage degradation and increased subchondral bone formation (osteosclerosis). Despite extensive pathophysiologic investigations, clinical management has not altered significantly and comprises administration of analgesics and non-steroidal anti-inflammatory agents and recourse upon joint failure to arthroplasty. No unifying pathogenetic model exists - suggested hypotheses encompass primary cartilage metabolic dysregulation, enthesial disease together with biomechanical dysregulation. Thus far, no critical checkpoint pathway has been identified that is essential for disease progression and which might by corollary represent a valid, disease-modifying OA therapeutic target. Protease-activated receptor-2 (PAR-2) is a G-protein coupled receptor whose 'tethered' ligand is activated by serine proteases. [3] PAR-2 is present in chondrocytes in cartilage from OA patients [4], and following its activation, matrix metalloproteases (MMPs) are generated. [5] However, these previous observations are associative and do not establish the role of PAR-2 in the pathogenesis of OA. We here sought direct evidence of a causal relationship between PAR-2 expression and cartilage and bone pathology in a murine model of OA.

Methods

Animals

Experiments were performed on adult wild-type (PAR-$2^{+/+}$) C57BL/6J mice (body weight 25-30 g), housed in standard cages, with food and water available *ad libitum*, and maintained in a thermoneutral environment. PAR-2-deficient (PAR-$2^{-/-}$) mice were

genetically modified [6] and maintained under the same conditions. All procedures were performed in accordance United Kingdom Home Office regulations.

Induction of experimental osteoarthritis

Two previously described models of joint instability were employed. [7] The principal model used involved destabilisation of the medial meniscus by section of the medial menisco-tibial ligament (MMTL), which results in development of a mild arthropathy resembling human OA.[7] A more severe model, involving anterior cruciate ligament (ACL) transection, was induced in a smaller group of mice. [7] All surgical procedures were performed by the same investigator who was blinded to genotype or treatment. Following surgery, animals were left for 4 or 8 weeks and thereafter, knee joints were harvested for subsequent histological analysis. Knee joint diameter was measured weekly for a month following MMTL surgery in a small cohort of mice but no significant difference occurred over time ($P=0.6$, 1-way ANOVA, $n = 6$). As similar results were obtained with the ACL section model, no further such measurements were performed.

Assessment of cartilage damage

Histological analysis of progression and severity of cartilage damage was undertaken on harvested joints, involving decalcification by incubating knee joints in 4% paraformaldehyde solution followed by phosphate buffer and then in 10% EDTA solution at 4^{o}C for 2 weeks. Joints were embedded in paraffin wax and frontal sections (6μm) cut at 200μm intervals, followed by staining with Haematoxylin, Safranin-O/fast green. Ten sections from each mouse were graded using a validated scoring system, [7] from 0 (normal) to 6 (>80% loss of cartilage) by two scorers blinded to the specimens, taking each quadrant of the joint separately and then summing scores. There was close

agreement between scorers (intraclass correlation coefficient 0.97; 95% CI 0.95 to 0.98) with the mean difference in score being -0.05 (95% CI 0.01 to -0.11).

Assessment of osteosclerosis

From the sections obtained above, two were chosen at random, digitally captured (Olympus BX51, DP70, AnalySIS software) and stored for computerised planimetry analysis using QS Onscreen v 3.1.19, (E-quantities Ltd; http://www.e-quantities.com). Only two photomicrographs per mouse were required for planimetric analysis as osteosclerosis was relatively consistent across sections (coefficient of variation 2.1%). The area of the medial and lateral aspects of the tibial plateau were traced, from the epiphyseal plate to the lower zone of cartilage adjacent to subchondral bone. The tibial plateau region was selected as cartilage damage was maximal at medial aspect with the lateral region never appearing to be affected in MMTL-sectioned wild-type mice, and so could be used as an internal reference. Controls were also performed in sham-operated as well as naïve mice. Once the overall area on each side of the tibial plateau was determined, areas containing marrow were traced, summed and subtracted from the overall area on each side to yield the area occupied by bone. Comparisons were made by expressing as the percentage area occupied by bone in the medial tibial compared to the lateral region in order to normalise for animal size, since analysis of naïve mice showed the difference between these areas to be minimal (1.1 ± 3.4%, mean ± sem, n = 5). Scores were independently verified by another blinded assessor and there was good agreement between scorers (intraclass correlation coefficient 0.91; 95% CI 0.77 to 0.97) with the mean difference in score being 2.7% (95% CI 5.45 to -0.06). Osteosclerosis was not assessed in the ACL model as it was previously reported that bone erosion can penetrate to the epiphyseal plate. [7]

Immunohistochemistry

Following decalcification, sections were deparaffinized, rehydrated and stained with the murine monoclonal antibody (mAb) SAM-11 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1μg/ml using the Dako (Ely, UK) ARK (Animal Research Kit) Peroxidase. SAM-11 is a highly specific mAb to human PAR-2 that binds the membrane-bound part of the receptor. Antigen–antibody complex was visualized utilizing diaminobenzidine (DAB) and counterstained with hematoxylin. The ARK was utilised to minimise reactivity of secondary anti-mouse antibody with endogenous immunoglobulin.

Statistical analysis

Data were tested for normality (Sigmastat 2.03, SPSS Inc) and expressed as mean ± sem with comparisons by one or two way ANOVA and multiple comparisons using Bonferroni correction. Non-normal data were transformed (square root) prior to statistical analysis and if normality was still not achieved, non-parametric methods were used. Agreement between scorers was assessed graphically using the Bland Altman plot [8] and expressed as the intraclass correlation coefficient.

Results

Four weeks following MMTL section, immunohistochemical analysis of cartilage revealed increased PAR-2 expression in superficial layer chondrocytes (Fig 1a), compared to sham-operated mice (Fig 1b). Having established enhanced expression of

the PAR-2 pathway with plausible tissue distribution, pathology was compared in wild-type mice (PAR-$2^{+/+}$; n = 5) and PAR-2 deficient littermates (PAR-$2^{-/-}$; n = 5), with sham-operated wild-type mice (n = 4) serving as a negative control. Compared to sham-operated mice (Fig 1d), PAR-$2^{+/+}$ mice showed cartilage damage and increased subchondral bone formation (Fig 1e) whereas such changes were minimal in PAR-$2^{-/-}$ mice (Fig 1f). Such differences could not be attributed to an underlying difference in bone phenotype of PAR-$2^{-/-}$ mice as the appearance of the unoperated knee did not differ from wild-type littermates (Supplementary Fig 1). To address the therapeutic utility of PAR-2, operated mice (n = 6) were treated with SAM-11 antibody, which we previously demonstrated inhibits PAR-2 activation. [9] In a further group (n = 5), we utilised p520, a selective PAR-2 antagonist. [10] Consistent with data in the PAR-2 deficient animals, mice treated with SAM-11 (Fig 1g) or p520 showed significantly reduced OA pathology four weeks post-operatively (Fig 1 h, i).

Two-way ANOVA revealed a significant difference in cartilage degradation between groups ($P < 0.0001$) and between regions ($P < 0.0001$). PAR-$2^{+/+}$ mice exhibited significantly greater cartilage degradation than sham-operated, PAR-$2^{-/-}$ mice, or wild-type mice treated with p520 (Fig 1h). Similarly, operated mice receiving an isotype antibody developed substantially greater cartilage degradation than SAM-11-treated mice. Consistent with previous work, [7] cartilage damage only occurred at the medial aspects of the tibia and femur. Analysis of subchondral bone revealed differences between treatment groups ($P = 0.006$, 1-way ANOVA). PAR-$2^{+/+}$ mice differentiated from sham-operated, PAR-$2^{-/-}$ mice, and wild-type recipients of p520 (Fig 1i). Consistent with this, mice receiving isotype control showed substantially greater osteosclerosis than SAM-11 recipients. Cartilage damage and osteosclerosis scores were significantly correlated (r =

0.7, $P < 0.0001$). Since genetic deficiency of PAR-2, or inhibition by two distinct routes, namely targeting the protease cleavage site with SAM-11 or directly by receptor antagonism, proved effective in preventing the development of experimental OA, we conclude that PAR-2 as a pivotal checkpoint in early progression of OA pathology.

The longer term effects of PAR-2 biology were examined using PAR-2 deficient mice by extending the postoperative period to eight weeks. Compared to wild-type littermates (Fig 2a) cartilage damage was still significantly ($P <0.0001$, 2-way ANOVA, n = 7-8) lower in PAR-$2^{-/-}$ mice (Fig 2b, c) and once again the latter showed no evidence of increased subchondral bone formation, whereas PAR-$2^{+/+}$ mice showed a significant increase compared to the 4 week model (Fig 2d). To further test our hypothesis, the more aggressive ACL transection model was investigated. PAR-$2^{+/+}$ mice developed almost complete cartilage erosion, particularly at the medial femoral condyle (Fig 3a, c), whereas PAR-$2^{-/-}$ mice exhibited significantly ($P < 0.0001$, 2-way ANOVA, n = 7-8) lower levels of damage (Fig 3b, c).

Discussion

Our observations in PAR-2 deficient mice using both MMTL and ACL transection models provide powerful proof of concept for a key role of PAR-2 in joint degradation associated with OA, and furthermore demonstrate the therapeutic potential of PAR-2 inhibition, which could have preventative clinical utility. The mechanism by which PAR-2 promotes OA has yet to be clarified. PAR-2 provides for sensing by cells of the protease environment and allows them to subsequently respond. For example, PAR-2 regulates synovial release of pro-inflammatory cytokines including IL-1β, [11] which is in turn a potent chondrocyte catabolin. [12] Activation of PAR-2 leads to inhibition of osteoclast differentiation [13] and could alter the balance between bone formation and

resorption and thereby contribute to osteosclerosis observed in PAR-$2^{+/+}$ but not PAR-$2^{-/-}$ mice. However, whether PAR-2 has a direct or indirect effect on the bone phenotype and its hierarchical role in OA are areas for further investigation. Prior studies revealed that selective PAR-2 activation of chondrocytes derived from human OA cartilage leads to generation of MMP-1 and -13, [5] both of which are known to play a key role in degradation of aggrecan and collagens. Although the initiating event in the development of OA lesions remains unknown, our data suggest that PAR-2 is a crucial checkpoint that mediates the effect of biomechanical instability upon cartilage degradation and osteosclerosis. PAR-2 inhibitors could therefore provide a new direction in the development of disease modifying osteoarthritis drugs (DMOADs), which following disappointing results of trials with MMP inhibitors, biphosphonates and cytokine inhibition, [14] still remain elusive.

ACKNOWLEDGMENTS

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References

1. Brooks PM. The burden of musculoskeletal disease- a global perspective. *Clin Rheumatol* 2006;25(6):778–781.
2. Lawrence RC, et al. Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum*1998;41(5):778–799.

3. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R Proteinase-activated receptors. *Pharmacol. Rev.* 2001;53(2):245-282.
4. Xiang Y, et al. Expression of proteinase-activated receptors (PAR)-2 in articular chondrocytes is modulated by IL-1beta, TNF-alpha and TGF-beta *Osteoarthritis Cartilage* 2006;14(11):1163-1173.
5. Boileau C, et al. Activation of proteinase-activated receptor 2 in human osteoarthritic cartilage upregulates catabolic and proinflammatory pathways capable of inducing cartilage degradation: a basic science study. *Arth. Res. Ther.* 2007;9(6)**:**R121.
6. Ferrell WR, et al. Essential role for proteinase-activated receptor-2 in arthritis.*J Clin Invest* 2003;111(1):35-41.
7. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartilage* 2007;15(9):1061-1069.
8. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1(8476):307-10.
9. Kelso EB, et al. Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. *J Pharmacol Expt Ther* 2006;316(3):1017–1024.
10. Hembrough T, et al. Anti-inflammatory antagonists of PAR-2 also have antitumor and antiangiogenic activity. *Proc Amer Assoc Cancer Res* 2005;46:2610.
11. Kelso EB, et al. Expression and proinflammatory role of proteinase-activated receptor 2 in rheumatoid synovium: ex vivo studies using a novel proteinase-activated receptor 2 antagonist. *Arthritis Rheum* 2007;56(3):765-771.

12. Daheshia M., Yao JQ. The interleukin 1 beta pathway in the pathogenesis of osteoarthritis. *J Rheumatol*. 2008;35(12):2306-2312.
13. Smith R, et al. Activation of protease-activated receptor-2 leads to inhibition of osteoclast differentiation. *J Bone Miner Res* 2004;19(3):507-516.
14. Qvist P, et al. The disease modifying osteoarthritis drug (DMOAD): Is it in the horizon? *Pharmacol Res* 2008;58(1):1-7.

Figure Legends

Fig 1. PAR-2 absence or inhibition ameliorates OA pathology in the four week MMTL model. Immunohistochemistry of cartilage in the medial tibial aspect using SAM-11 to detect PAR-2 shows increased surface expression in chondrocytes of the wild-type mouse following MMTL section (a), compared to a sham-operated animal (b), with isotype control (c). Bar indicates 50µm. Histological appearances of the medial aspect of the knee joint four weeks following sham-operation (d), or following MMTL section in either a wild-type (e) or PAR-2 deficient mouse (f), or wild-type mouse treated daily with SAM-11 (1µg i.p.) immediately prior to and after induction of OA (g). Arrowheads in (e) denote cartilage damage and * indicates osteosclerosis. Scale bar in panel f indicates 500µm and applies to all four photomicrographs. Cartilage degradation scores (h) for the medial tibial (MT), medial femoral (MF), lateral tibial (LT) and lateral femoral (LF) regions following MMTL section in PAR-$2^{+/+}$ and PAR-$2^{-/-}$ mice, PAR-$2^{+/+}$ mice treated with PAR-2 antagonist p520 (5mg i.p. daily), SAM-11 (1µg i.p. daily) or isotype (IgG2a) control antibody (ISO; 1µg i.p. daily), along with sham operated controls. Data are expressed as mean ± sem, n = 4 - 6. Analysis by one-way ANOVA with multiple comparisons using the Bonferroni correction, * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. † = $P < 0.005$, Mann-Whitney rank sum test. Percentage difference in bone area (i) comparing the medial and lateral tibial regions following MMTL transection in the above groups of mice along with a naïve control group. Analysis by one-way ANOVA with multiple comparisons using the Bonferroni correction, * = $P < 0.05$; ** = $P < 0.02$; *** = $P < 0.01$. § = $P < 0.001$, t-test. NS = not significant, t-test.

Fig 2. PAR-2 deficiency still confers protection eight weeks following MMTL section. Examination of the medial tibial and femoral regions of the PAR-$2^{+/+}$mouse (a) compared to its PAR-$2^{-/-}$ littermate (b) eight weeks following MMTL section shows continued protection in the latter. Scale bars indicate 500µm. This was confirmed for these two groups (n = 7 and 8 respectively) in terms of cartilage damage scores (c). ** = $P < 0.01$, *** = $P < 0.001$. Osteosclerosis, comparing % difference in bone area between the medial and lateral tibial areas, showed a significant increase in the eight week model (MMTL8) compared to 4 weeks (MMTL4) in PAR-$2^{+/+}$ mice but no difference between the PAR-$2^{-/-}$ groups (d). * = $P < 0.02$, ** = $P < 0.0001$.

Fig 3. Eight weeks following ACL transection, substantial damage was evident in multiple areas of the knee in PAR-$2^{+/+}$ (a), but less so in PAR-$2^{-/-}$ mice (b). Scale bar indicates 1000µm. Cartilage damage scores confirm the more extensive nature of damage in this model of arthritis, but there remain significant differences compared to PAR-$2^{-/-}$ mice (c). *** = $P < 0.001$, ** = $P < 0.005$, * = $P < 0.05$.

Supplementary Fig 1. Comparison of the unoperated knee joint from the wild-type mouse (a) with its PAR-$2^{-/-}$ littermate (b) shows no apparent difference in cartilage or bone phenotype. Scale bars indicate 500µm. Medial tibial bone areas in (a) and (b) were 44.8% and 43.2%, respectively.

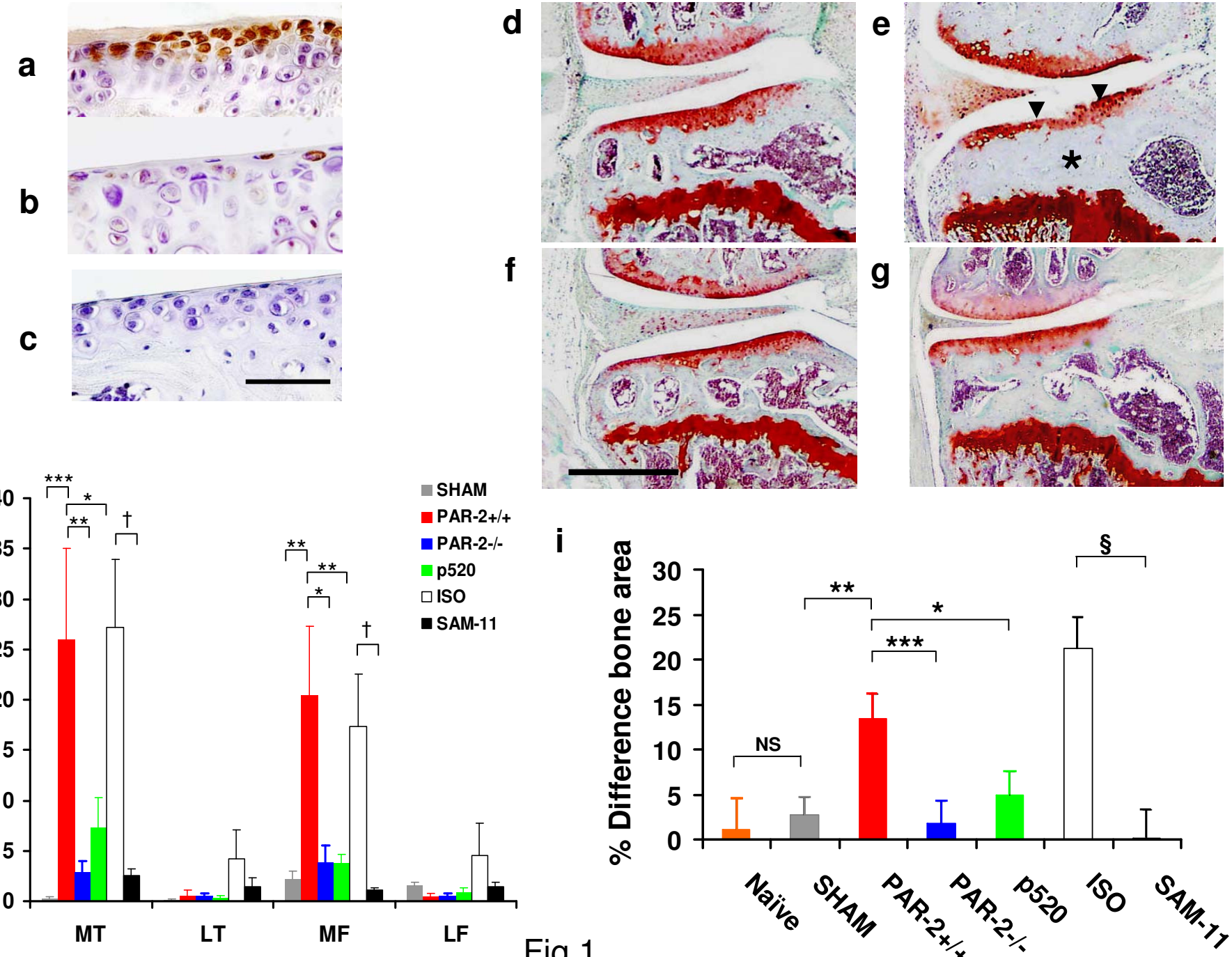
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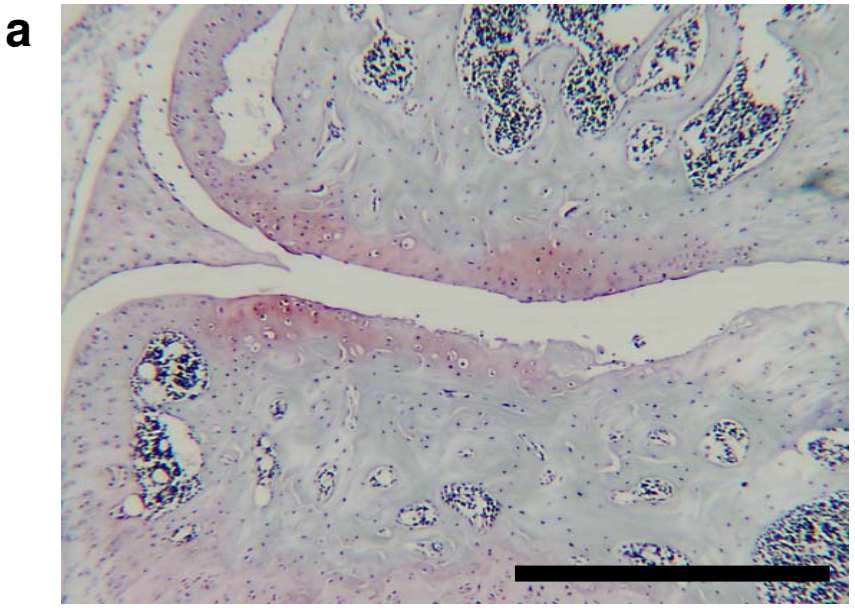
Fig 1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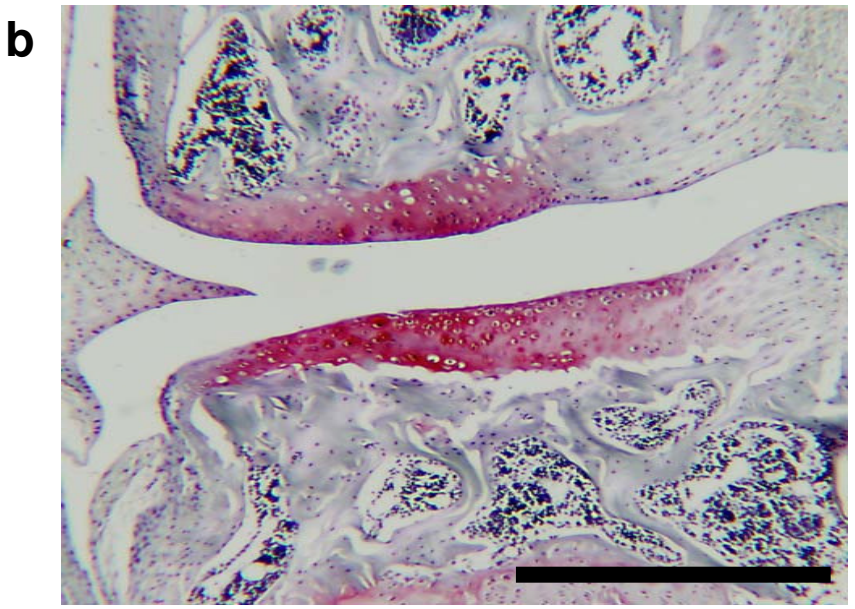
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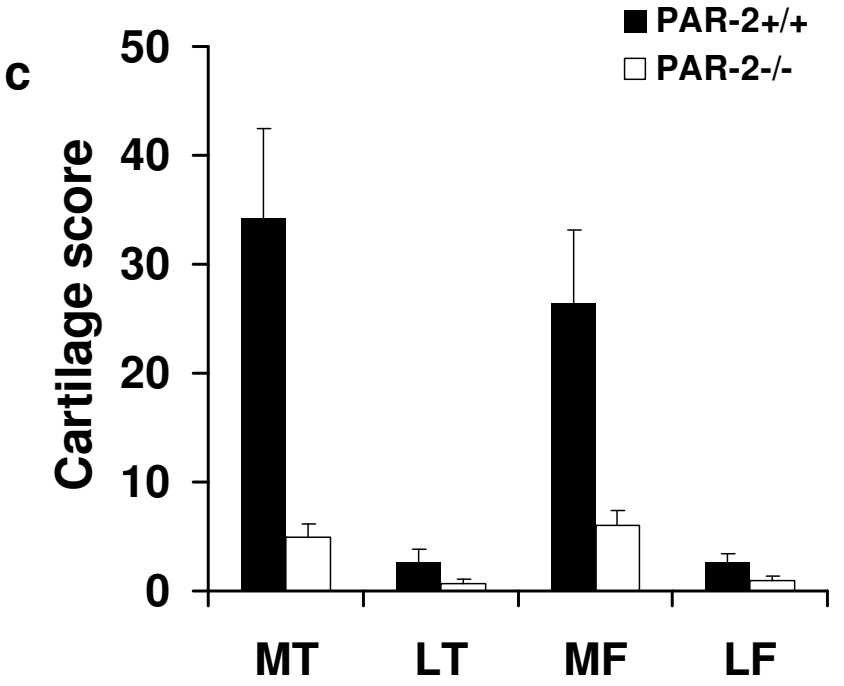
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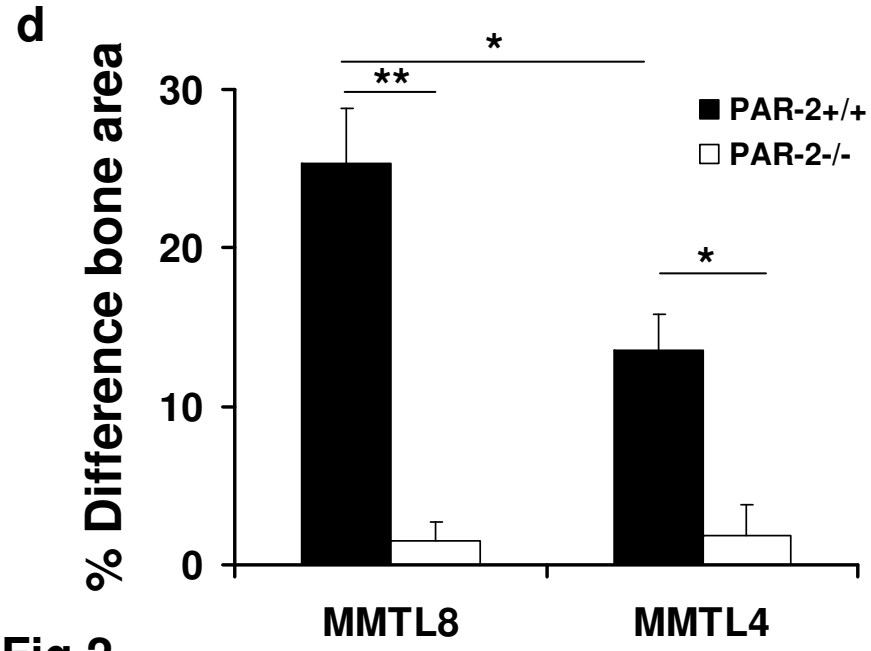
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Fig 2

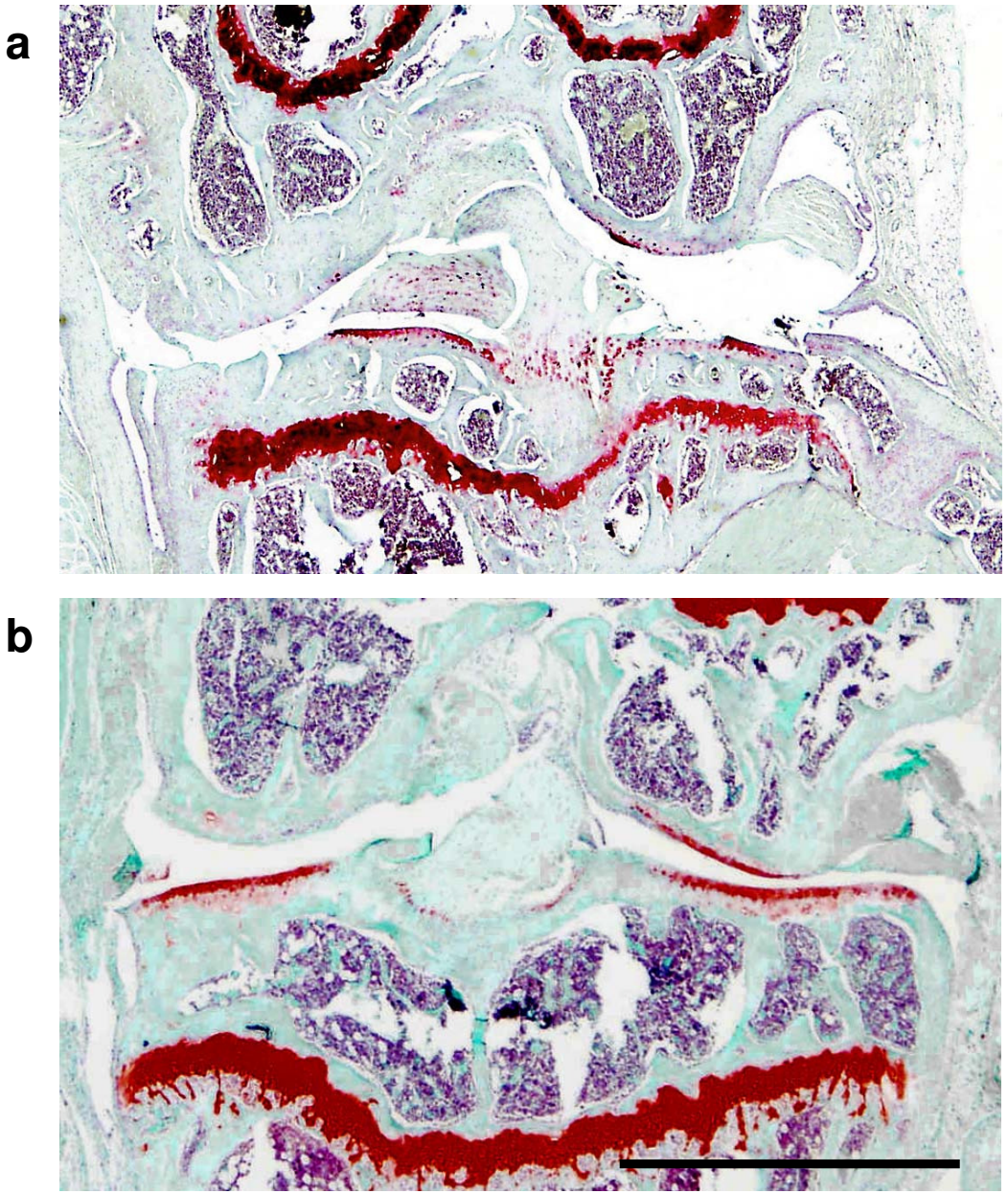
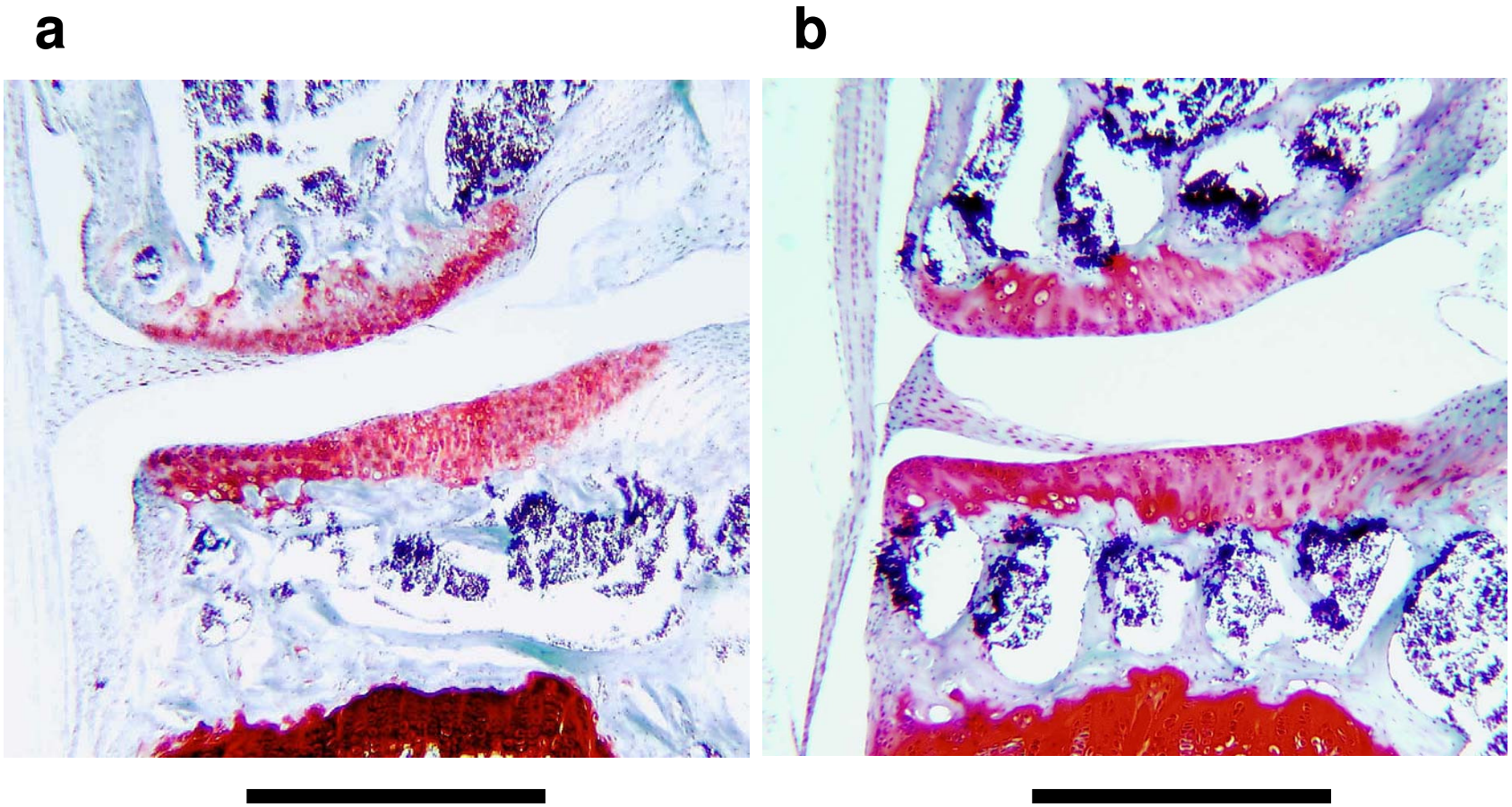


Fig 3



Supplemental Figure 1