Bone & Joint Research

Supplementary Material

10.1302/2046-3758.143.BJR-2024-0251.R1

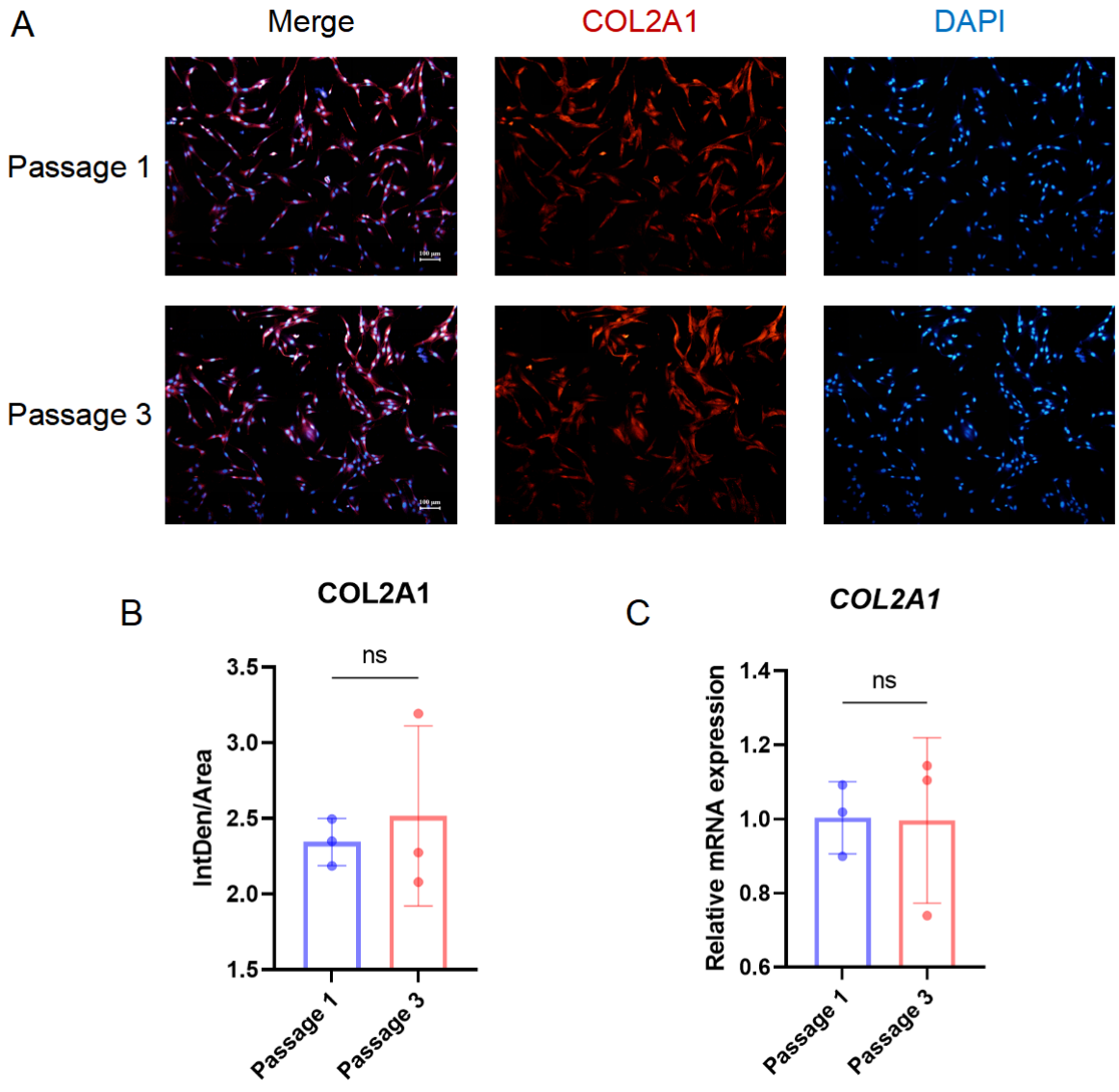

Fig. a. Phenotypic validation of primary (passage 1) and passaged (passage 3)

chondrocytes. a) and b) Immunofluorescence staining and c) reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis show high COL2A1 expression in both passage 1 and passage 3 chondrocytes, with no statistically significant differences. These results indicate that the cells retain their chondrocytic phenotype without undergoing de-differentiation. COL2A1, collagen, type II, alpha 1; DAPI, 4',6-diamidino-2-phenylindole; mRNA, messenger RNA; ns, not significant.

Table i. Quantitative polymerase chain reaction primer sequences for target gene.

Primer	Forward (5′–3′)	Reverse (5′–3′)
rPFKM	GCGGAGGAGAGCTAAAACTACA	CCGCAGCATTCATACCTTGG
rUSP8	GCGCTGCGCAAGAATCA	TGGTGCTGGTTTTCTCAGGTT
rSMAD3	CTGGGCAAGTTCTCCAGAGTT	AAGGGCAGGATGGACGACAT
rINO80D	CCAGGCATTGTTTCCAGCAC	CAAACACTGGCACAGAGCAC
rMST1R	GCCATCGCACTGGTCTGTAA	TGTTGTCGGGGAGAGGGAAT
rMAPK3	AACCCAAACAAGCGCATCAC	CTCCTCAGCCACTGGTTCATC
rGCAT	GTGGGTCAACTTCTTGTGGC	GATCACCCGCTCACTCTTCC
rKCNH2	ACACCTTCCTCGACACCATCA	CGAGCGTTAGCGATGATGAA
rRAD9A	CGTCCCGGTCTGGTTGTAAA	TTTCCCAGCACCTTCACGTT
rCOLGALT2	AGGGGTGAAATCGGTTGCTT	TCTTCTCCAGCTCTCGGTCA
rβ-actin	CTCTGTGTGGATTGGTGGCT	CGCAGCTCAGTAACAGTCCG

Table ii. Characteristics of clinical groups.

Parameter	Normal group	OA group	p-value
Median age, yrs (IQR)	75.2 (73 to 77)	75.6 (75 to 76)	0.841*
Sex, n			0.999†
Male	2	1	
Female	3	4	
Kellgren-Lawrence grade, n			0.008*
0	5	-	
1	-	-	
2	-	-	
3	-	1	
4	-	4	

Side of involvement, n			0.999†
Left	3	2	
Right	2	3	

*Non-parametric tests.

†Fisher's exact test.

Table iii. Results of the enrichment analysis for transcriptome-wide association study-derived genes ($p < 0.01$).

Panel	Term	p-value	OR	Combined score	Genes
GO biological process	Regulation of triglyceride biosynthetic process (GO:0010866)	2.95E-04	11.06	89.88	*CTDNEP1;SCARB1;KAT5;NR1H3;SIK1*
GO biological process	Positive regulation of triglyceride metabolic process (GO:0090208)	5.42E-04	9.36	70.36	*CTDNEP1;SCARB1;KAT5;NR1H3;PNPLA2*
GO biological process	Positive regulation of triglyceride biosynthetic process (GO:0010867)	6.51E-04	13.89	101.90	*CTDNEP1;SCARB1;KAT5;NR1H3*
GO biological process	Regulation of cellular catabolic process (GO:0031329)	8.11E-04	4.07	28.94	*VPS29;MTMR3;USP10;LRRK2;USP33;ITPR1;ABL2;TYSND1;TPCN2*
GO biological process	Quaternary ammonium group transport (GO:0015697)	9.45E-04	12.15	84.62	*SLC22A4;SLC22A5;SLC25A19;SLC44A2*
GO biological process	DNA damage response, signal transduction by P53 Class mediator resulting in transcription of P21 class mediator (GO:0006978)	3.01E-03	14.56	84.58	*MUC1;KAT5;RPS27L*
GO biological process	Response to vitamin D (GO:0033280)	3.01E-03	14.56	84.58	*CDKN2D;FES;SPP1*
GO biological process	Positive regulation of actin filament bundle assembly	3.83E-03	3.87	21.56	*CDC42;PLEK;SERPINF2;TESK1;LPAR1;RHOA;RAPGEF3*

	(GO:0032233)				
GO biological process	Protein modification process (GO:0036211)	4.19E-03	1.59	8.70	*GPI;CSTA;MTMR3;CTDP1;UBA7;PRKDC;LRRK2;PLOD2;PTPRJ;DUSP16;TRIOBP;GMPPB;UGCG;RPS6KA5;PDPR;OGFOD1;CHEK1;ST3GAL4;ABL2;MAPK1;PTK2B;MARK3;MAP4K4;MAPK3;PRMT6;YES1;ST8SIA1;IGFBP3;KRT1;TAF11;PASK;CDC42BPA;CTDNEP1;CTH;TSSK6;PFDN1;SIK1;BMPR1B;PTPN5;TRIB2;CSNK1G2;MAP3K11;ATG4D*
GO biological process	DNA damage response, signal transduction resulting in transcription (GO:0042772)	4.38E-03	12.14	65.92	*MUC1;KAT5;RPS27L*
GO cellular component	Microbody lumen (GO:0031907)	3.04E-03	4.06	23.52	*AOC1;AGPS;TYSND1;CRAT;ACOT4;PAOX;DHRS4*
GO cellular component	Peroxisomal matrix (GO:0005782)	3.04E-03	4.06	23.52	*AOC1;AGPS;TYSND1;CRAT;ACOT4;PAOX;DHRS4*
GO cellular component	Amphisome (GO:0044753)	8.10E-03	9.10	43.83	*LRRK2;CHMP2B;CHMP7*
GO cellular component	Cytoplasmic side of plasma membrane (GO:0009898)	1.05E-02	2.37	10.83	*YES1;JUP;DLG4;FES;TRAF3;TRAF2;TRAF1;LITAF;CHMP7;RHOA;CYTH1*
GO cellular	Caveola (GO:0005901)	1.12E-	3.10	13.92	*SCARB1;SMPD2;LRRK2;PTCH1;*

component		02			*MAPK1;EMP2;MAPK3*
GO cellular component	Extrinsic component of cytoplasmic side of plasma membrane (GO:0031234)	1.12E-02	2.82	12.67	*GNA14;YES1;DLG4;FES;GNA12;GNB5;RHOA;CYTH1*
GO cellular component	tRNA methyltransferase complex (GO:0043527)	2.12E-02	12.12	46.70	*WDR4;TRMT61A*
GO cellular component	Lytic vacuole (GO:0000323)	3.30E-02	1.76	6.00	*ARSA;SCARB1;CD164;USP4;SLC11A1;LRRK2;EPDR1;TPCN2;BACE1;MFHAS1;SNX1;CHMP2B;PLEKHM1;ACP2;LAMTOR1*
GO cellular component	Dendrite cytoplasm (GO:0032839)	3.76E-02	8.08	26.51	*DLG4;LRRK2*
GO cellular component	Platelet dense tubular network membrane (GO:0031095)	3.76E-02	8.08	26.51	*DMTN;ITPR1*
GO cellular component	Clathrin adaptor complex (GO:0030131)	3.76E-02	4.55	14.92	*SGIP1;AP1B1;AP1M2*
GO molecular function	Thioesterase binding (GO:0031996)	2.65E-04	19.44	160.16	*CDC42;TRAF3;TRAF2;TRAF1*
GO molecular function	Pyridoxal phosphate binding (GO:0030170)	9.18E-04	8.11	56.70	*PYGB;SHMT1;CTH;CISD1;ACCS*
GO molecular function	Quaternary ammonium group transmembrane transporter activity (GO:0015651)	3.88E-03	7.48	41.50	*SLC22A4;SLC22A5;SLC44A2;SLC25A19*
GO molecular function	Tumor necrosis factor receptor binding (GO:0005164)	4.38E-03	12.14	65.92	*TRAF3;TRAF2;TRAF1*

GO molecular function	RNA cap binding (GO:0000339)	5.93E-03	6.48	33.22	*EIF4E3;EIF4E2;DCPS;SNUPN*
GO molecular function	RNA 7-methylguanosine cap binding (GO:0000340)	1.05E-02	8.09	36.87	*EIF4E3;EIF4E2;DCPS*
GO molecular function	Mitogen-activated protein kinase binding (GO:0051019)	1.20E-02	5.11	22.63	*MAPKAPK3;PTPRJ;DUSP16;CDK5RAP3*
GO molecular function	Phosphatase binding (GO:0019902)	1.37E-02	2.39	10.26	*MTMR3;SMAD3;JUP;DLG4;TRAF3;MAPK1;TRAF2;TRPC4AP;PDLIM4;MAPK3*
GO molecular function	FAD binding (GO:0071949)	1.60E-02	4.63	19.12	*AOC1;NQO2;MICAL1;MMACHC*
GO molecular function	Tumor necrosis factor receptor superfamily binding (GO:0032813)	2.09E-02	4.22	16.33	*TRAF3;TNFSF4;TRAF2;TRAF1*
KEGG	Fructose and mannose metabolism	1.70E-03	5.41	34.50	*GMPPB;ENOSF1;MPI;PFKM;PFKP;KHK*
KEGG	Glycolysis/gluconeogenesis	1.67E-02	2.84	11.61	*LDHC;GPI;G6PC3;AKR1A1;PFKM;PFKP;ALDH9A1*
KEGG	Adherens junction	2.23E-02	2.66	10.11	*CDC42;SMAD3;YES1;MAPK1;PTPRJ;RHOA;MAPK3*
KEGG	Glutathione metabolism	2.50E-02	2.86	10.55	*GSTM4;GPX1;GSTP1;MGST3;CHAC2;PRDX6*
KEGG	Circadian entrainment	3.90E-02	2.19	7.09	*ADCYAP1;GNG10;RPS6KA5;PER3;ITPR1;MAPK1;GNB5;MAPK3*
KEGG	Sphingolipid signaling pathway	4.75E-	1.99	6.07	*SMPD2;ADORA3;GNA12;ADORA*

		02			*1;MAPK1;TRAF2;S1PR5;RHOA;MAPK3*
KEGG	Axon guidance	5.94E-02	1.72	4.85	*CDC42;EFNB3;SEMA4D;DPYSL5;FES;PTCH1;MAPK1;SRGAP3;BMPR1B;SSH3;RHOA;MAPK3*
KEGG	Lysosome	6.89E-02	1.84	4.92	*ARSA;CD164;GGA1;SLC11A1;AP1B1;AP4E1;ACP2;LITAF;AP1M2*
KEGG	Glycine, serine and threonine metabolism	7.30E-02	2.70	7.06	*SHMT1;CTH;AMT;GCAT*
KEGG	Long-term depression	8.92E-02	2.21	5.33	*GNA12;ITPR1;MAPK1;CRHR1;MAPK3*
GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; OR, odds ratio.					

Table iv. Results of the high-confidence genes identified by multiple transcriptome-wide association study methods.

Tissue	Site	Gene name	Druggable	CHR	P0	P1	TWAS Z	TWAS p-value	Best SNP	SNP Z	Model *	Model p-value	Perm p-value	Cond p-value	PP.H0	PP.H1	PP.H2	PP.H3	PP.H4
Muscle skeletal	Hip	*LEPREL1*	No	3	190122436	190122437	5.34	9.48E-08	rs10513845	-6.13	susie	4.80E-08	7.48E-03	5.00E-08	0.000	0.000	0.000	0.148	0.852
Muscle skeletal	Spine	*MST1R*	Yes	3	49903843	49903844	-5.09	3.65E-07	rs2280406	-5.09	top1	2.10E-12	0.00E+00	3.60E-07	0.000	0.001	0.000	0.006	0.992
Muscle skeletal	Knee	*RNF212*	No	4	1113561	1113562	-4.79	1.70E-06	rs7659624	5.38	susie	6.20E-16	8.21E-03	1.70E-06	0.000	0.001	0.000	0.007	0.992
Muscle skeletal	Hip	*RAD9A*	Yes	11	67317870	67317871	4.90	9.35E-07	rs7952436	4.94	susie	6.70E-38	2.88E-03	9.30E-07	0.000	0.003	0.000	0.000	0.997
Whole blood	Hip	*SSSCA1-AS1*	No	11	65570422	65570423	5.06	4.26E-07	rs1152620	5.50	top1	6.80E-09	8.67E-03	4.30E-07	0.000	0.002	0.000	0.014	0.984
Muscle skeletal	Hip	*SSSCA1-AS1*	No	11	65570422	65570423	5.02	5.14E-07	rs1152620	5.49	susie	8.50E-16	4.67E-03	1.00E-07	0.000	0.002	0.000	0.016	0.982
Muscle skeletal	Knee	*PFKM*	Yes	12	48105138	48105139	-5.65	1.62E-08	rs7967762	-6.12	top1	1.60E-07	3.75E-02	2.00E-02	0.000	0.000	0.008	0.073	0.919

Muscle skeletal	Hip	*SMAD3*	Yes	15	67065844	67065845	5.43	5.57E-08	rs12901499	7.10	enet	3.30E-21	1.48E-03	5.60E-08	0.000	0.000	0.000	0.011	0.989
Whole blood	Knee	*USP8*	Yes	15	50424379	50424380	5.60	2.11E-08	rs4380013	-6.02	top1	2.40E-13	3.73E-02	2.10E-08	0.000	0.000	0.000	0.020	0.980
Whole blood	Knee	*MAPK3*	Yes	16	30123505	30123506	5.08	3.69E-07	rs7201384	5.11	enet	3.50E-38	5.55E-03	3.70E-07	0.000	0.006	0.000	0.021	0.973
Muscle skeletal	Knee	*RP11-231C14.4*	No	16	29505998	29505999	-4.96	7.07E-07	rs4420550	4.97	lasso	7.30E-07	6.16E-03	7.10E-07	0.000	0.004	0.000	0.009	0.987
Muscle skeletal	Knee	*RP11-419C5.2*	No	16	69996187	69996188	5.97	2.38E-09	rs2270842	6.57	enet	4.60E-70	4.41E-02	2.40E-09	0.000	0.000	0.000	0.013	0.987
Muscle skeletal	Knee	*ILF3-AS1*	No	19	10653843	10653844	4.76	1.92E-06	rs3843751	-5.61	susie	1.30E-11	3.77E-03	1.90E-06	0.000	0.000	0.000	0.016	0.983
Muscle skeletal	Hip	*GCAT*	Yes	22	37807904	37807905	-5.00	5.86E-07	rs11089854	-5.44	top1	1.70E-16	3.18E-02	5.90E-07	0.000	0.002	0.000	0.045	0.953

Chr, chromosome; Cond, conditional analysis; GWAS, genome-wide association studies; Perm, permutation test; PP, poster probability; SNP, single nucleotide polymorphism.

Notes: As highlighted in red, genes were considered to be identified as high confidence if they effectively passed a sequence of assessments, including TWAS analysis ($p < 1E-5$), permutation testing ($p < 0.05$), model evaluation ($p < 0.05$), conditional analysis ($p < 0.05$), and colocalization analysis (PP.H4 > 0.8).

*Gene expression models: enet, elastic-net regression with mixing parameter of 0.5; lasso, LASSO regression; susie, sum of single effects model; top1, single best eQTL.

NOTE: Please save this file locally before filling in the table, DO NOT work on the file within your internet browser as changes will not be saved. Adobe Acrobat Reader (available free here) is recommended for completion.

The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	functional mechanisms underlying osteoarthritis (OA) through a transcriptome-wide association study (TWAS). The experiment included both in vitro and in vivo OA models, as well as human histological sections, to validate the involvement of key genes such as MAPK3 and SMAD3 in OA pathogenesis. n tissue sections, in vitro cultured cells, and in vivo animal models (likely rodents). Different models were compared to assess gene expression and validate the identified candidate genes.
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	12 rodents were used for the in vivo validation model, and 10 different human histological sections were analyzed. The sample size was determined based on previous studies and available resources for in vivo and in vitro experiments. No a priori sample size calculation was performed.
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	es were healthy, adult rodents with no previous surgical procedures. For in vitro models, only cells with over 90% viability were included. Human tissue samples were included based on histological evidence of OA. No animals or tissue samples were excluded from the study post-experimentation. The total number of animals used in the analysis was 6, and the total number of human tissue sections was 2.
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Animals in the in vivo model were randomly assigned to experimental and control groups. Randomization of tissue samples was not necessary due to the focus on OA-affected tissues. Confounding factors such as the time of sample collection and the environmental conditions of animal housing were controlled by standardizing experimental conditions across groups.
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Blinding was employed during the histological analysis of human tissue sections and during the outcome assessment in the in vivo models. The researchers performing the gene expression analysis were not aware of the group allocations.
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	vels of gene expression (MAPK3 and SMAD3) in the OA models, as well as the activity of these genes in human histological sections. Functional assays, including Western blotting and RT-qPCR, were used to quantify gene expression. The primary outcome measure for sample size estimation was the expression level of MAPK3 and SMAD3, which were hypothesized to be significantly elevated in OA samples.
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	standard t-tests or ANOVA to compare gene expression between experimental and control groups. Post-hoc tests were conducted where necessary. All statistical analyses were conducted using R software or GraphPad Prism. The assumptions of normality and homogeneity of variance were tested prior to statistical analysis. If these assumptions were not met, non-parametric tests were employed (e.g., Mann-Whitney U test).
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Adult male rodents (e.g., SD rats, aged 6-8 weeks, weighing 180-200g) were used for in vivo experiments. They were housed in a controlled environment with a 12-hour light/dark cycle and ad libitum access to food and water. The animals were procured from an accredited supplier and were acclimatized for 7 days prior to the initiation of the experiment. No previous procedures were performed on these animals.
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	while in vitro procedures involved culturing cells under OA-like conditions. Tissue samples were collected for gene expression analysis using Western blotting and RT-qPCR. r animal, and the animals were euthanized 4 weeks post-surgery. In vitro experiments were carried out in triplicates, with the same time intervals for OA induction. The animals were acclimatized for 7 days prior to the start of the experiment. the involvement of MAPK3 and SMAD3 in OA pathogenesis. The rationale for each procedure was based on previous literature showing their involvement in OA and other degenerative joint diseases.
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	re corroborated these findings in histological sections of rat and human knee joints. Immunofluorescence results indicated heightened expression of MAPK3 in OA joint tissues, while immunohistochemistry results revealed an enrichment of SMAD3 in inflamed joint tissues. Effect sizes were calculated where appropriate, with confidence intervals reported for the key outcome measures (e.g., fold changes in gene expression).