Bone & Joint Research

Supplementary Material

10.1302/2046-3758.119.BJR-2021-0443.R2

Haematoxylin and eosin staining

The specimens were fixed in 10% neutral formalin at room temperature for 24 hours. For decalcification, the samples were placed in ethylenediaminetetraacetic acid (EDTA) buffer solution (ZSGB-Bio, China) for four weeks. The decalcification solution was changed twice a week. Subsequently, rat cartilage tissues were fixed in 10% formaldehyde buffer, dehydrated with ethanol in xylene, embedded in paraffin, and cut into 5-μm sections. Then, serial sections were mounted on slides and stained with haematoxylin and eosin (H&E). For histological examination, the slides were examined under an optical microscope by a histopathologist who was unaware of the grouping details. According to the criteria, each section was scored on a scale of 0 to 4, which shows that the higher the score, the more severe the injury.

Toluidine blue staining

After routine dewaxing, tissue sections were stained with 0.5% toluidine blue solution for 30 minutes and washed under tap water. After washing with 0.5% glacial acetic acid solution for five seconds, the sections were washed under distilled water, dehydrated by gradient ethanol, cleared with xylene, and sealed. The average optical density (OD) value of toluidine blue was then analyzed.

Enzyme-linked immunosorbent assay

Rats were anaesthetized by intraperitoneal injection of ketamine hydrochloride (0.08 mg/100 g) and xylazine (0.04 mg/100 g), and fixed in the left lateral position. The right knee joint cavity was punctured and lavaged with an irrigation cannula containing 1.8 ml of phosphate-buffered saline (PBS) at the space on the anterolateral tibial plateau, and the lavage fluid was collected. One week later, the lavage fluid was taken again in the same way, and 100 μl of diluted synovial fluid was harvested. The levels of MCP-1 (#DY3144-05) in synovial fluid of rats or macrophages were measured using the DuoSet sandwich enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (USA). In brief, Capture Antibody was diluted to working concentration. After that, 100 μl of sample or standard was added to each well and incubated for two hours at room temperature, followed by another two-hour incubation with the antibody. Afterwards, the samples were incubated with Streptavidin-horseradish peroxidase (HRP) working dilution for 20 minutes at room temperature and with the substrate solution for 20 minutes. The OD value of each well was read using an ELISA reader set to 450 nm.

The levels of tumour necrosis factor alpha (TNF-α) (#RTA00), interleukin-10 (IL-10) (#R1000), IL-6 (#R6000B), and transforming growth factor beta (TGF-β) (#MB100B) were measured using Quantikine ELISA Kit (R&D Systems). The 96-well plates were rinsed three times in 0.05% Tween 20 in PBS and sealed with 1% bovine serum albumin (BSA) in PBS for 1.5 hours at ambient temperature. Standards and samples were then added for a two-hour incubation at room temperature. Next, the reaction was detected using 100 μl of coupling compound. Tetramethylbenzidine (TMB) was then added as a substrate solution, followed by reaction termination using H_2SO_4. The OD value at 450 nm was immediately measured with an automatic ELISA reader at a calibrated wavelength of 570 nm. The concentration was calculated from the standard curve.

Changes in IL-12 levels were detected using the Rat IL-12 p40/IL12B ELISA kit (#ELR-IL12p40-1, RayBiotech Life, USA). In a 96-well plate, standards and samples were added to each well and incubated for 2.5 hours at room temperature, incubated with the prepared biotin antibody for one hour, with streptavidin solution for 45 minutes at room temperature and with TMB substrate for 30 minutes. The OD value of each well was determined using an ELISA reader set to 450 nm.

Immunohistochemistry

The sections were dewaxed, detached with 0.1% trypsin for ten minutes at 37°C, followed by quenching of the endogenous peroxidase activity using 3% H_2O_2 for ten minutes. After that, 5% BSA was added to the sections to block non-specific binding, and the sections were incubated with antibodies against inducible nitric oxide synthase (iNOS) (ab178945, Abcam, USA) or Arg (ab133543, Abcam) at 4°C overnight. The sections were incubated with HRP-conjugated secondary antibody (SA1022, Wuhan Boster Biological Technology, China). The percentage of positively stained cells was determined by the ImageJ software (USA). Positive cells (%) = positive cells/total cells × 100%, and five random fields were selected to assess the positive cell rate.

Terminal deoxynucleotidyl transferase (TdT)-mediated 2'-Deoxyuridine 5'-Triphosphate (dUTP) nick end labelling (TUNEL)

Apoptosis in cartilage tissues was examined according to the protocol of the TUNEL kit (11684817910, Roche Applied Science, Germany). The sections were dewaxed, treated with Proteinase K working solution for 15 to 30 minutes, and incubated for 20 minutes at room temperature in 3% hydrogen peroxide solution prepared with PBS. The samples were incubated with 50 μl of in-situ cell death assay for one hour at 37°C in the dark and with 0.3 ml of reaction termination solution for ten minutes at room temperature. Afterwards, the samples were incubated at room temperature with Streptavidin-HRP working solution and DAB colour development solution,

respectively. Nuclei were stained with haematoxylin staining solution. The data were visualized using a Nikon ECLIPSE Ti microscope (Japan).

Collagen deposition assay

For the detection of collagen deposition in rat cartilage tissues, we used the Abcam Total Collagen Assay Kit (Perchlorate-Free, ab222942) in strict accordance with the instructions. The cartilage tissues were cut into small pieces of about 1 cm^3, placed in liquid nitrogen for two minutes, and grounded with the denaturing solution and β-mercaptoethanol to obtain the homogenized cartilage tissue, followed by a ten-minute centrifugation at 1,407 g to remove the pellet. The extracted supernatant was supplemented with 10 N sodium hydroxide for alkaline hydrolysis for 30 minutes. Oxidation reagent mix was then added and incubated for 25 minutes at 37°C. P-dimethylaminobenzaldehyde was added and incubated for 45 minutes at 65°C. The OD560 was measured on a microplate reader.

RNA-sequencing (seq)

Sequencing of RNA samples (500 ng) was conducted in the Finnish Institute of Molecular Medicine sequencing core using the Illumina HiSeq 2500 sequencing platform. Sequencing depth was 15 million paired-end reads 100 bp in length. Read quality was first evaluated using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the reads were trimmed using Trimmomatic. Trimmed reads were aligned to the full reference human genome with STAR. Count matrices were prepared with the featureCounts program. Differential expression was assessed with DESeq2 using patient number as an additional experimental factor for pairwise comparisons. Gene expression was given as DESeq2-normalized counts, and genes with a mean normalized count 5 or less across all samples were excluded from further analysis. Moreover, genes with a minimum of 2.0-fold change in abundance and false discovery rate-corrected p-value < 0.05 were regarded as statistically significant.

Flow cytometry

We evaluated expression of CD133, CD86, and CD206 using flow cytometry according to the manufacturer's instructions. The prepared macrophages (M0) (5×10^5 cells) were resuspended in 100 µl cell staining buffer and suspended in a 12 mm × 75 mm plastic tube. The macrophage suspension was ice-bathed with 1 µg antibodies against CD133 (ab252129, Abcam), CD206 (ab270647, Abcam), and CD86 (ab77276, Abcam). The cells were centrifuged at 350 g for five minutes at 4°C to discard the supernatant. The pellet was then added with 500 µl PBS buffer to remove the excess unbound antibody. The cells were fixed in 0.5 ml of fixing buffer for 20 minutes in the dark, then centrifuged, resuspended in 2 ml of staining buffer, loaded on a flow cytometer, and analyzed by Flow Jo v10.0.7 software (BD Biosciences, USA).

Immunofluorescence staining

Chondrocytes grown on coverslips were fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0.3% Triton X-100 for 15 minutes at ambient temperature. After incubation with 3% BSA for 30 minutes, the cells were incubated with antibodies specific to MMP1 (#GTX100534, GeneTex, USA), MMP9 (#GTX01747, GeneTex), Collagen II (#28459-1-AP, ProteinTech Group, USA), and Aggrecan (sc-33695, Santa Cruz Biotechnology, USA) at 4°C overnight, followed by Alexa Fluoro 568-labeled goat anti-rabbit IgG (ab175471, Abcam) incubation for two hours at ambient temperature. Nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) prior to imaging with a laser scanning confocal microscope (LSM, Carl Zeiss, Germany). Quantification of the number of positive cells was performed by ImageJ.

Double-labelled immunofluorescence

To gain insight into the binding relationship between fat mass and obesity-associated protein (FTO) and osteoarthritis non-coding transcript (OANCT) in cells and tissues, double-labelled immunofluorescence was performed using anti-rabbit FTO (#GTX131517, GeneTex) and OANCT-Cys probe (GenePharma, China). Cartilage tissue

sections from rats or macrophages were sequentially reacted with OANCT-Cys probe and anti-FTO and incubated overnight at 4°C. A fluorescein-coupled secondary antibody (Histofine Simple Stain MAX PO; Nichirei, Japan) was then applied. Finally, nuclei were stained with DAPI for three minutes for cell localization. Fluorescence images were captured at 405 and 594 nm using excitation spectral laser lines under an LSM 5 PASCAL confocal laser scanning microscope (Carl Zeiss).

Fluorescence in situ hybridization (FISH) assays

The prepared cell samples were fixed with 5% formaldehyde for 25 minutes. Then, the cells or cartilage tissues were incubated with the OANCT-Cys probe at 42°C overnight. After the sample was washed with sodium chloride-sodium citrate buffer, the nuclei of the cells were stained with DAPI. The staining results were observed under a confocal laser scanning microscope.

Monodansylcadaverine (MDC) staining

First, the macrophages in each well were added with MDC staining solution (G0170, Beijing Solarbio Life Sciences, China). After 15 minutes of staining under warm and dark conditions, the cells were rinsed three times with PBS, and observed under a fluorescence microscope.

TEM

TEM was used to monitor the process of autophagosome formation. Briefly, 24 hours after dysfunctional chondrocyte-derived exosomes (DC-exo) treatment, the macrophages were pre-fixed with 2.5% glutaraldehyde at ambient temperature and fixed with 1% osmium tetroxide for two hours. Subsequently, the samples were gradually dehydrated with increasing concentrations of ethanol and acetone, and embedded in sodium feldspar. Finally, 50 to 60 nm sections were prepared on an LKB-1 ultra-thin slicer, transferred to a copper grid, and photographed with a JEM-1400 plus TEM (JEOL, Japan).

Red fluorescent protein-green fluorescent protein-microtubule associated protein light chain 3 (RFP-GFP-LC3)

First, the macrophages were loaded onto 15 mm glass bottom cell culture dishes. Cells were infected with adenovirus-mRFP-GFP-LC3 (MOI 300; Hanbio, China) at 30% to 50% confluence according to the manufacturer's instructions. At 24 hours after infection, the fresh complete medium was renewed, and the cells were observed under a fluorescence microscope. When > 95% of the viable cells were confirmed, exosome treatment and short hairpin RNA (shRNA) plasmid transfection were carried out. Images were captured with the aid of a laser scanning confocal microscope (LSM, Carl Zeiss) where green fluorescence (dots) represented autophagosomes. The number of autophagosomes was determined by manually counting the fluorescent dots in five high magnification fields of view (×60, LSM, Carl Zeiss).

Western blot

After lysis with lysis buffer containing phosphatase and protease inhibitors (Beyotime, China), proteins from macrophages or rat cartilage tissues were collected. Total protein concentration was determined using bicinchoninic acid assay protein assay reagent (Beyotime). Protein extracts were subjected to 10% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transblotted to polyvinylidene difluoride membranes (Millipore, USA). The membranes were sealed in Tris-buffered saline with Tween containing 5% blotting-grade milk (Boster) for one hour and then incubated with primary antibodies against p62 (ab109012, Abcam), Atg4B (#15131-1-AP, Proteintech), Beclin-1 (#66665-1-Ig, Proteintech), PIK3R5 (OTI4G9, Novus Biological, USA), PI3K (#GTX111068, Genetex), phos-PI3K (#GTX132597, Genetex), AKT1 (ab233755, Abcam), phos-AKT1 (ab133458, Abcam), mTOR (ab32028, Abcam), phos-mTOR (ab109268, Abcam), CD63 (#PA5-92370, Thermo Fisher Scientific, USA), CD9 (#GTX76184, Genetex), CD81 (#MA5-13548, Thermo Fisher), ALIX (#MA1-83977, Thermo Fisher), and GAPDH (#MA5-15738-D680, Thermo Fisher) overnight. The membrane was

incubated with the secondary antibody in a sealing solution for two hours at room temperature and then detected using chemiluminescence (Millipore). Finally, the membranes were exposed to X-ray film to visualize the bands (Amersham Pharmacia, USA). ImageJ was used to quantify the results.

RNA pull-down

A biotin-tagged PIK3R5 mRNA probe (GenePharma) was synthesized to validate the binding relationship of FTO protein to PIK3R5 mRNA. RNA probes contain m6A bases, adenine bases, or mutated guanine bases. RNAiMAX reagent (Thermo Fisher, USA) was used to transfect RNA probes into cells. After 48 hours, the macrophages were lysed in 500 μl immunoprecipitation (IP) lysis buffer containing 1× protease inhibitor mixture and 5 μl RNase inhibitor. One-tenth of the supernatant served as input sample. Then, 40 μl streptavidin beads (Thermo Fisher) were added to the remaining supernatant, and the samples were incubated for 120 minutes at room temperature. After washing with IP wash buffer (Thermo Fisher), the unbound protein was eluted with 95% formamide at 95°C for two minutes. Protein samples were denatured by LDS buffer (Thermo Fisher) and analyzed by western blot.

RNA IP (RIP)

To detect the binding of FTO protein to PIK3R5 mRNA, an EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore) was used according to the manufacturer's protocol. Briefly, the macrophages (1×10^7) were lysed in 500 μl IP lysis buffer containing 1× protease inhibitor mixture and 5 μl RNase inhibitor. The supernatant was harvested after centrifugation, and 10% of which was retained as input sample. Anti-Flag antibody (5 μg) and magnetic bead complex (40 μl) were added to the remaining 90% supernatant and incubated at 4°C overnight. The beads were washed with wash buffer and eluted using an RNA purification kit (Qiagen, USA) according to the manufacturer's protocol. The enrichment of PIK3R5 mRNA was quantified using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

mA-specific methylated RIP (MeRIP)

Total RNA was isolated from macrophages by the TRIzol method, and mRNA was isolated and purified from total RNA using the PolyATtract mRNA isolation system (A-Z5300, A&D Technology Corporation, China). The m6A antibody (ab151230, Abcam) or IgG antibody (ab109489, Abcam) was added to IP buffer containing 20 mM Tris (pH = 7.5), 140 mM NaCl, 1% NP-40, and 2 mM EDTA and incubated with protein A/G beads for one hour for binding. The purified mRNA and magnetic bead-antibody complexes were then added to IP buffer with ribonuclease inhibitors and protease inhibitors and incubated at 4°C overnight. The RNA was eluted using elution buffer, then extracted and purified by phenol-chloroform. Quantitative analysis was performed using RT-qPCR.

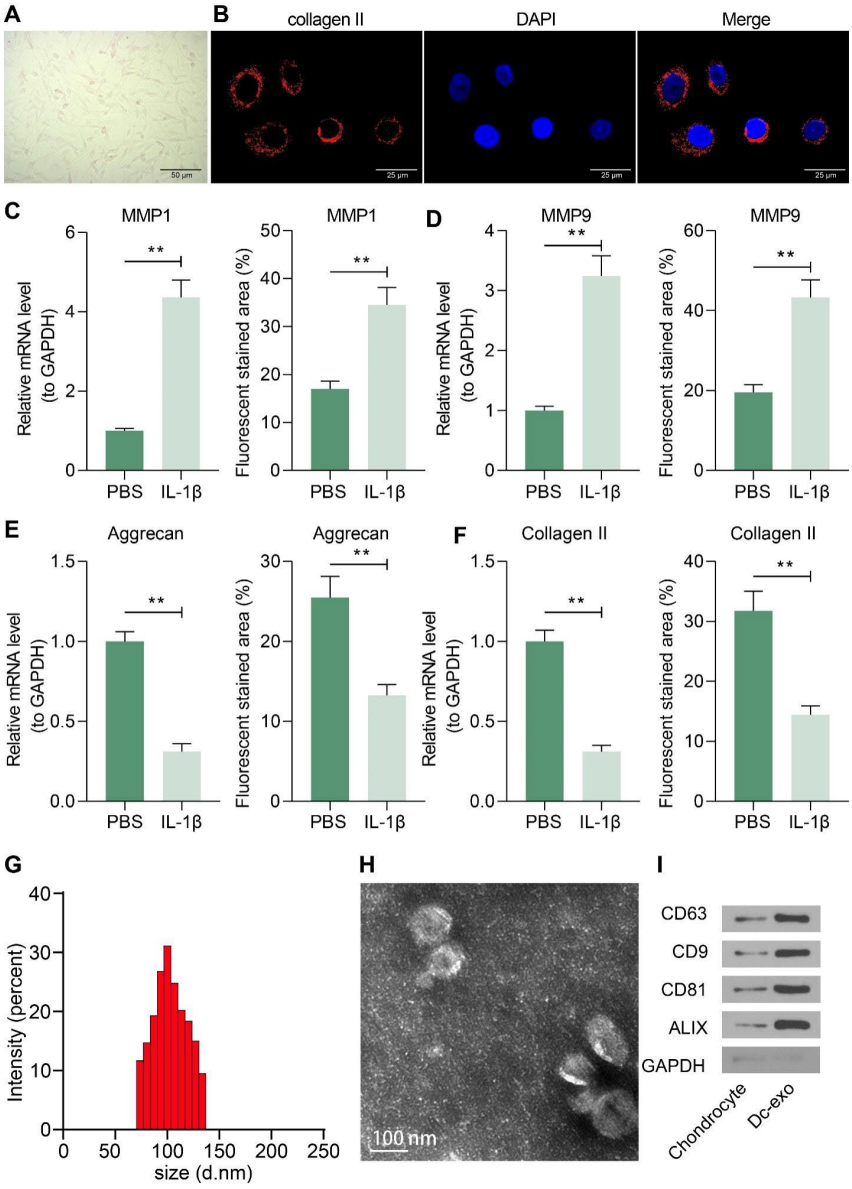

Fig a. Isolation and culture of chondrocytes and identification of exosomes. a) Morphology of chondrocytes under light microscopy. b) Immunofluorescence detection of collagen II expression and distribution in chondrocytes; c) to f) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence detection of c) matrix metalloproteinase (MMP)1, d) MMP9, e) aggrecan, and f) collagen II expression in chondrocytes after treatment with interleukin (IL)-1β. g) Nanosight LM10 nanoparticle tracking analysis of extracted exosomes. h) Transmission electron microscope (TEM) observation of the morphology of exosomes released by IL-1β-treated chondrocytes. i) The expression of exosomal marker proteins CD63, CD9, CD81, and ALIX determined by western blot. The results are representative of three independent experiments. All data are represented as mean and standard deviation. Data were analyzed using independent-samples *t*-test. **p < 0.01. DAPI, 4′,6-diamidino-2-phenylindole; Dc-exo, dysfunctional chondrocyte-derived exosome; GAPDH, glyceraldehyde3-phosphate dehydrogenase; mRNA, messenger RNA; PBS, phosphate-buffered saline.

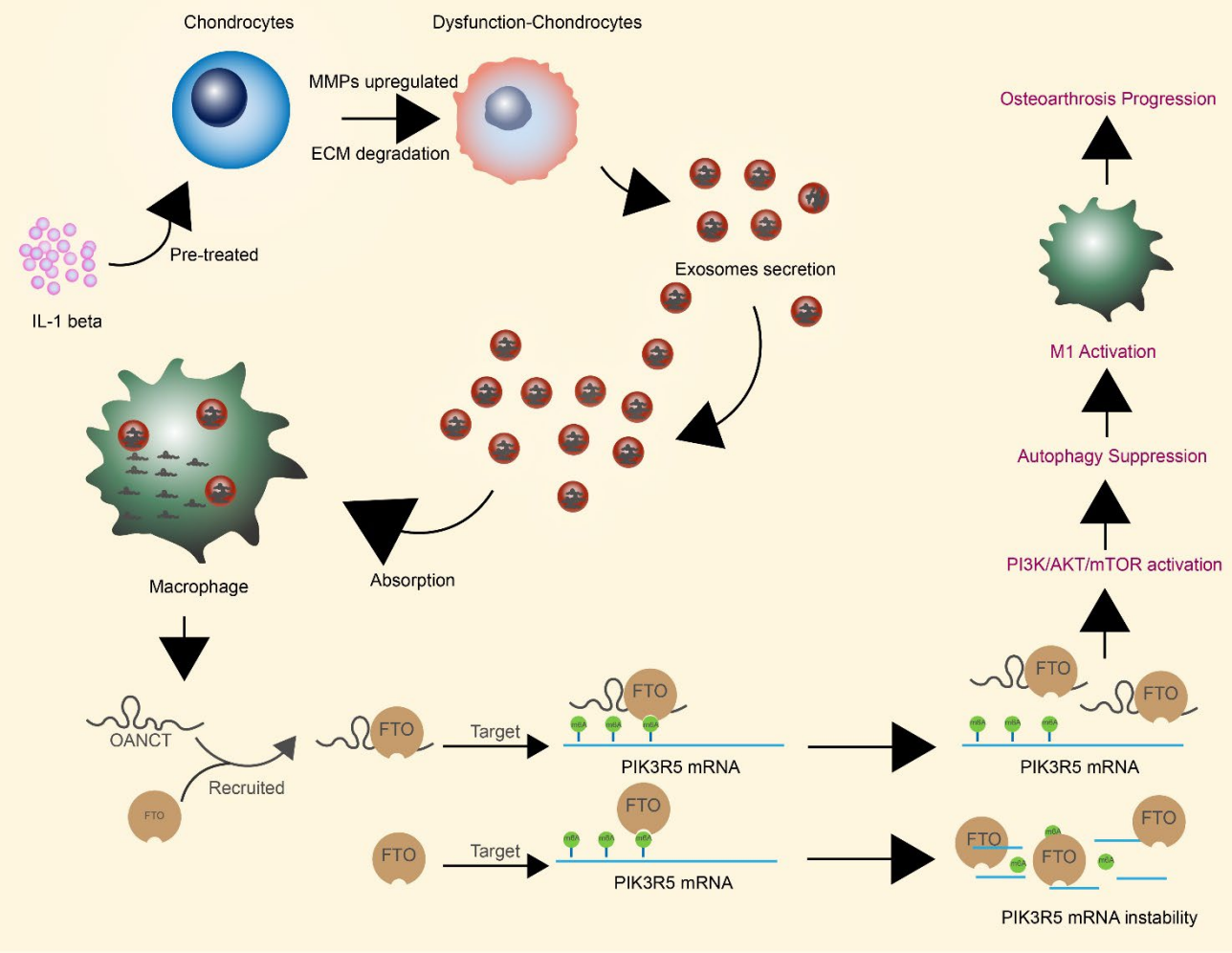

Fig b. Underlying mechanism of dysfunctional chondrocyte-derived exosome (Dc-exo) on osteoarthritis (OA). Exosomal osteoarthritis non-coding transcript (OANCT) inhibited the ability of fat mass and obesity-associated protein (FTO) protein to demethylate and modify, thereby maintaining the messenger RNA (mRNA) stability of phosphoinositide-3-kinase regulatory subunit 5 (PIK3R5) and activating the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway to inhibit macrophage autophagy and promote M1-type polarization of macrophages. ECM, extracellular matrix; IL, interleukin; MMP, matrix metalloproteinase.

Table i. Primer sequences.

Gene symbol	Forward (5′-3′)	Reverse (5′-3′)
OANCT	TGCCTCCTTTCCCTCGTT	TTCAGCGTTGCCCTTTGT
MMP1	ATGAAGCAGCCCAGATGTGGAG	TGGTCCACATCTGCTCTTGGCA
MMP9	TCTATGGTCCTCGCCCTGAA	TTGTATCCGGCAAACTGGCT
Collagen II	CCTGGCAAAGATGGTGAGACAG	CCTGGTTTTCCACCTTCACCTG
Aggrecan	CAGGCTATGAGCAGTGTGATGC	GCTGCTGTCTTTGTCACCCACA
FTO	CCAGAACCTGAGGAGAGAATGG	CGATGTCTGTGAGGTCAAACGG
PIK3R5	AGAAGACCCGAGAGGTCCAG	GCTTCCCTGGTTTTGCAGTG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
Atg4b	AGAGCTGCCAAGTCCAGATTC	TTCAGGTTTCCCACACACCC
Beclin-1	CTGGACACTCAGCTCAACGTCA	CTCTAGTGCCAGCTCCTTTAGC
p62	CCATCCAGTGGCAGGACAAA	GCTATGGAGTCCCATCCAGC

FTO, fat mass and obesity-associated protein; GAPDH, glyceraldehyde3-phosphate dehydrogenase; MMP, matrix metalloproteinase; OANCT, osteoarthritis non-coding transcript.

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).	Supplementary methods paragraph 4 Supplementary methods paragraph 4
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.	Supplementary methods paragraph 1 Supplementary methods paragraph 1
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.	Supplementary methods paragraph 4 Supplementary methods paragraph 4 Supplementary methods paragraph 4
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.	Supplementary methods paragraph 4 Supplementary methods paragraph 4
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).	Supplementary methods paragraph 4
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.	Supplementary methods paragraph 4 Supplementary methods paragraph 4
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.	Methods paragraph 4 Methods paragraph 4
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.	Supplementary methods paragraph 1 Supplementary methods paragraph 4
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).	Supplementary methods paragraph 4 Supplementary methods paragraph 4 Supplementary methods paragraph 4 Supplementary methods paragraph 4
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.	Result paragraph 3,8,11 Result paragraph 3,8,11

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	Abstract paragraph
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach. b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	Aim paragraph Aim paragraph
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	Aim paragraph
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	Ethical statement paragraph
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	Supplementary methods paragraph 4
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	Supplementary methods paragraph 4 Supplementary methods paragraph 4 Supplementary methods paragraph 4
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	Discussion paragraph 1-5 Discussion paragraph 1-5
Generalisability/ translation	18	Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	Discussion paragraph 5
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	Ethical paragraph
Data access	20	Provide a statement describing if and where study data are available.	Data Availability paragraph
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	ICMJE COI statement paragraph Funding statement