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Original Article

Therapeutic potential of dedifferentiated fat cells in a rat model of osteoarthritis of the knee

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ABSTRACT

Introduction: Mature adipocyte-derived dedifferentiated fat cells (DFATs) represent a subtype of multipotent cells that exhibit comparable phenotypic and functional characteristics to adipose-derived stem cells (ASCs). In this study, we assessed the chondroprotective properties of intra-articularly administrated DFATs in a rat model of osteoarthritis (OA). We also investigated in vitro the expression of anti-inflammatory and chondroprotective genes in DFATs prepared from the infrapatellar fat pad (IFP) and subcutaneous adipose-tissue (SC) of human origin.
Methods: In the cell transplantation experiment, rats were assigned to the DFAT and Control group (n = 10 in each group) and underwent anterior cruciate ligament transection (ACLT) accompanied by medial meniscus resection (MMx) to induce OA. One week later, they received intra-articular injections of 1×10^6 DFATs (DFAT group) or PBS (control group) four times, with a weekly administration frequency. Macroscopic and microscopic evaluations were conducted five weeks post-surgery. In the in vitro experiments. DFATs derived from the IFP (IFP-DFATs) and SC (SC-DFATs) were prepared from donor-matched tissue samples (n = 3). The gene expression of *PTGS2*, *TNFAIP6*, *PRG4*, *BMP2*, and *BMP6* under TNF-α or IFN-γ stimulation in these cells was evaluated using RT-PCR. Furthermore, the effect of co-culturing synovial fibroblasts with DFATs on the gene expression of *ADAMTS4* and *IL-6* were evaluated.
Results: Intra-articular injections of DFATs significantly inhibited cartilage degeneration in the rat OA model induced by ACLT and MMx. RT-PCR analysis revealed that both IFP-DFATs and SC-DFATs upregulated the expression of genes involved in immune regulation, anti-inflammation, and cartilage protection such as *PTGS2*, *TNFAIP6*, and *BMP2*, under stimulation by inflammatory cytokines. Co-culture with DFATs suppressed the expression of *ADAMTS4* and *IL6* in synovial fibroblasts.
Conclusions: The intra-articular injection of DFATs resulted in chondroprotective effects in the rat OA model. Both SC-DFATs and IFP-DFATs induced the expression of anti-inflammatory and

Abbreviations: ACLT, anterior cruciate ligament transection; Adamts, a disintegrin and metalloproteinase with thrombospondin motifs; ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs 4; ASCs, adipose-derived stem cells; BMP2, bone morphogenetic protein 2; BMP6, bone morphogenetic protein 6; cDNA, complementary deoxyribonucleic acid; DFATs, dedifferentiated fat cells; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; IFN-γ, interferon-γ; IFP, infrapatellar fat pad; IFP-DFATs, DFATs derived from IFP; IL6, Interleukin 6; MMP, matrix metalloproteinase; MMx, medial meniscus resection; MSCs, mesenchymal stem cells; N-SFs, synovial fibroblasts derived from normal donors; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; OA-SFs, synovial fibroblasts derived from OA patients; PBS, phosphate-buffered saline; PGE2, prostaglandin E2; PRG4, proteoglycan 4; PTGS2, prostaglandin-endoperoxide synthase 2; RNA, ribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction; SC, subcutaneous adipose tissue; SC-DFATs, DFATs derived from subcutaneous adipose tissue; SFs, synovial fibroblasts; TIMP-1, tissue inhibitor of metalloproteinase 1; TKA, total knee arthroplasty; TNF-α, tumor necrosis factor-α; TNFAIP6, tumor necrosis factor alpha induced protein 6; TSG-6, tumor necrosis factor-α stimulated gene/protein 6.

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chondroprotective genes in vitro. These results indicate that DFATs appear to possess therapeutic potential in inhibiting cartilage degradation and could serve as a promising cellular resource for OA treatment.

1. Introduction

Osteoarthritis (OA) is a pathological condition in which joint damage is not repaired due to stresses that can be caused by abnormalities in any joint or periarticular tissue. The symptoms of knee OA specifically include joint pain, restricted range of motion, and muscle weakness, ultimately resulting in diminished activities of daily living. Both physiological and mechanical mechanisms play a causative role in the etiology of OA. Advanced age, body weight, sex, history of trauma, and prior arthritis episodes have been identified as risk factors associated with OA onset [1]. It is anticipated that the prevalence of OA will continue to increase due to the progressive aging of societies worldwide. OA represents a prevalent ailment afflicting a significant proportion of the global population aged 60 years and above [2].

The disease process of OA begins with the degradation and wear of articular cartilage followed by the progression of synovitis caused by the presence of cartilage fragments floating in the joint space. Synovitis contributes to the upregulation of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). These inflammatory cytokines induce the production of interleukin 6 (IL6), matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and cause cartilage degeneration and synovitis [3,4]. Although conventional treatments such as exercise, physical therapy, and analgesic medications can transiently ameliorate symptoms, they fail to halt or reverse the progression of OA. While surgical treatment, including total knee arthroplasty (TKA), is effective in severe cases, the artificial joint has a limited lifespan and is not suitable for young individuals with early-onset OA. Consequently, there has been growing interest in the field of therapeutic strategies targeting the underlying pathological processes of OA, with cell-based therapies emerging as a particularly promising avenue. Among the various types of cells investigated for OA therapy, mesenchymal stem cells (MSCs) have received considerable attention due to their inherent ability to differentiate into chondrocytes and their immunomodulatory properties [5]. In particular, MSCs derived from adipose tissue, commonly known as adipose-derived stem cells (ASCs), have gained significant clinical application in recent years due to their minimally invasive collection from patients. In fact, numerous clinical trials have effectively demonstrated the safety and consistent efficacy of intra-articular injections of ASCs for patients suffering from OA [6,7]. However, it should be noted that MSCs, including ASCs derived from elderly individuals, have been observed to possess diminished proliferative and multipotent differentiation capacities [8,9]. Therefore, there is still a need for alternative cell sources suitable for autologous cell therapy for OA in the elderly.

Dedifferentiated fat cells (DFATs) are mature adipocyte-derived ASC-like cells with high proliferative activity and multilineage differentiation potential [10]. DFATs hold significant promise in the field of cell-based therapy, as they can be generated in abundant quantities from minimal volumes of adipose tissue, regardless of the donor's age or underlying medical conditions. Experimental evidence has shown the therapeutic potential of DFATs in animal models of various diseases, including osteochondral injuries [11–13]. In addition, in vitro and in vivo investigations have established the immunosuppressive effects of DFATs through TNF-α stimulated gene/protein 6 (TSG-6) [14,15]. DFATs can be prepared not only from subcutaneous adipose tissue (SC) but also from the infrapatellar fat pad (IFP) extracted during TKA. Notably, DFATs derived from the IFP exhibit superior potential for chondrogenic differentiation compared to IFP-derived ASCs [16]. The putative mechanisms of MSC-based therapy for OA have been proposed as paracrine effects of humoral factors with chondroprotective, immunomodulatory and anti-inflammatory activities [17]. Transplanted MSCs also suppress the secretion of inflammatory cytokines and cartilage matrix degradation enzymes from the synovium and cartilage [18,19]. Although it has been speculated that DFATs may have therapeutic efficacy against OA via mechanisms similar to ASCs, to our knowledge no such reports have been published. Therefore, the aim of this study was to investigate the chondroprotective effects of intra-articular DFAT injections in a rat model of OA and to compare the anti-inflammatory and chondroprotective properties of DFATs derived from IFP (IFP-DFATs) with those of DFATs derived from SC (SC-DFATs).

2. Materials and methods

2.1. Animal model of OA

Male Wistar rats, aged between 10 and 12 weeks, were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). All animal experiments were approved by Animal Research and Care Committee at the Nihon University School of Medicine (approval numbers: AP17M001 and AP17M034). The OA model in rats was created by anterior cruciate ligament transection (ACLT) in combination with medial meniscus resection (MMx) was created as previously described [20]. Briefly, the rats were anesthetized using isoflurane and a medial parapatellar approach was used to laterally dislocate the patella. The anterior cruciate ligament was transected, and the medial meniscus was completely resected using micro-scissors. The surgical procedure was concluded by repairing the joint capsule and closing the skin in layers. Subsequently, all rats were returned to their respective cages and allowed unrestricted movement post-surgery.

2.2. Preparation of rat DFATs

Rat DFATs were prepared from SC obtained from Wistar rats, aged between 10 and 12 weeks, using the ceiling culture technique outlined in the previous publication by Matsumoto et al. [10]. Briefly, approximately 1 g of SC was finely chopped and enzymatically digested with 0.1% collagenase solution (Sigma-Aldrich, St. Louis, MO) at 37 °C for 30 min with gentle agitation. Following filtration and centrifugation at 135×g for 1 min, the floating top layer containing lipid-filled adipocytes was collected. To prevent stromal cell contamination, the dissociated fat cells were repeatedly pipetted,

washed with phosphate-buffered saline (PBS), and centrifuged two times. The cells were then plated in T-12.5 culture flasks (NUNC, Roskilde, Denmark) filled completely with Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS, JRH bioscience, Lenexa, KS, Lot 6G2146) and incubated at 37 °C in 5% CO_2. Over time, the cells floated to the top and adhered to the upper inner surface of the flask. After 7 days, the medium was removed, and the flasks were inverted to position the cells at the bottom. The medium was changed every 4 days until the cells reached confluence. For passage, the cells were detached using a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen), after which they were seeded in 100-mm dishes at a density of 1×10^6 cells per dish, and further cultured. Experiments were performed using cells up to passage 5.

2.3. Cell transplantation experiments

The rats underwent ACLT and MMx in the right knee to induce OA. Starting 1 week after surgery, the rats then received intra-articular injection of 50 μl of PBS alone (Control group, n = 10) or 1×10^6/50 μl DFATs suspended in PBS (DFAT group, n = 10), a total of four times, with a weekly administration frequency. The cell suspension was administered into the joint cavity using a syringe fitted with a 27G needle and was injected from the anterior knee joint toward the intercondylar area. Four weeks after the injections, the rats were sacrificed and evaluated for cartilage degeneration.

2.4. Evaluations of cartilage degeneration

Both the femoral condyle and the tibial plateau were isolated and examined using India Ink staining for macroscopic observation, following the previously described method [21]. For histological analysis, both femoral condyle and the tibial plateau were fixed in 4% paraformaldehyde solution and decalcified in 20% EDTA solution, followed by embedding in paraffin wax. The specimens were then sectioned in the sagittal plane at a thickness of 5 μm and stained with Safranin-O. The assessment of cartilage degeneration was performed using Mankin's score [22] and the Osteoarthritis Research Society International (OARSI) scoring system [23] in the medial region of the femoral condyle and the tibial plateau.

2.5. Human tissue samples

Samples of human IFP and SC were obtained from three female patients (average age 79.7 ± 1.5 years, average body mass index 24.1 ± 4.7 kg/m^2) diagnosed as having knee OA (Kellgren-Lawrence grade III or IV) during TKA performed at the Department of Orthopedic Surgery, Nihon University Itabashi Hospital (Tokyo, Japan). The patients provided written informed consent, and the Ethics Committee of Nihon University School of Medicine approved the study (approval number: RK-160209-6). Human DFATs were prepared from IFP and SC using the previously described ceiling culture method [24]. Briefly, approximately 1 g of IFP and SC was

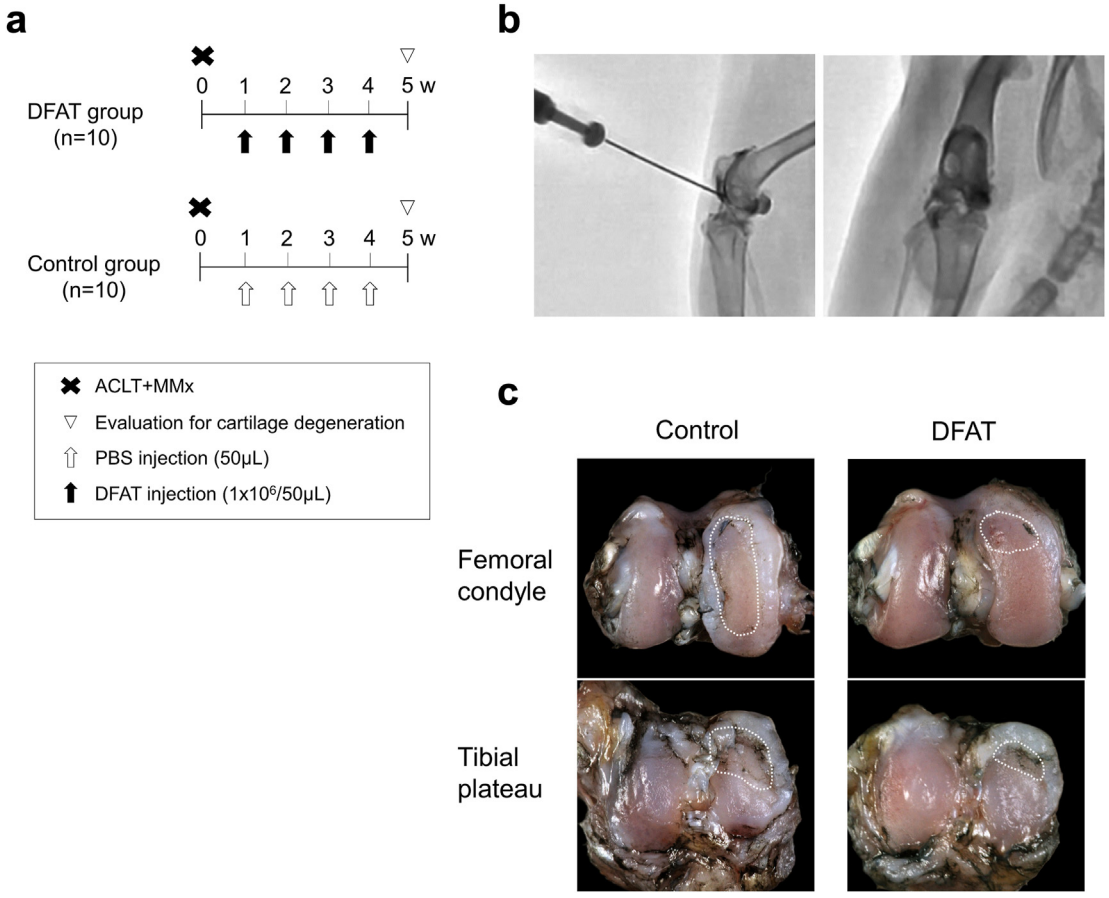

Fig. 1. The effect of intra-articular injection of dedifferentiated fat cells (DFATs) in a rat model of osteoarthritis (OA). Rats were divided into two groups (DFAT and Control groups) and received anterior cruciate ligament transection (ACLT) with medial meniscus resection (MMx). One week later, they received intra-articular injections of DFATs or PBS four times a week. Macroscopic and microscopic evaluation was performed 5 weeks after ACLT + MMx. (a) The experimental design. (b) Radiographic findings of intra-articular injections of contrast medium. (c) Representative macroscopic features of the femoral condyle and tibial plateau in each group. Cartilage erosion is indicated by the white dashed line.

finely minced and digested with 0.1% collagenase solution at 37 °C for 30 min with gentle agitation. After filtration and centrifugation, the floating lipid-filled adipocytes were collected and seeded in T-12.5 culture flasks filled completely with DMEM supplemented with 20% FBS. Subsequently, the cells were incubated at 37 °C in 5% CO_2. After 7 days, the medium was removed, and the flasks were inverted. The medium was changed every 4 days. For cell passage, the cells were treated with a trypsin-EDTA solution. Subsequently, the cells were seeded in 100-mm dishes at a density of 1×10^6 cells per dish and cultured. Cells at the second passage were used for the experiments.

2.6. Cell culture experiments

IFP-DFATs and SC-DFATs, each consisting of 1×10^5 cells, were seeded on a 100-mm dish and incubated in DMEM containing 10% FBS. At 80% confluence, the cells were exposed to either 10 ng/ml TNF-α (Sigma-Aldrich) or 150 U/ml IFN-γ (Sigma-Aldrich), or remained untreated for a duration of 12–72 h. Total ribonucleic acid (RNA) was extracted at the indicated time points, and gene expression of anti-inflammatory and chondroprotective genes was measured using real-time reverse transcription-polymerase chain reaction (RT-PCR).

Coculture experiments of DFATs with synovial fibroblasts (SFs) were carried out based on a previous study [25] with a modification. Briefly, SFs derived from normal donors (N-SFs) and SFs derived from OA patients (OA-SFs) (both from Articular Engineering, Northbrook, IL) were cultured in DMEM supplemented with 10% FBS and were used up to the passage 6 for the experiments. N-SFs or OA-SFs, at a density of 1×10^5 cells, were cultured alone or cocultured with IFP-DFATs or SC-DFATs, also at a density of 1×10^5 cells, in a 6-well plate equipped with a cell culture insert with 0.4-μm pores (BD Falcon, Franklin Lakes, NJ). After 24 h of coculture, the cells were either stimulated with 10 ng/ml TNF-α or left unstimulated and further incubated for 12 h. The SFs were harvested, and total RNA was extracted. Subsequently, the samples were evaluated for expression of genes associated with cartilage degeneration through real-time RT-PCR.

2.7. Real-time RT-PCR

Total RNA was extracted from cells using an RNeasy Mini kit (Qiagen). Subsequently, 1 μg of total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using random 9-mers and a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio, Ohtsu, Japan). The primers and probe utilized for the quantification of *PTGS2* (prostaglandin-endoperoxide synthase 2) (Hs00153133_m1), *TNFAIP6* (tumor necrosis factor alpha-induced protein 6) (Hs00200180_m1), *PRG4* (proteoglycan 4) (Hs00981633_m1), *BMP2* (bone morphogenetic protein 2) (Hs00154192_m1), *BMP6* (bone morphogenetic protein 6) (Hs01099594_m1), *ADAMTS4* (a disintegrin and metalloproteinase with thrombospondin motifs 4) (Hs00192708_m1), and *IL6* (interleukin 6) (Hs00174131_m1) were obtained from TaqMan Pre-Developed Assay Reagents (Applied

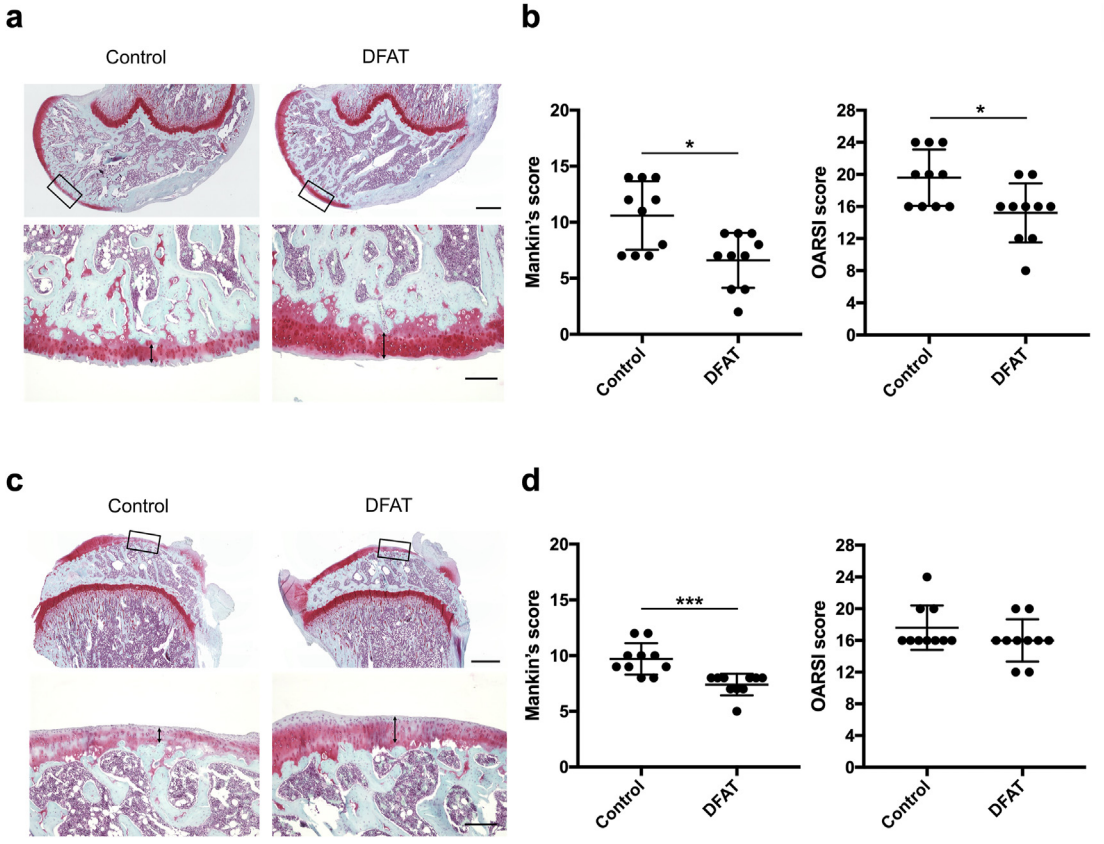

Fig. 2. Histological evaluation of the femoral condyle and tibial plateau after DFATs injection in the rat OA model. Histological evaluation was performed 5 weeks after ACLT + MMx by Safranin O staining. (a) Representative histological sections of the femoral condyle in each group. The lower panels indicate the higher magnification views of the open rectangles in the upper panels. Double arrows indicate the cartilage layer. Scale bars: 1000 μm (upper panels), 200 μm (lower panels). (b) Mankin's scores and OARSI scores of the femoral condyle in each group are shown. Data are presented as mean ± SD. *P < 0.05. (c) Representative histological sections of the tibial plateau. The lower panels indicate the higher magnification views of the open rectangles in the upper panels. Double arrows indicate the cartilage layer. Scale bars: 1000 μm (upper panels), 200 μm (lower panels). (d) Mankin's scores and OARSI scores of the tibial plateau in each group are shown. Data are presented as mean ± SD. ***P < 0.001.

Biosystems, Foster City, CA). 18S ribosomal RNA for human (HS99999901_S1, Applied Biosystems) was included as endogenous normalization controls to adjust for unequal amounts of RNA. Quantification of mRNA was performed using an ABI Prism 7300 system (Applied Biosystems). Each sample (each reaction, 5 μl cDNA; total volume, 25 μl) was run in triplicate. The cycling parameters consisted of an initial step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. Relative gene expression was determined using the comparative Ct method, with 18S ribosomal RNA as the endogenous control, after confirming that the amplification efficiencies of the target genes and the endogenous control were approximately equal. The results are presented as normalized target gene expression relative to that of 18S ribosomal RNA.

2.8. Statistical analysis

Values are expressed as the mean ± the standard deviation. The Mann-Whitney's *U* test was used to analyze comparisons between the two groups. For comparisons among multiple groups, the ordinary one-way ANOVA and Tukey's multiple comparisons test were conducted. A significance level of $P < 0.05$ was considered to indicate statistical significance. Statistical analysis was performed using GraphPad Prism Version 5 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Intra-articular injections of DFATs inhibit OA progression

We first examined whether intra-articular injections of DFATs inhibit OA progression in a rat model of OA. In preliminary experiments, gross observation of joint sites in rats treated with ACLT + MMx revealed partial cartilage degeneration on the articular surfaces, commencing at 5 weeks post-treatment in the femur and 1 week post-treatment in the tibia (Supplementary Fig. 1). The extent of cartilage degeneration gradually increased and persisted without amelioration until 17 weeks after treatment. Histologically, by Safranin O staining, thinning of the cartilage matrix was observed from 5 weeks post-treatment, followed by matrix loss at 7 weeks and exposure of subchondral bone at 9 weeks (Supplementary Fig. 2). These changes persisted until the 17 weeks.

The experimental design for cell transplantation is shown in Fig. 1a. We initially confirmed that 50 μl of contrast agent was reliably administered and retained in the joint cavity (Fig. 1b).

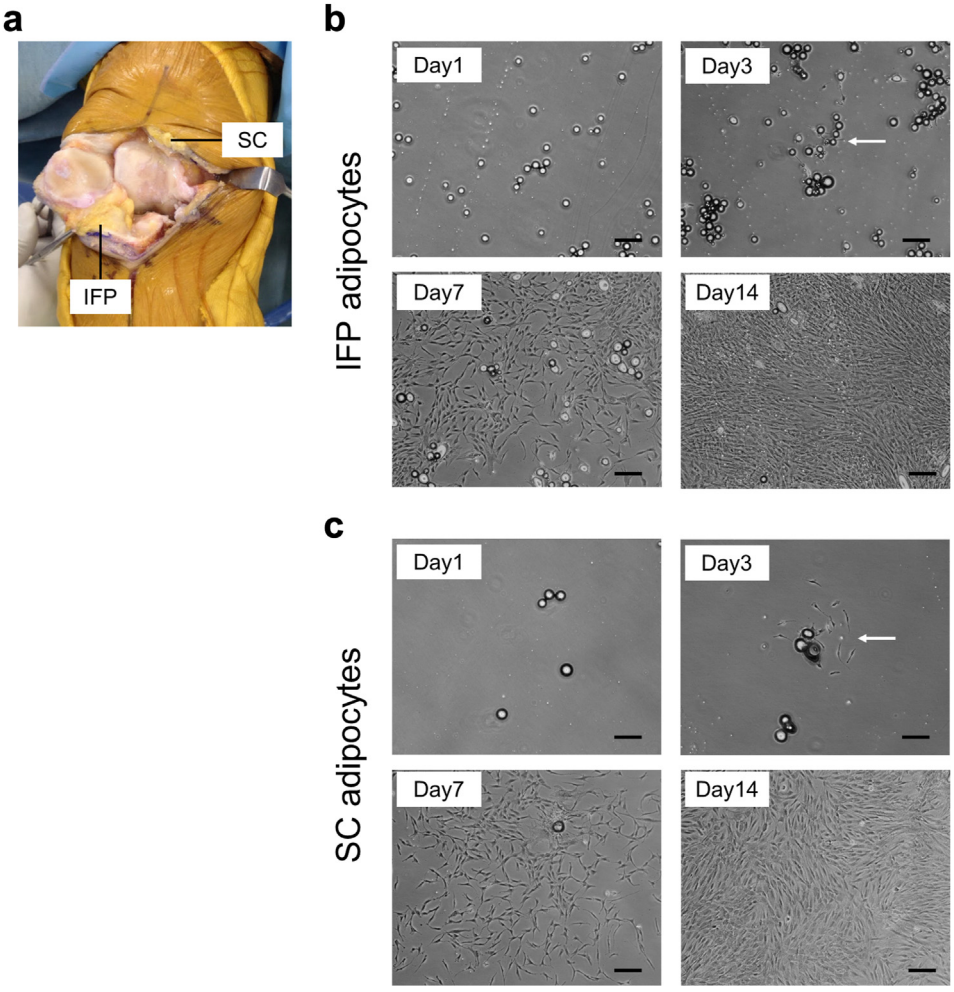

Fig. 3. Morphological changes of adipocytes isolated from infrapatellar fat pad (IFP) and subcutaneous adipose tissue (SC) during ceiling culture. IFP and SC were provided by knee OA patients undergoing total knee arthroplasty. Mature adipocytes isolated from IFP and SC were incubated by the ceiling culture method to prepare DFATs. (a) A macroscopic image of IFP and SC in a OA patient. (b, c) Phase-contrast microscopic images of cultured adipocytes isolated from IFP (b) and SC (c) on days 1, 3, 7, and 14 of the ceiling culture. Arrows indicate DFATs. Scale bars: 200 μm.

Following ACLT + MMx treatment, cell transplantation was performed four times per week. Consequently, macroscopic observation showed that the group receiving DFAT transplantation exhibited a tendency towards reduced cartilage degeneration on the femoral and tibial articular surfaces compared to the control group receiving saline injections (Fig. 1c). Histological analysis of the distal femoral articular surface with Safranin O staining revealed irregular cartilage surfaces, cartilage fissures, and diminished hyaline cartilage matrix in the Control group (Fig. 2a). In contrast, the DFAT group exhibited mild irregularities in the cartilage surface with few cartilage fissures and preserved hyaline cartilage matrix. Quantitative analysis indicated that both Mankin's score and the OARSI score, which are histological scores of cartilage degeneration, were significantly lower ($P < 0.05$) in the DFAT group compared to the Control group (Fig. 2b). Similarly, histology in the proximal articular surface of the tibia revealed a reduction in the hyaline cartilage matrix with chondrocyte loss in the Control group, whereas the DFAT group exhibited a tendency towards preserving the hyaline cartilage matrix without chondrocyte loss (Fig. 2c). Quantitative analysis showed that Mankin's score was significantly lower ($P < 0.05$) in the DFAT group compared to the Control group (Fig. 2d). These findings suggested that the intra-articular administration of DFATs in a rat model of knee OA inhibits cartilage degeneration.

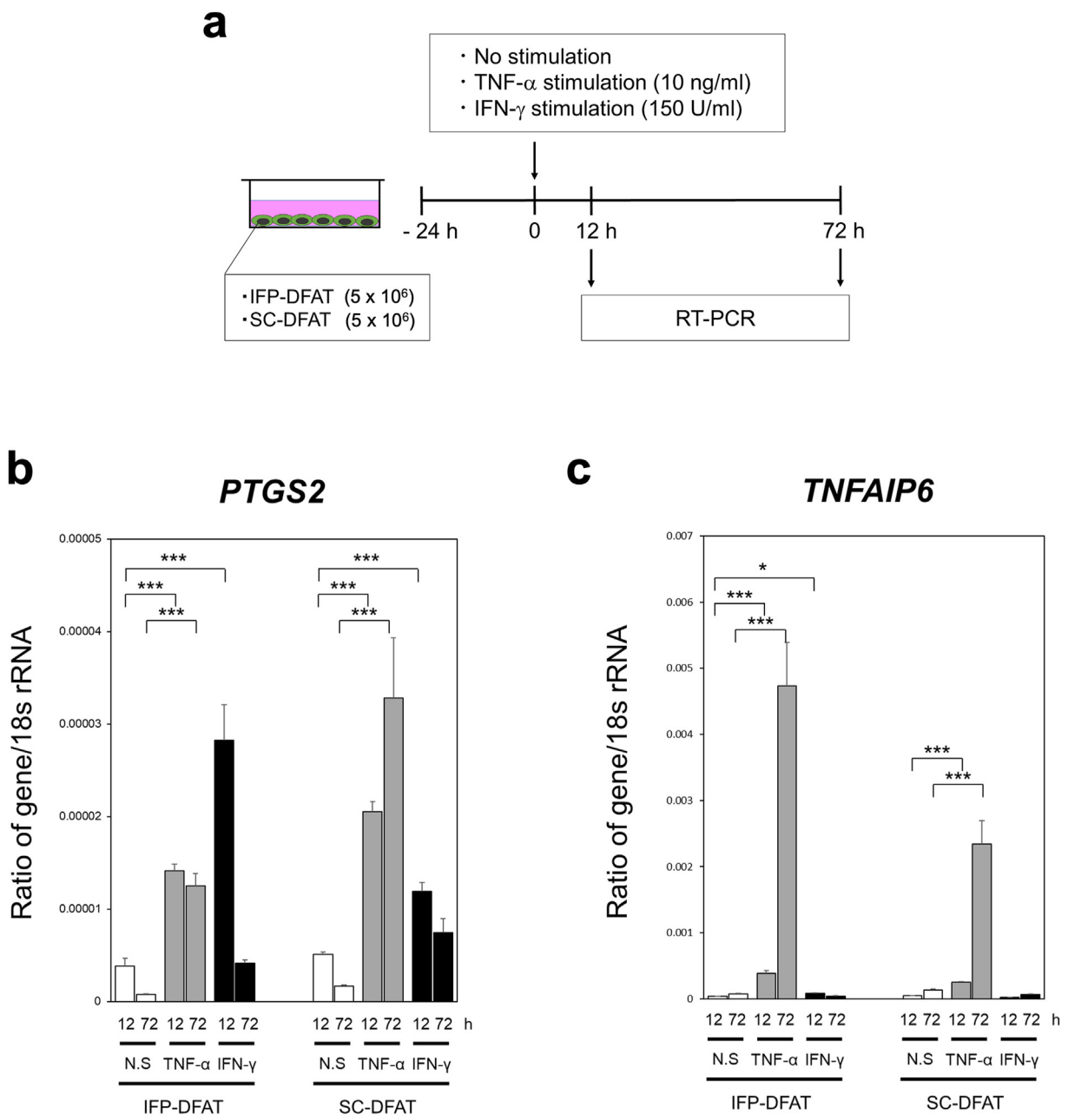

Fig. 4. DFATs expressed anti-inflammation-related genes in response to inflammatory cytokine stimulation. IFP-DFATs and SC-DFATs were incubated with or without 10 ng/ml TNF-α or 150 U/ml IFN-γ for 12–72 h. Total RNA was extracted at the indicated time points, and gene expression of the anti-inflammation-related genes *PTGS2* and *TNFAIP6* was measured by real-time RT-PCR. (a) Experimental scheme for gene expression analysis in DFATs. (b, c) Expression levels of *PTGS2* (b) and *TNFAIP6* (c) in each type of DFATs. Data are presented as mean ± SD. *P < 0.05 and ***P < 0.001. N.S.: no stimulation.

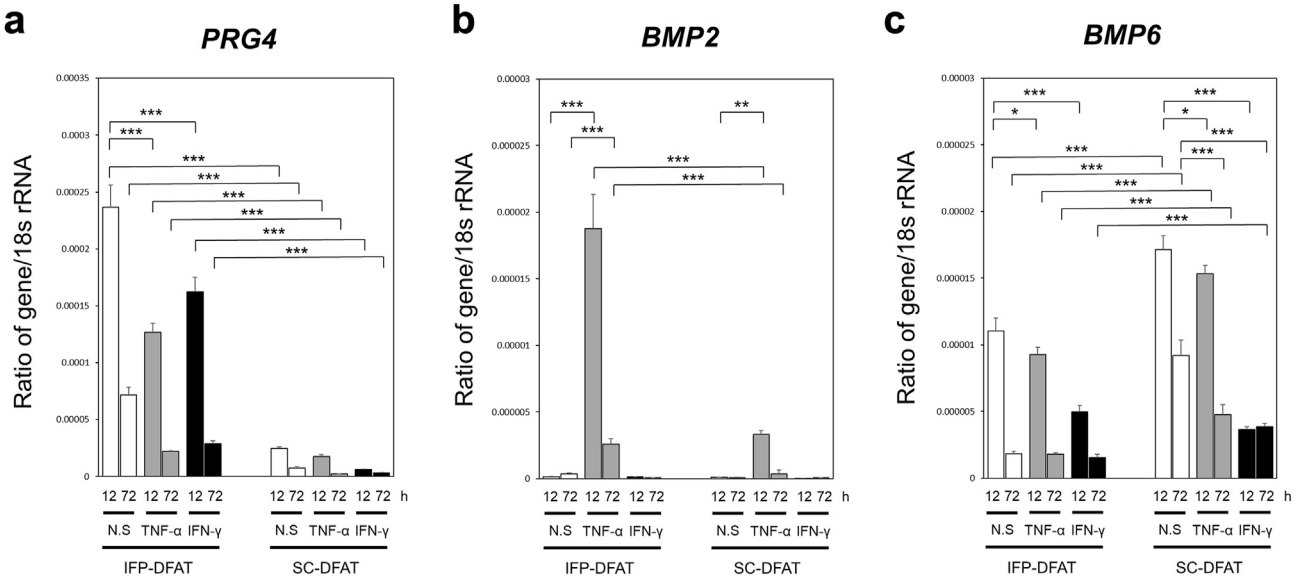

Fig. 5. DFATs expressed chondroprotective genes. IFP-DFATs and SC-DFATs were incubated with or without 10 ng/ml TNF-α or 150 U/ml IFN-γ for 12–72 h. Total RNA was extracted at the indicated time points, and gene expression of chondroprotective genes *PRG4, BMP2, and BMP6* was measured by real-time RT-PCR. (a, b, c) Expression levels of *PRG4* (a), *BMP2* (b), *and BMP6* (c) in each type of DFATs. Data are presented as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001. N.S.: no stimulation.

3.2. DFATs exhibit the upregulation of genes associated with anti-inflammatory responses upon stimulation with inflammatory cytokines

To elucidate the mechanisms underlying the suppression of cartilage degeneration by DFATs, we next examined in vitro the expression of genes associated with anti-inflammatory and chondroprotective properties in human DFATs. As our previous study had shown the superior potential of IFP-DFATs for chondrogenic differentiation in comparison to SC-DFATs [24], we evaluated the gene expression profiles of both IFP-DFATs and donor-matched SC-DFATs in this experimental context (Fig. 3a). We initially confirmed comparable efficacy in preparing DFATs from both the IFP and SC depots (Fig. 3b, c). Subsequently, both types of cells were either stimulated or left unstimulated with TNF-α or IFN-γ, followed by assessment of gene expression levels using RT-PCR (Fig. 4a). The expression of *PTGS2*, a mediator of immune regulation, significantly increased in both types of DFATs following stimulation with TNF-α and IFN-γ (Fig. 4b). The expression of *TNFAIP6*, which is associated with anti-inflammatory effects, was significantly upregulated in both types of DFATs, especially at 72 h post-stimulation with TNF-α (Fig. 4c). The expression of *PRG4*, a type of proteoglycan involved in cartilage protection, was observed in both types of DFATs under both non-stimulated and cytokine-stimulated conditions (Fig. 5a). Interestingly, the expression level of *PRG4* in IFP-DFATs was significantly higher than that in SC-DFATs, regardless of either cytokine-stimulated and unstimulated conditions. In IFP-DFATs, the expression of *PRG4* decreased at 12 h following stimulation with TNF-α or IFN-γ. The expression of *BMP2*, a cytokine associated with cartilage homeostasis and repair, significantly increased in both IFP-DFATs and SC-DFATs, especially at 12 h after post-stimulation with TNF-α (Fig. 5b). The expression of *BMP2* in IFP-DFATs was significantly higher than that in SC-DFATs at 12 and 72 h after stimulation with TNF-α. The expression of *BMP6*, another cytokine associated with cartilage protection, was observed in both types of DFATs under both non-stimulated and cytokine-stimulated conditions (Fig. 5c). Notably, the expression level of *BMP6* in SC-DFATs was significantly higher than that in IFP-DFATs, regardless of cytokine stimulation or its absence. The expression level of *BMP6* in both types of DFATs decreased at 12 h following stimulation with TNF-α or IFN-γ. Collectively, these findings indicate that both types of DFATs possess anti-inflammatory and chondroprotective effects, although there are certain disparities in the expression levels of chondroprotective genes.

3.3. DFATs exert a suppressive effect on ADAMTS4 expression in SFs

Because humoral factors released from synovial tissue in OA patients have been shown to promote cartilage degeneration, we hypothesized that intra-articularly administrated DFATs could inhibit cartilage degeneration through their interaction with synovial cells in patients. Therefore, we next examined whether DFATs modulate the expression of genes associated with cartilage degeneration in SFs through in vitro coculture experiments. The experimental design is shown in Fig. 6a. *ADAMTS4*, also known as aggrecanase-1, was found to be expressed under steady-state conditions in both N-SFs and OA-SFs, and its expression was significantly suppressed upon coculture with both IFP-DFATs and SC-DFATs (Fig. 6b left panel). TNF-α stimulation increased expression of *ADAMTS4* in both types of SFs, but this expression was also suppressed by coculture with both types of DFATs (Fig. 6b right panel). The expression of *IL-6*, a pro-inflammatory cytokine implicated in cartilage degeneration, was also detected in both types of SFs under steady-state conditions. Coculture with both types of DFATs significantly suppressed the expression of *IL-6* in N-SFs (Fig. 6c left panel). TNF-α stimulation also induced the expression of *IL-6* in both types of SFs, and this expression was not affected by coculture with either type of DFATs (Fig. 6c right panel). These findings suggested that the both IFP-DFATs and SC-DFATs possess inhibitory effects on the expression of factors associated with cartilage degenerative in SFs.

4. Discussion

This is the first study, to our knowledge, to show the chondroprotective effects of DFAT transplantation in an experimental animal model of knee OA. To assess the effects of cell transplantation, the rat ACLT + MMx model was used as a paradigm for knee OA. This model has been reported to exhibit irreversible

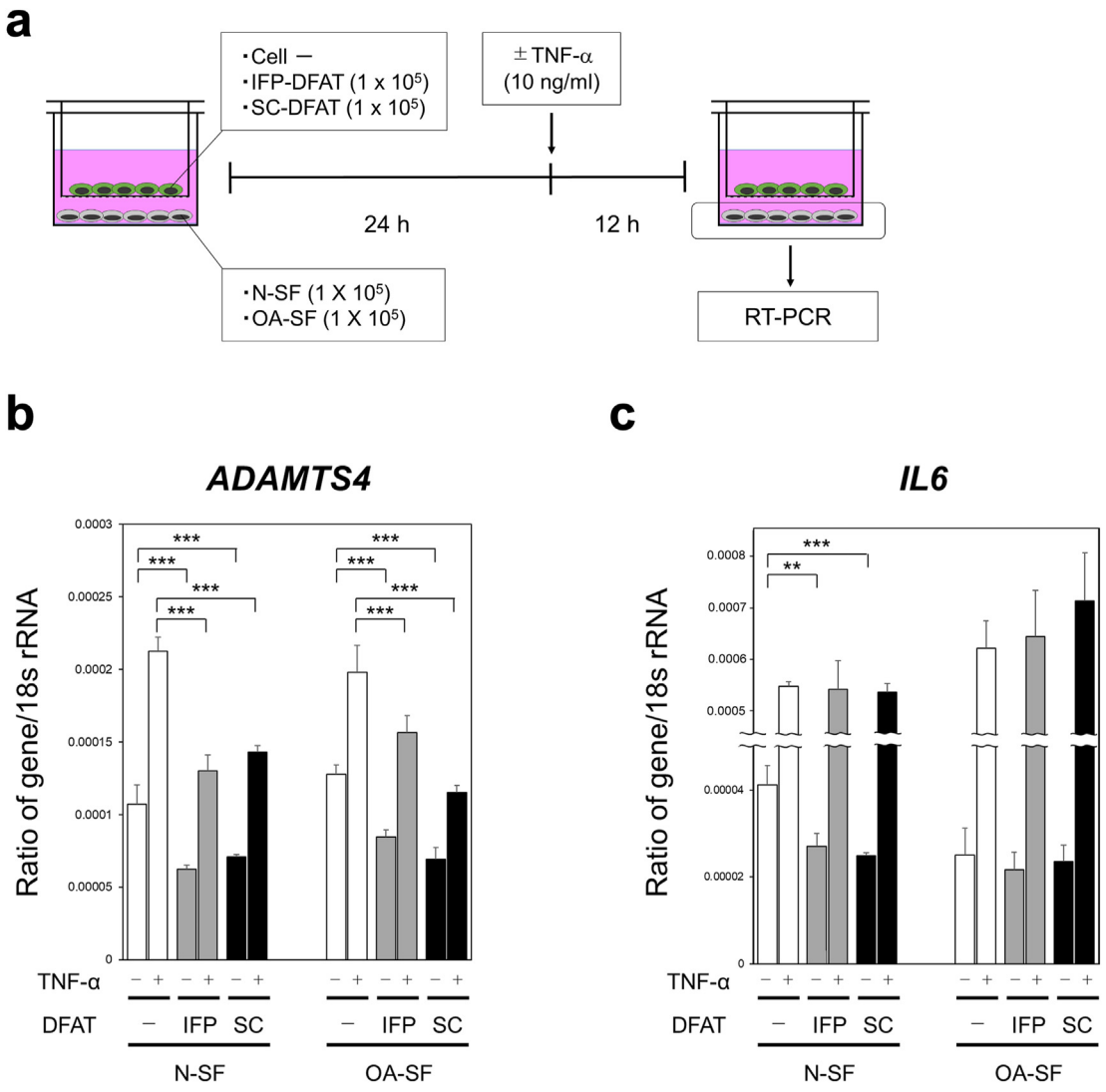

Fig. 6. Co-culture of DFATs decreased the expression of cartilage degeneration related genes in synovial fibroblasts (SFs). Human SFs derived from normal donors (N-SF) or from OA patients (OA-SF) were co-cultured with IFP-DFATs or SC-DFATs for 24 h using a trans-well culture system. The cells were then stimulated with or without 10 ng/ml TNF-α for 12 h and analyzed for the expression of cartilage degeneration-related genes *ADAMTS4* and *IL6* by real-time RT-PCR. (a) Experimental scheme for gene expression analysis in SFs co-cultured with DFATs. (b, c) Expression levels of *ADAMTS4* (b) and *IL6* (c) in N-SFs and OA-SFs. Data are presented as mean ± SD. **P < 0.01 and ***P < 0.001.

lesions similar to human OA, including articular cartilage degradation, subchondral bone sclerosis, and osteophyte formation. Notably, cartilage degradation occurs at a more accelerated rate in this model compared to the ACLT alone model [20]. Through careful observation, we confirmed that the temporal progression after ACLT + MMx treatment closely followed previously reported findings [20], with reproducible thinning of articular cartilage and irreversible progression within 5 weeks of treatment. It has been noted that the number of MSCs transplanted into the knee joint cavity diminishes rapidly within two weeks [26,27]. Therefore, we performed DFAT transplantation four times at weekly intervals to evaluate the therapeutic efficacy of DFATs for OA. The results showed that the DFAT group displayed attenuated articular cartilage thinning compared to the control group and was accompanied by a significant reduction in pathological OA scores. These findings suggest that DFATs may have a potent inhibitory effect on cartilage degradation, making them a promising potential therapeutic intervention for OA.

To investigate the underlying mechanism of action of DFATs in the context of inflammatory conditions, we performed an analysis of gene expression related to cartilage protection with DFATs upon stimulation with TNF-α or IFN-γ. We observed robust induction of gene expression for *PTGS2* and *TNFAIP6* in response to TNF-α, whereas *PTGS2* expression was also stimulated by IFN-γ in both IFP- and SC-DFATs (Fig. 4b). *PTGS2* encodes for prostaglandin E2 (PGE2), which serves as an important factor in the anti-inflammatory effects of MSCs on OA [25]. Contrastingly, *TNFAIP6* encodes for TSG-6, which is known for its anti-inflammatory properties. It has been reported that transplanted MSCs reduce inflammatory damage mainly via the secretion of TSG-6 in animal models of acute myocardial infarction, peritonitis, and corneal injury [28–30]. Our previous study showed that intravenous injection of DFATs ameliorated immunological glomerulonephritis via increased secretion of TSG-6 [14]. Notably, TSG-6 has been shown to suppress the expression of inflammatory cytokines such as IL-6 and IL-1β, as well as cartilage matrix-degrading enzymes such as MMP-9 [30]. These biological actions of TSG-6 are likely to contribute to its inhibitory effects on cartilage degradation.

We showed that DFATs express several genes associated with cartilage protection, including *PRG4, BMP2,* and *BMP6*. PRG4

represents a synovial glycoprotein that regulates the expression of various OA-associated catabolic enzymes and inhibits synovial overgrowth and cartilage destruction [31]. Notably, the intra-articular administration of recombinant PRG4 inhibited cartilage degeneration in a rat model of OA [32]. BMP2 and BMP6 play an important role in articular chondrogenesis [33–35]. In an OA rat model, the transplantation of synovial MSCs into the joint cavity led to the suppression of cartilage degradation, concomitant with increased gene expression of MSC-derived *TSG-6*, *PRG4*, *BMP2, BMP6,* and *TIMP-1* (tissue inhibitor of metalloproteinase 1) at the transplantation site [21]. Our RT-PCR data suggest that the inhibitory effect of DFATs on cartilage degradation is also mediated by the paracrine action of these trophic factors, as in the case of MSCs. Interestingly, our data revealed that IFP-DFATs showed higher *PRG4* expression and lower *BMP6* expression compared to SC-DFATs (Fig. 5a, c). IFP-DFATs also showed a significantly higher induction of *BMP2* expression by TNF-α stimulation compared to SC-DFATs (Fig. 5b). These findings suggest that IFP-DFATs and SC-DFATs may exert therapeutic activity in OA through different mechanisms.

It is known that cartilage matrix degrading enzymes and pro-inflammatory cytokines secreted by SFs and chondrocytes play an important role in the progression of cartilage degradation in OA patients. Indeed, we observed that stimulation of cultured SFs derived from healthy subjects or OA patients with TNF-α increased the expression of *ADAMTS4* and *IL6* (Fig. 6b, c). ADAMTS4 is a major member of the ADAMTS metalloproteinase family that degrades aggrecan and promotes cartilage degradation [36]. IL-6 is a type of pro-inflammatory cytokine and has been implicated in the progression of OA [37]. Our co-culture experiments showed that both types of DFATs suppressed *ADAMTS4* expression regardless of the origin of the SFs (healthy subjects or OA patients) or the presence of TNF-α stimulation. These results suggest that both IFP- and SC-DFATs have an inhibitory effect on chondrogenic degradation in OA patients via suppression of ADAMTS4 expression from the patients' damaged SFs. However, the inhibitory effect of DFATs on IL-6 expression was only observed in SFs from healthy subjects without TNF-α stimulation, suggesting that the therapeutic effect of DFATs may be limited in IL-6-mediated pathological conditions. Further studies are needed to clarify the difference in therapeutic effect and mechanism of action between IFP-DFATs and SC-DFATs in OA.

This study has several limitations in this study. First, the mechanism of action of intra-articular administration of DFATs on OA, as well as the temporal kinetics and localization changes of the cells, remains unclear. In the case of synovial MSCs, it has been shown that transplanted cells migrate to the synovial membrane and promote anti-inflammatory effects through TSG-6 expression, increased lubrication through PRG-4 expression, and cartilage matrix synthesis through BMP expression, which is the mode of action for OA [38]. Second, although the present study has evidenced that DFATs suppress cartilage degradation, it remains unclear whether DFATs also suppress local inflammation and pain associated with OA. Further studies using appropriate animal models such as a monoiodoacetic acid-induced OA model, in which local inflammation and pain can be evaluated, are needed. Third, it remains unclear how much advantage DFATs have over other cell types as a cell source for the treatment of OA. Our group has previously shown that DFATs are less susceptible to donor age and underlying diseases [10]. Furthermore, DFATs have a greater capacity for chondrogenic differentiation than ASCs [16]. Future human cell transplantation experiments in immunodeficient animals will be needed to identify the type of cells with the best therapeutic effect for OA. Moreover, it is desirable to compare the therapeutic effects of IFP-DFATs and SC-DFATs through in vivo transplantation studies. This study showed that IFP-DFATs exhibit higher expression of *PRG4* and *BMP2* compared to SC-DFATs. Our previous report showed that IFP-DFATs have a more robust capacity for chondrogenic differentiation than SC-DFATs [24]. These findings suggest that IFP-DFATs possess enhanced therapeutic activity for OA compared to SC-DFATs. Due to difficulties in collecting IFP from rats and inability to prepare rat IFP-DFATs, clinical trials or preclinical animal studies using human IFP-DFATs would be required.

5. Conclusions

Our study showed that intra-articular injections of DFATs significantly inhibited cartilage degeneration in a rat model of OA induced by ACLT + MMx. DFATs expressed genes associated with cartilage protection such as *PRG4* and *BMP6* and induced the upregulation of genes involved in immune regulation, anti-inflammation, and cartilage protection, such as *PTGS2*, *TNFAIP6*, and *BMP2*, upon stimulation by inflammatory cytokines. Co-culturing with DFATs suppressed the expression of *ADAMTS4* and *IL6* in SFs. These results suggest that DFATs may possess a therapeutic capacity to inhibit cartilage degradation and would present themselves as a compelling cellular source for the treatment of OA.

Conflict of interest disclosure statement

The authors declare that there is no conflict of interests in this article.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.05.006.

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