The role of EDIL3 in maintaining cartilage extracellular matrix and inhibiting osteoarthritis development

a potential novel therapy

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Aims

Therapeutic agents that prevent chondrocyte loss, extracellular matrix (ECM) degradation, and osteoarthritis (OA) progression are required. The expression level of epidermal growth factor (EGF)-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) in damaged human cartilage is significantly higher than in undamaged cartilage. However, the effect of EDIL3 on cartilage is still unknown.

Methods

We used human cartilage plugs (ex vivo) and mice with spontaneous OA (in vivo) to explore whether EDIL3 has a chondroprotective effect by altering OA-related indicators.

Results

EDIL3 protein prevented chondrocyte clustering and maintained chondrocyte number and SOX9 expression in the human cartilage plug. Administration of EDIL3 protein prevented OA progression in STR/ort mice by maintaining the number of chondrocytes in the hyaline cartilage and the number of matrix-producing chondrocytes (MPCs). It reduced the degradation of aggrecan, the expression of matrix metalloproteinase (MMP)-13, the Osteoarthritis Research Society International (OARSI) score, and bone remodelling. It increased the porosity of the subchondral bone plate. Administration of an EDIL3 antibody increased the number of matrix-non-producing chondrocytes (MNCs) in cartilage and exacerbated the serum concentrations of OA-related pro-inflammatory cytokines, including monocyte chemotactic protein-3 (MCP-3), RANTES, interleukin (IL)-17A, IL-22, and GROα. Administration of β1 and β3 integrin agonists (CD98 protein) increased the expression of SOX9 in OA mice. Hence, EDIL3 might activate β1 and β3 integrins for chondroprotection. EDIL3 may also protect cartilage by attenuating the expression of IL-1β-enhanced phosphokinase proteins in chondrocytes, especially glycogen synthase kinase 3 alpha/beta (GSK-3α/β) and phospholipase C gamma 1 (PLC-γ1).

Conclusion

EDIL3 has a role in maintaining the cartilage ECM and inhibiting the development of OA, making it a potential therapeutic drug for OA.

Bone & Joint Research

Article focus

- We investigated the impact of EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) on cartilage.
- We used human cartilage plugs (ex vivo) and mice with spontaneous osteoarthritis (OA) (in vivo) to explore whether EDIL3 has a chondroprotective effect, by altering OA-related indicators, including histological analysis, Osteoarthritis Research Society International (OARSI) score, chondrocyte count, micro-CT analysis, OA-related cytokine measurement, and downstream signalling pathways.

Key messages

- EDIL3 prevents chondrocyte clustering and maintains chondrocyte number and SOX9 expression in human cartilage plugs.
- Administration of EDIL3 protein prevented OA progression in STR/ort mice, including maintaining the cartilage extracellular matrix and reducing the expression of matrix metalloproteinase (MMP)-13 and the OARSI score.
- EDIL3 may activate β1 and β3 integrins and attenuate interleukin (IL)-1β-enhanced glycogen synthase kinase 3 alpha/beta (GSK-3α/β) and phospholipase C gamma 1 (PLC-γ1) phosphokinase proteins in chondrocytes to protect cartilage.

Strengths and limitations

- We used a human cartilage plug and STR/ort mice to evaluate the therapeutic effect of OA. This is a more reliable research model than in vitro experiments.
- It is difficult to explore the possible mechanism of EDIL3 cartilage protection using animal experiments.

Introduction

The rapid ageing of the human population is a global problem. The prevalence of bone- and joint-related diseases, such as osteoarthritis (OA), increases markedly with age,[1] affecting 10% to 15% of older adults (> 60 years old) worldwide.[2,3] It is a degenerative joint disease caused by the breakdown of cartilage covering the ends of the bone joints due to chronic inflammation, leading to localized pain and limited joint movement.[2-4] It results in disability and reduced quality of life. OA treatment aims to reduce pain, maintain or improve joint function, and minimize disability.[5] Artificial joint replacement is currently the only definitive solution.[6]

In healthy individuals, chondrocytes in the articular cartilage maintain a dynamic balance between the generation and degradation of the extracellular matrix (ECM), including collagen, proteoglycans, and aggrecan.[7-9] During the early progression of OA, disruption of ECM balance ultimately leads to the catabolic rate exceeding the anabolic rate.[2,10] This induces inflammation and increases the number of reactive oxygen species (ROS). The resulting high oxidative stress can cause cell death and ECM destruction. In clinical OA, this is accompanied by articular cartilage inflammation, pain, and disability.[11] According to recent studies, many long non-coding RNAs (lncRNAs) and circular RNAs are differentially expressed in various pathological processes of OA, such as ECM degradation.[12-14] The organized molecular crosstalk of ligand-receptor pairs in OA and strategies to interfere with candidate ligands and receptors in OA pathophysiology may help to discover molecular targets for future OA therapy.[15-18]

In addition, according to the American College of Rheumatology guidelines, knee OA should be managed with a combination of exercise and lifestyle changes, analgesics, non-steroidal anti-inflammatory drugs, and corticosteroid injections.[19-21] However, pharmacological therapies provide only short-term benefits and cause side effects.[22-25] Therefore, novel molecular targets that can be used to reduce chondrocyte loss during OA treatment are urgently needed.

Proteomic and secretome analysis is the global profile of secreted proteins.[26] Secreted proteins are promising therapeutic targets that can be blocked using antibodies. We performed large-scale secretome analyses to compare OA and control chondrocytes. We identified a secreted protein, EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3), which was significantly increased in damaged versus non-damaged cartilage.

EDIL3 is a 52-kilodalton glycoprotein, also referred to as developmental endothelial locus-1 (Del-1). EDIL3 protects chondrocytes from apoptosis. This effect is mediated primarily through the integrin αVβ3.[27] EDIL3 knockout mice develop severe OA associated with increased susceptibility of chondrocytes to apoptosis.[28] EDIL3 also inhibits osteoclastogenesis and inflammatory bone loss in nonhuman primates.[29] Thus, it represents a new class of endogenous therapeutics that can be used to manage inflammation-associated bone loss. Hence, EDIL3 is a potential therapeutic target for OA. However, the role of EDIL3 in the maintenance of hyaline cartilage remains unclear. Thus, in this study, we investigated the impact of EDIL3 on cartilage, using various clinically relevant resources including human OA cartilage plugs, primary chondrocytes, and STR/ort spontaneous OA mouse models. We believe that this will facilitate the development of novel strategies for the treatment of OA and contribute to the field of chondrocyte biology.

Methods

Histological assessment and culture of human OA cartilage plugs

This study was approved by the Institutional Review Board (IRB) of Chang Gung Memorial Hospital. Informed consent was obtained from all patients prior to cartilage collection. The OA cartilage specimens were harvested from the femoral condyles of patients who underwent TKA. For Figure 1, cartilage tissues were fixed for seven days in 10% neutral buffered formalin, decalcified for two weeks in a rapid decalcifier solution, and embedded in paraffin. Then 5 μm sections were cut and mounted for histological and immunoflourescence (IF) staining. For Figure 2, fresh cartilage plugs were cut into pieces of approximately 8 mm^3 in volume using a Premier Uni-Punch Disposable Biopsy trephine (8 mm diameter; Premier Medical, USA). Osteochondral explants were obtained, and the bone was flushed with phosphate-buffered saline (PBS) to remove all bone marrow and blood cells.[30] Plugs were then cultured overnight in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 1% fetal bovine serum (FBS; both Gibco, Thermo Fisher Scientific, USA) at 5% CO_2 and 37°C. The next morning, plugs were treated with either vehicle PBS or recombinant EDIL3 protein (2 μg/ml, 6046-ED; R&D Systems,

USA). After 48 hours and six days, the plugs were fixed in 10% neutral-buffered formalin and used for histological analysis.

Safranin O and immunofluorescence staining

Bone samples were incubated in a rapid decalcification solution, trimmed, and embedded in paraffin. The 5 μm-thick sections were stained with: 1) Safranin O; 2) aggrecan fragments (1:100, AF1220; R&D Systems); 3) EDIL3 (1:50, sc-293337; Santa Cruz Biotechnology, USA); 4) SOX9 (1:50, sc-166505; Santa Cruz Biotechnology); and 5) matrix metalloproteinase (MMP)-13 (1:100, GTX100665; GeneTex, USA). For Safranin O staining, sections were incubated in Weigert's iron haematoxylin (HT1079), 0.08% fast green (F7252), and 0.1% Safranin O solution (S2255; all MilliporeSigma, USA). Primary antibodies against aggrecan fragments, SOX9, and MMP-13 were used for immunofluorescence (IF) staining. Samples were subsequently incubated with a secondary antibody, namely Alexa Fluor 488-conjugated anti-goat immunoglobulin G (IgG) (1:500, SA5-10086), Alexa Fluor 488-conjugated anti-mouse IgG (1:500, R37114), and Alexa Fluor 488-conjugated anti-rabbit IgG (1:500, A21206; Invitrogen, Thermo Fisher Scientific, USA), for 60 minutes at 25°C. Then 4',6-diamidino-2-phenylindole (DAPI) staining was used for nuclear staining (1:1,000, D1306; Invitrogen). Safranin O-stained slides were digitalized using a NanoZoomer S360 digital slide scanner (Hamamatsu Photonics, Japan). Images were acquired using a microscope (DFC7000 T; Leica Microsystems, Germany). IF slides were digitized using a TissueFAXS System (TissueGnostics, Austria) coupled onto a Zeiss Axio Imager Z1 microscope (Zeiss, Jena, Germany). Image acquisition was performed using the TissueFAXS System (TissueGnostics). The IF signal occurred around the articular cartilage. We used TissueQuest software (TissueGnostics) to quantify the fluorescent signal.

Animal studies

All animal procedures complied with the guidelines established by the National Institutes of Health (USA), and were approved by the Institutional Animal Care and Use Committee. In addition, the ARRIVE guidelines for animal research and submission of studies were followed, and an ARRIVE checklist is included in the Supplementary Material.[31] STR/ort mice acquired from RIKEN BioResource Research Center (Japan) were maintained in environmentally controlled rooms (controlled room temperature: 25°C ± 1°C) and subjected to 12-hour light-dark cycles. Male STR/ort mice were randomly divided into four groups comprising five to six mice each. Past studies have demonstrated that the incidence of degenerative joint disease is higher in male mice than in female mice, and that the medial condyle is much more affected than the lateral condyle.[32,33] Therefore, male mice were used in this study.

The STR/ort mice were administered tail-vein injections of 1 μg/mouse anti-EDIL3 IgG1 (MABS1976; MilliporeSigma), 2 μg/mouse recombinant EDIL3 protein (6046-ED; R&D Systems), 2 μg/mouse CD98 protein (50813-M07H; Sino Biological, China), 1 μg/mouse isotype-matched control IgG1 monoclonal antibody (mAb) (mabg1-ctrlm; InvivoGen, USA), or vehicle PBS solution. Mice were anaesthetized using an intraperitoneal injection of a 0.01 ml/kg mixture (1:1 v/v) of tiletamine-zolazepam (Zoletil 50, France) and xylazine hydrochloride (Rompun; Bayer HealthCare, Germany), and then euthanized. The tibiofemoral joint was immediately fixed in 10% formaldehyde, subjected to micro-CT, incubated in a rapid decalcifier solution, trimmed, and embedded in paraffin. Each protocol was performed by an investigator (KLC) who was blinded to the experimental conditions.

Osteoarthritis Research Society International score

Damage to the knee joint cartilage was scored by two blinded observers (KLC, MFC) using the Osteoarthritis Research Society International (OARSI) scoring system described by Glasson et al.[34] Serial coronal sections (5 μm) obtained from the right and left knee were stained by safranin-O. Blinded observers (KLC, MFC) scored the cartilage pathology in all four quadrant compartments (medial femoral condyle (MFC), lateral femoral condyle (LFC), medial tibial plateau (MTP), and lateral tibial plateau (LTP)) of the histological sections.

Micro-CT bone imaging

Non-destructive ultrastructural bone analysis was performed using a SkyScan 1176 micro-CT scanner (Bruker, Belgium). Samples were wrapped in saline-soaked gauze and subsequently scanned with the following parameters: pixel size of 9 μm, voltage at 60 kVp, current at 417 μA, filter as 0.5 mm aluminium, and exposure time of 1,000 ms. The cross-sectional images were reconstructed using GUP-NRecon software (version 1.7.4.6; Bruker, Belgium) and analyzed with the Skyscan CTAn programme (version 1.20.8.0; Bruker). The articular cartilage was further analyzed by distinguishing specific regions of the subchondral bone, namely epiphyses. Epiphyseal trabeculae from the subchondral bone plate were automatically isolated using the CTAn software. Subchondral bone plate thickness (Pl.Th), trabecular parameters (trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp)), bone volume (BV/TV), and total porosity (Po(tot)) in the MTP and LTP were calculated using the CTAn software. Illustrative images were obtained using SkyScan DataViewer (version 1.6.0.0; Bruker). These methods were also reported in the study by Chen et al.[32]

Cytokine detection

Serum cytokine concentration was measured using mouse 48-plex ProcartaPlex Panel (EPX480-20834-901; Invitrogen). Luminex xMAP magnetic-bead fluorescent immunoassays (Invitrogen) were performed on MAGPIX (MilliporeSigma). Measurements were conducted according to the manufacturer's protocol. Mouse serum was added to the plate containing the mixed antibody-linked beads and incubated for two hours at 25°C. Each sample was measured in singlets. The reading beads were placed on a Luminex 100/200 instrument with a lower bound of 100 beads per sample per cytokine/chemokine.

Isolation, culture of chondrocytes, and C20A4 cells

C20A4 cells were cultured in DMEM containing 10% FBS. Three types of small interfering RNA (siRNA), which covered different regions of the human EDIL3 (sense 5'-GGUGAUAUUUGUGAUCCCAtt-3', antisense 5'-ttCCACUAUAAACACUAGGGU-3'; sense 5'-GUGGAAUUAAUAUCAAACCAtt-3', antisense 5'-ttCACCUUAAUAUAGUUUGGU-3'; sense 5'-CAUCUAUGCACGACACAUAtt-3', antisense 5'-ttGUAGAUACGUGCUGUGUAU-3'), and the non-specific scrambled control siRNA (sense 5'-UUCUCCGAACGUGUCACGUtt-3', 5'-ttAAGAGGCUUGCACAGUGCA-3')

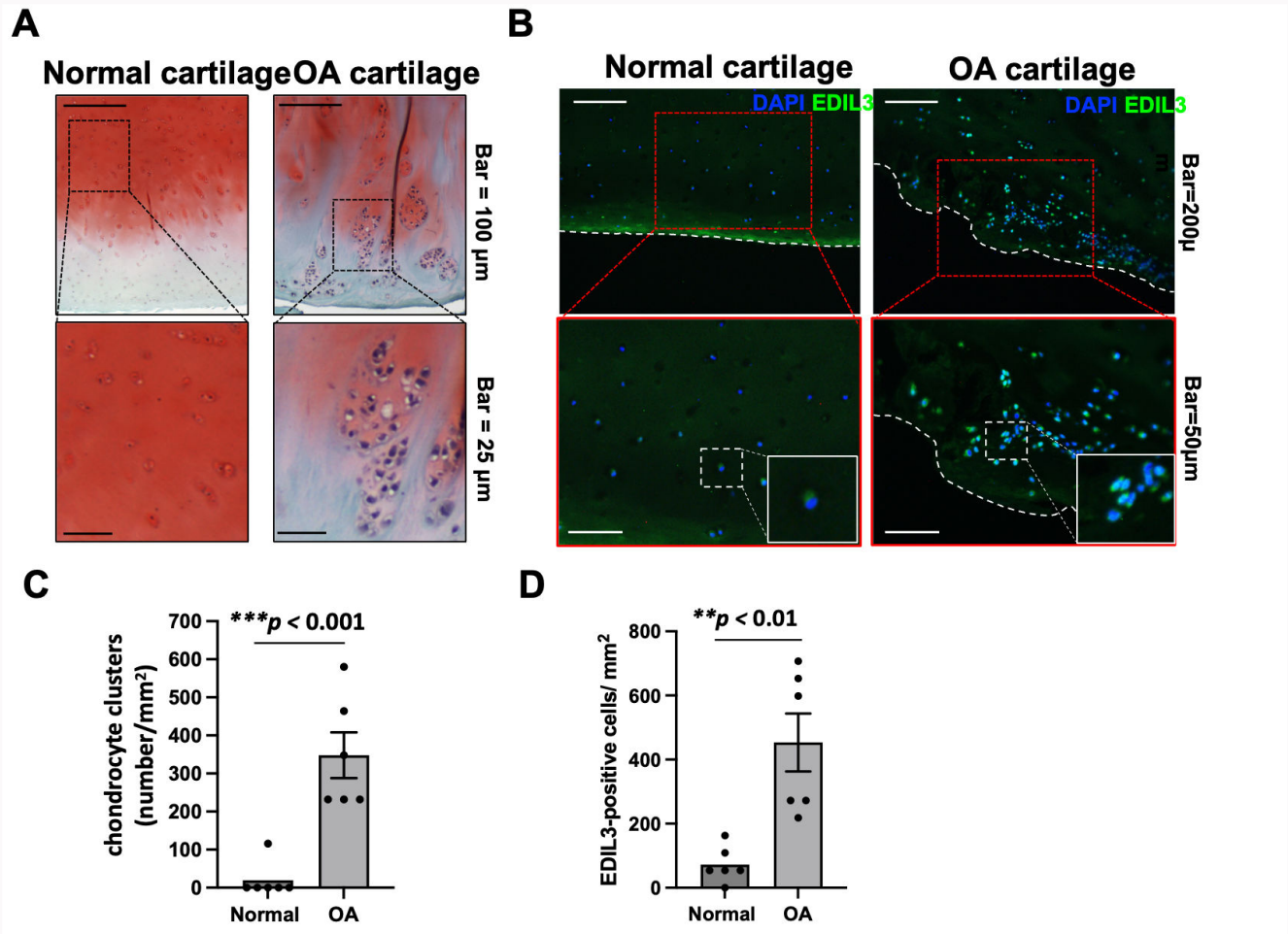

Fig. 1
The expression of EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) is higher in human osteoarthritis (OA) cartilage compared with normal cartilage. Paraffin tissue sections were established from both pathological and normal cartilage. Safranin O and immunofluorescence staining were performed for the human articular cartilage sections. a) and b) Compared with normal cartilage, OA cartilage demonstrated chondrocyte cell clustering, empty lacunae morphology, and increased EDIL3 fluorescence signal. c) and d) The chondrocyte cluster and EDIL3-positive cells in the articular cartilage were quantified. Data are presented as means and standard errors. **p < 0.05, ***p < 0.001; independent-samples *t*-test. DAPI, 4′ ,6-diamidino-2-phenylindole.

were custom-designed and synthesized by MDbio (Taiwan). A mixture containing the three types of EDIL3 siRNA and control siRNA was used to knockdown EDIL3 messenger RNA (mRNA). The control scrambled siRNA served as a negative control. siRNAs were introduced into cultured C20A4 cells using Lipofectamine 2000 (Invitrogen) in six-well culture dishes (Corning, USA) in accordance with the manufacturer's instructions. Human chondrocytes were isolated from OA cartilage specimens. The cartilage fragments were minced and digested with 0.15% type II collagenase at 37°C for 16 hours. The primary chondrocytes were resuspended in DMEM containing 20% FBS. All cells were maintained in a humidified incubator containing 5% CO_2 at 37°C.

Protein extraction and phosphokinase antibody array analysis

C20A4 cells were plated in a six-well plate (density of 2×10^5 cells per well) and cultured as previously described. The cells were divided into four experimental conditions: control group, interleukin (IL)-1β-treated group, IL-1β+ recombinant EDIL3 protein group, and recombinant EDIL3 protein group. IL-1β and recombinant EDIL3 protein were added in growth medium supplemented with 5 ng/ml and 2 μg/ml, respectively. After 24 hours of incubation, the C20A4 cell lysate was used to survey the phosphorylated proteins using a phosphokinase antibody array (#ARY003; R&D Systems). The signal intensities of the spots in the protein array were quantified using Image J software (National Institutes of Health). Total protein extraction was performed using radioimmunoprecipitation assay (RIPA) extraction buffer (MilliporeSigma), containing a complete protease inhibitor cocktail (no. 04693132001; Roche, Switzerland) and phosphatase inhibitor cocktails 2 (Invitrogen) and 3 (MilliporeSigma).

Statistical analysis

The results indicated in Figures 2b and 2d were analyzed using two-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test for selected pairs of groups. The data presented in Figures 3c to 3e were analyzed using one-way ANOVA followed by Tukey's multiple comparison test for selected pairs of groups. Data in Figure 3g were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. The data in Figure 4 were analyzed using two-way ANOVA followed by Tukey's multiple comparison test.

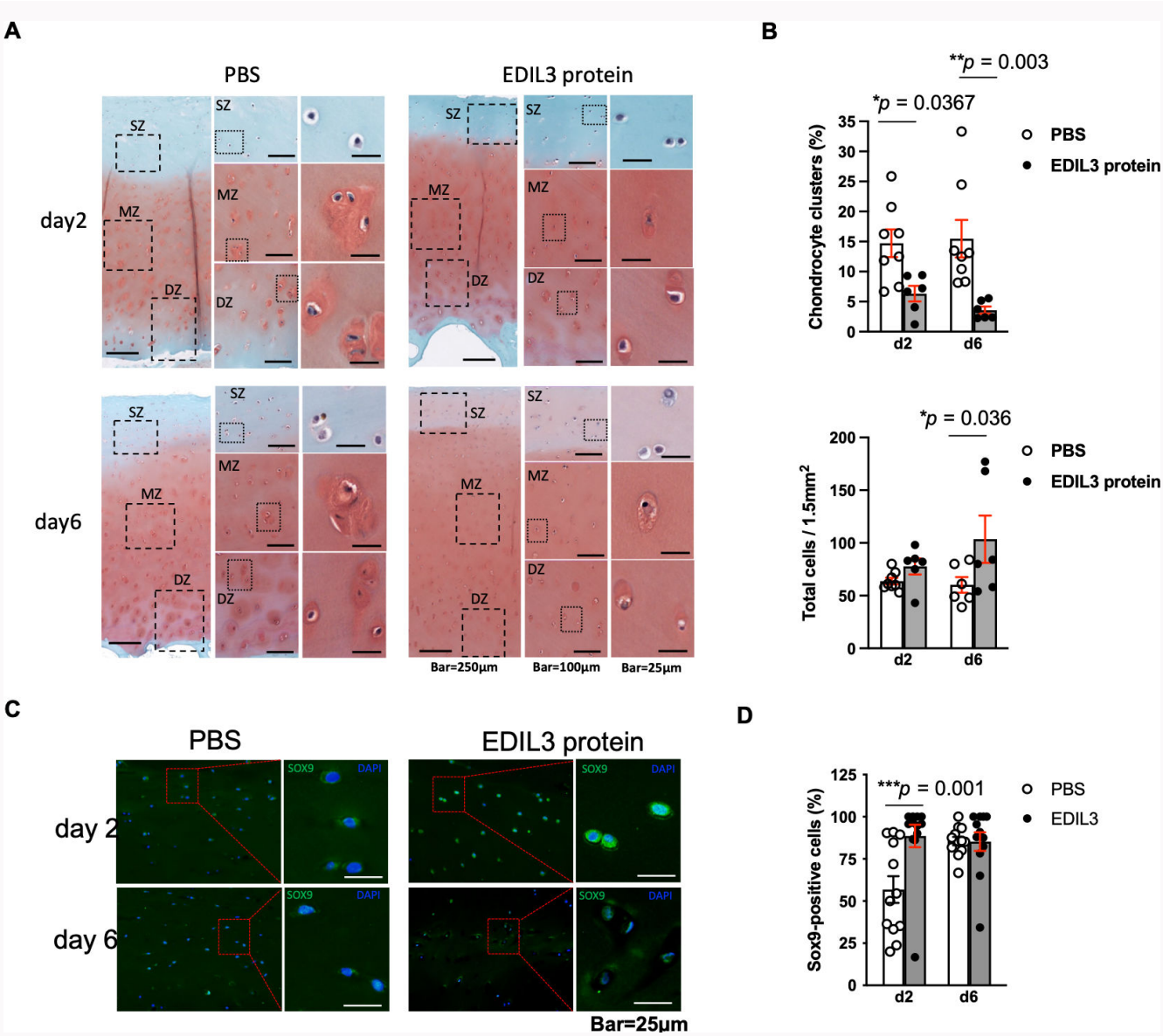

Fig. 2

EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) prevents chondrocyte clustering and chondrocyte loss, and maintains enhanced SOX9 expression in human osteoarthritis (OA) cartilage. a) Osteochondral plugs obtained from patients were cultured ex vivo and treated with phosphate-buffered saline (PBS) (vehicle) or recombinant EDIL3 proteins for six days. Representative images of Safranin O staining and magnified images of regions include the superficial zone (SZ), middle zone (MZ), and deep zone (DZ). Compared with the vehicle control group, the EDIL3 protein-treated group demonstrated prevention of chondrocyte clustering, cell loss, and lacunae morphology in SZ, MZ, and DZ regions. b) The percentage of clustered chondrocytes and chondrocyte number in the cartilage were quantified. The EDIL3 protein treatment reduces the severity of chondrocyte clustering and chondrocyte loss. c) and d) SOX9 protein is displayed in green, and 4′ ,6-diamidino-2-phenylindole-stained nuclei are in blue. The EDIL3 protein treatment increased the expression level of SOX9. Data are presented as means and standard errors. *p < 0.05, **p < 0.01, ***p < 0.001; two-way analysis of variance, followed by Bonferroni's multiple comparison test for selected pairs of groups for multiple comparisons.

The data presented in Figures 5c and 6f were analyzed using one-way ANOVA followed by Tukey's multiple comparison test for selected pairs of groups. Data in Figure 6c were analyzed using an independent-samples *t*-test. Statistical analyses were performed using GraphPad Prism version 9.3.1 (GraphPad Software, USA). Two-tailed p-values < 0.05 were considered statistically significant.

Results

Increased EDIL3 expression prevents chondrocyte loss in human OA cartilage

We collected cartilage tissues from patients undergoing TKA, and isolated primary chondrocytes for culture. We treated primary human chondrocytes with IL-1β and collected the culture medium at 12 and 24 hours. The culture medium was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis; we detected the expression level of many secreted proteins increased in IL-1β-stimulated human chondrocytes (data not presented). Among them, compared with the vehicle control group, EDIL3 demonstrated the highest expression in the IL-1β-stimulated group. We confirmed the expression level of EDIL3 protein in OA cartilage. Compared with normal cartilage, OA cartilage demonstrated severe chondrocyte clustering, empty lacunae morphology, and increased EDIL3 fluorescence signal (Figures 1a to 1d).

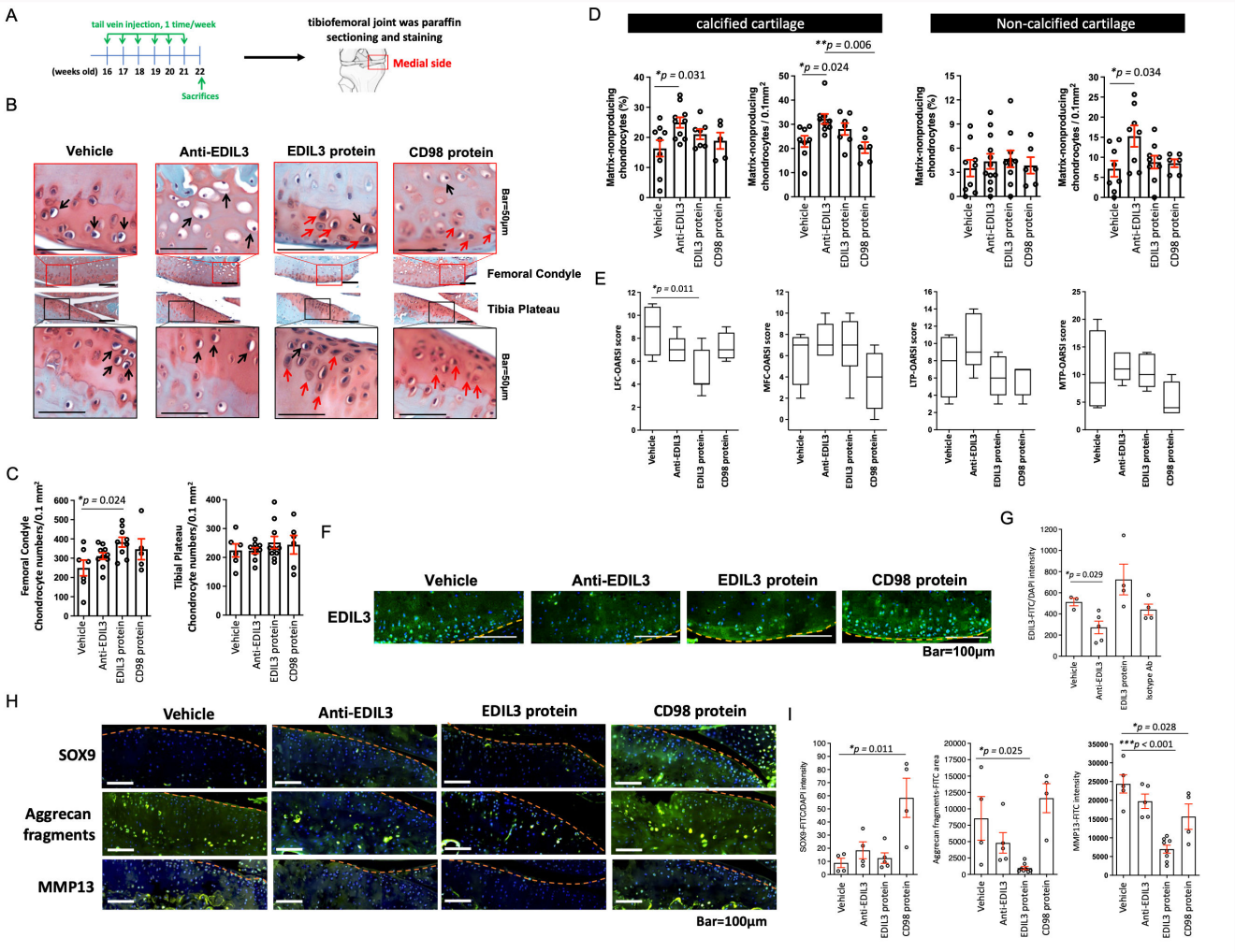

Fig. 3

EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) has beneficial effects on cartilage maintenance in mice with osteoarthritis (OA). a) Schematic representation of the timeline of the antibody drug or recombinant protein (EDIL3 or CD98) treatment during 16 to 21 weeks of age. STR/ort mice were injected weekly through the tail vein with phosphate-buffered saline (PBS, vehicle) combined with either the antibody or the protein for six consecutive weeks. Mice were killed at 22 weeks. This was followed by paraffin tissue sections and Safranin O and immunofluorescence (IF) staining in knee articular cartilage. b) Representative images include articular cartilage in the tibial plateau knee joints. Arrows indicate representative matrix-producing (red) and matrix-non-producing (black) chondrocytes. c) The total chondrocyte number in the articular cartilage was quantified. EDIL3 protein treatment increased the number of chondrocytes. d) The number and percentage of matrix-non-producing chondrocytes (MNCs) in the articular cartilage were quantified. EDIL3 antibody treatment increased the number of MNCs. e) EDIL3 protein treatments decreased the Osteoarthritis Research Society International (OARSI) score in the LFC. f) Representative images of IF staining (green) in whole articular cartilage obtained from STR/ort mice for the EDIL3; 4',6-diamidino-2-phenylindole (DAPI) (blue) stained nuclei; orange dashed lines define the cartilage region. EDIL3 antibody treatments decreased the EDIL3 contents in the cartilage region. g) The fluorescence of EDIL3 was quantified in the whole articular cartilage region. EDIL3 antibody significantly decreased EDIL3 expression. h) Representative images of IF staining (green) in whole articular cartilage obtained from STR/ort mice for the indicated OA markers; DAPI (blue) stained nuclei; orange dashed lines define the cartilage region. i) Fluorescence was quantified in the whole articular cartilage region. Data are presented as means and standard errors in Figures 3c, 3d, 3g, and 3i. Data are presented as mean and maximum with 95% confidence intervals in Figure 3e. CD98 protein significantly increased SOX9 expression, and EDIL3 protein significantly reduced aggrecan fragments. LTP, lateral tibial plateau; MFC, medial femoral condyle; MMP, matrix metalloproteinase; MTP, medial tibial plateau. Data presented in Figures 3c to 3e were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for selected pairs of groups for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001. Data in Figures 3g to 3i were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Isotype Ab, immunoglobulin G (IgG)1 antibody.

We investigated the biological effects of recombinant EDIL3 protein in human cartilage by examining the effects of chondrocyte clustering and cell number on ex vivo-cultured human osteochondral plugs (Figure 2a). Treatment of osteochondral plugs with recombinant EDIL3 protein prevented chondrocyte clustering, cell loss, and lacuna morphology (Figures 2a and 2b). SOX9 transcription factor is essential for chondrogenesis, and is downregulated in OA.[35,36] Moreover, SOX9 gene transfer to the sites of osteochondral defects repairs articular cartilage and reduces perifocal osteoarthritic changes in OA animal models.[37] Therefore, we investigated whether EDIL3 protein altered the expression level of SOX9. We observed that EDIL3 protein treatment directly enhanced SOX9 protein expression in cartilage tissues (Figures 2c and 2d). EDIL3 protein maintains chondrocyte morphology and cartilage health.

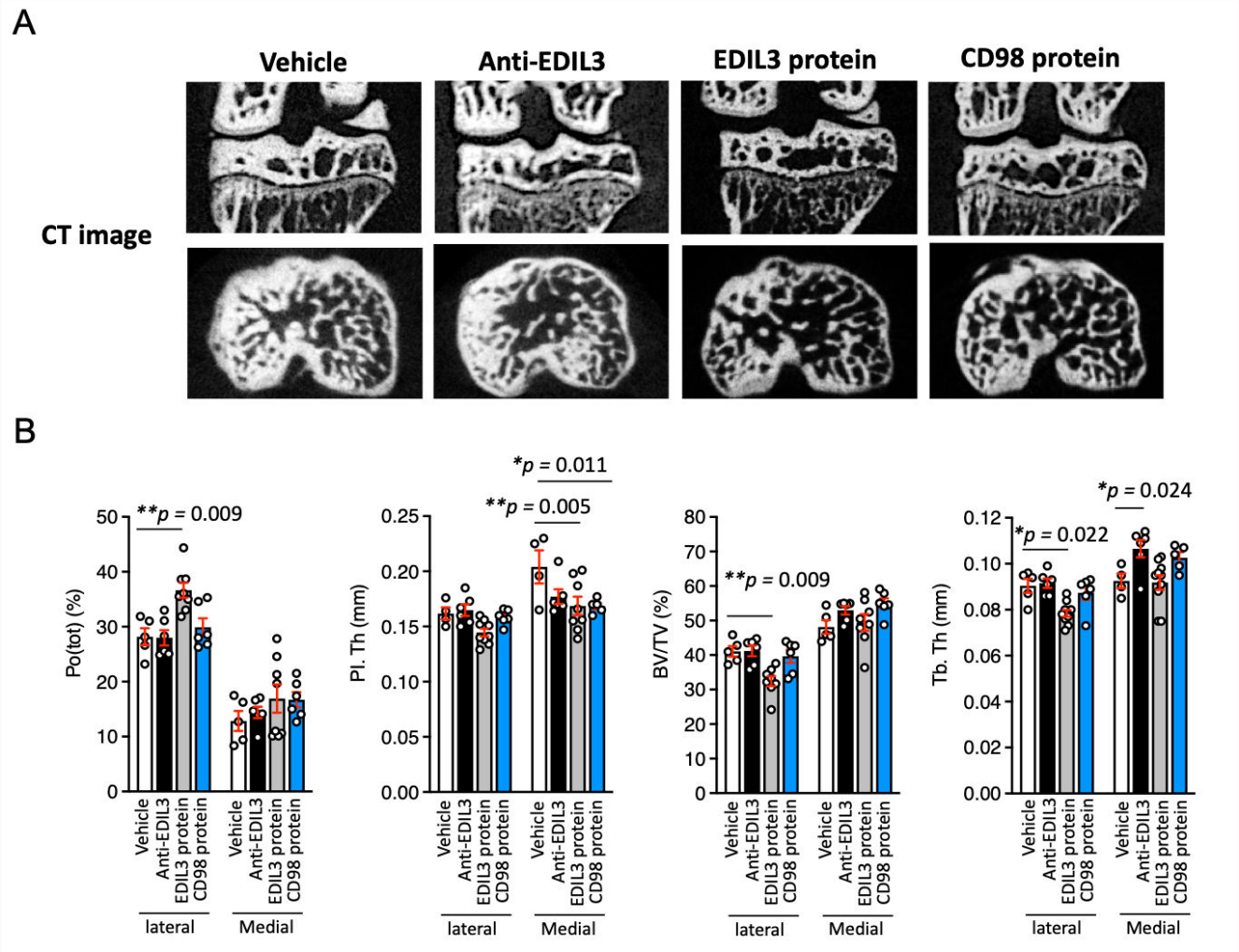

Fig. 4

EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) treatment prevents subchondral bone plate thickness (Pl.Th) and epiphyseal trabecular mineralization. a) Representative cross and horizontal sections of knee joints obtained from STR/ort mice. The 3D reconstruction of a proximal tibia with a plane indicates the location from which the cross and horizontal sections were obtained. The vehicle control mice exhibited uneven trabecular bone distribution; moreover, EDIL3 antibody treatment was observed to worsen this phenomenon. However, EDIL3 and CD98 protein prevented subchondral bone mineralization. b) Trabecular bone morphometric parameters were calculated using micro-CT analysis. The medial and lateral subchondral bone plate and underlying epiphyseal trabecular bone were analyzed separately. Epiphysis was manually selected as representative of subchondral bone. The epiphyseal trabeculae were split from the subchondral bone plate, and trabecular bone morphometric parameters were calculated. EDIL3 protein treatment prevented osteoarthritis (OA)-associated reduction in subchondral bone total porosity (Po(tot)). EDIL3 protein treatment also decreased the subchondral Pl.Th, bone volume (BV/TV), and trabecular parameters (trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp)). Analyses were conducted with two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Data are presented as means and standard deviations. *p < 0.05, **p < 0.01; analyzed using a two-way analysis of variance followed by Tukey's multiple comparison test.

EDIL3 protein has beneficial effects on cartilage maintenance in mice with OA

STR/ort mice spontaneously exhibit an OA phenotype and numerous OA characteristics such as damaged articular cartilage. We evaluated the effects of EDIL3 on histological features and subchondral Pl.Th and quantified epiphyseal trabecular parameters in STR/ort OA mice. We also clarified whether the EDIL3 protein and its corresponding receptor have chondroprotective effects in OA mice. αvβ3 and α5β1 integrin are known as receptors for EDIL3, and CD98 regulates β1 and β3 integrin-mediated cell signalling.[38,39] STR/ort mice were injected weekly via the tail vein with PBS (vehicle) and either an antibody or recombinant protein (EDIL3 or CD98) for six consecutive weeks (Figure 3a). Mice were killed at 22 weeks of age. Safranin O staining depicts the overall cell morphology, cartilaginous ECM distribution, and organization of in vivo constructs. We analyzed Safranin O images, calculated chondrocyte numbers, and measured the OARSI scores of the knee joint in each group (Figures 3b to 3e). Compared with the vehicle control group, the EDIL3 antibody-treated group demonstrated sparse chondrocytes and more lacunar cells. Both EDIL3 and CD98 protein treatments maintained an even distribution of chondrocyte numbers and had more matrix-producing chondrocytes (MPCs) (Figure 3b). We calculated the total number of chondrocytes in the articular cartilage and observed that EDIL3 protein-treated mice demonstrated an increased number of chondrocytes, especially in the femoral condyle region (Figure 3c). In addition, EDIL3 and CD98 protein treatments did not alter the number of matrix-non-producing chondrocytes (MNCs),

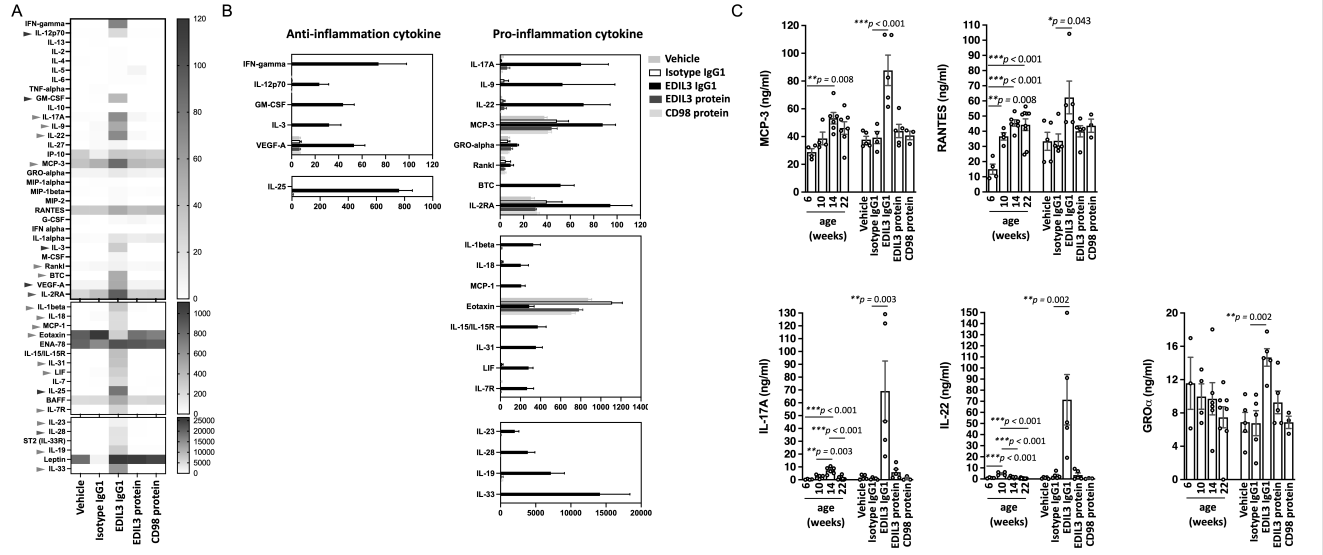

Fig. 5

EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) inhibition promotes pro-inflammatory cytokine production in STR/ort mice. a) The variable expressions of 48 cytokines among vehicle control, isotype IgG1, EDIL3 IgG1, recombinant EDIL3 protein, and CD98 protein groups were identified by comparing the expression pattern in the serum of STR/ort mice and those with vehicle control group by heat-map analysis. Arrows indicate representative anti-inflammatory cytokines (red) and pro-inflammatory cytokines (green). b) Quantitation of each cytokine demonstrated serum samples from different groups using Luminex xMAP technology. Six anti-inflammatory cytokines and 20 pro-inflammatory cytokines were identified with the most significant difference between the five groups. c) Notably, many pro-inflammatory cytokines in serum increase with ageing, including monocyte chemotactic protein-3 (MCP-3), RANTES, interleukin (IL)-17A, IL-22, and GRO-alpha. EDIL3 antibody significantly promoted the increase of these pro-inflammatory cytokines. Analyses were conducted with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for selected pairs of groups. Data are presented as means and standard errors. *p < 0.05, **p < 0.01, ***p < 0.001. Data presented in Figure 5c were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test for selected pairs of groups for multiple comparisons. IgG, immunoglobulin G.

whereas EDIL3 antibody treatment effectively increased the number of MNCs in the MTP, including both uncalcified and calcified cartilage zones (Figure 3d). The OARSI scores were measured for the MTP, LTP, MFC, and LFC. Compared with the vehicle control group, EDIL3 protein-treated mice demonstrated decreased OARSI scores in the LFC (Figure 3e). However, the OARSI scores remained unchanged in the MFC, LTP, and MTP.

To further investigate the effect of EDIL3 on articular chondrocyte hypertrophy-related molecules in the STR/ort knee, we performed IF staining for the matrix degradation marker aggrecan fragments and MMP-13, and for the chondrogenic transcription factor SOX9 (Figure 3h). Quantification of the fluorescent area indicated that EDIL3 protein treatment prevented matrix degradation and reduced MMP-13; moreover, CD98 protein treatment increased SOX9 expression (Figure 3i).

EDIL3 protein treatment prevents subchondral bone plate thickness and epiphyseal trabecular mineralization

Micro-CT 3D reconstructions of knee joints in STR/ort mice demonstrated significantly uneven trabecular bone distribution in the tibia, especially in the vehicle control group. Our CT scans demonstrated subchondral bone mineralization in the vehicle control group, particularly at the tibial plateau (Figure 4a). EDIL3 antibody treatment enhanced subchondral bone mineralization; however, EDIL3 and CD98 prevented subchondral bone mineralization (Figure 4a). Subchondral bone changes are OA-associated pathological features with decreased bone Po(tot) and increased BV/TV seen, especially at the tibial plateau.[40] Thus, the epiphysis was manually selected as a representative of the subchondral bone from the MTP and LTP, the epiphyseal trabeculae were separated from the subchondral bone plate, and trabecular bone morphometric parameters were analyzed. The quantified subchondral region by μCT analysis indicated that, compared with vehicle control mice, EDIL3 protein-injected mice demonstrated a significant increase in subchondral bone Po(tot) (Figure 4b). In addition, the subchondral Pl.Th, subchondral trabecular parameters (Tb.Th), and subchondral BV/TV density decreased following EDIL3 protein treatment (Figure 4b). Moreover, CD98 protein treatment reduced the thickness of the subchondral bone plate in mice (Figure 4b). Thus, EDIL3 treatment significantly prevented OA-associated subchondral bone changes, including increased subchondral bone porosity and decreased subchondral bone thickness.

EDIL3 acts as a potential anti-inflammatory factor in mice with OA

Ageing and age-related systemic inflammation are important factors affecting the incidence and severity of OA in humans.[3] EDIL3 regulates inflammatory cell recruitment and osteoclastogenesis to attenuate the severity of periodontitis.[29,41] We explored the role of EDIL3 in OA mice by measuring whether the administration of EDIL3 Ab or EDIL3 protein to STR/ort mice altered the expression of pro-inflammatory and anti-inflammatory cytokines in the blood. We simultaneously quantified the serum concentrations of 48 cytokine, chemokine, and growth factor targets for efficient immune response profiling and validation (Figure 5a). Studies have

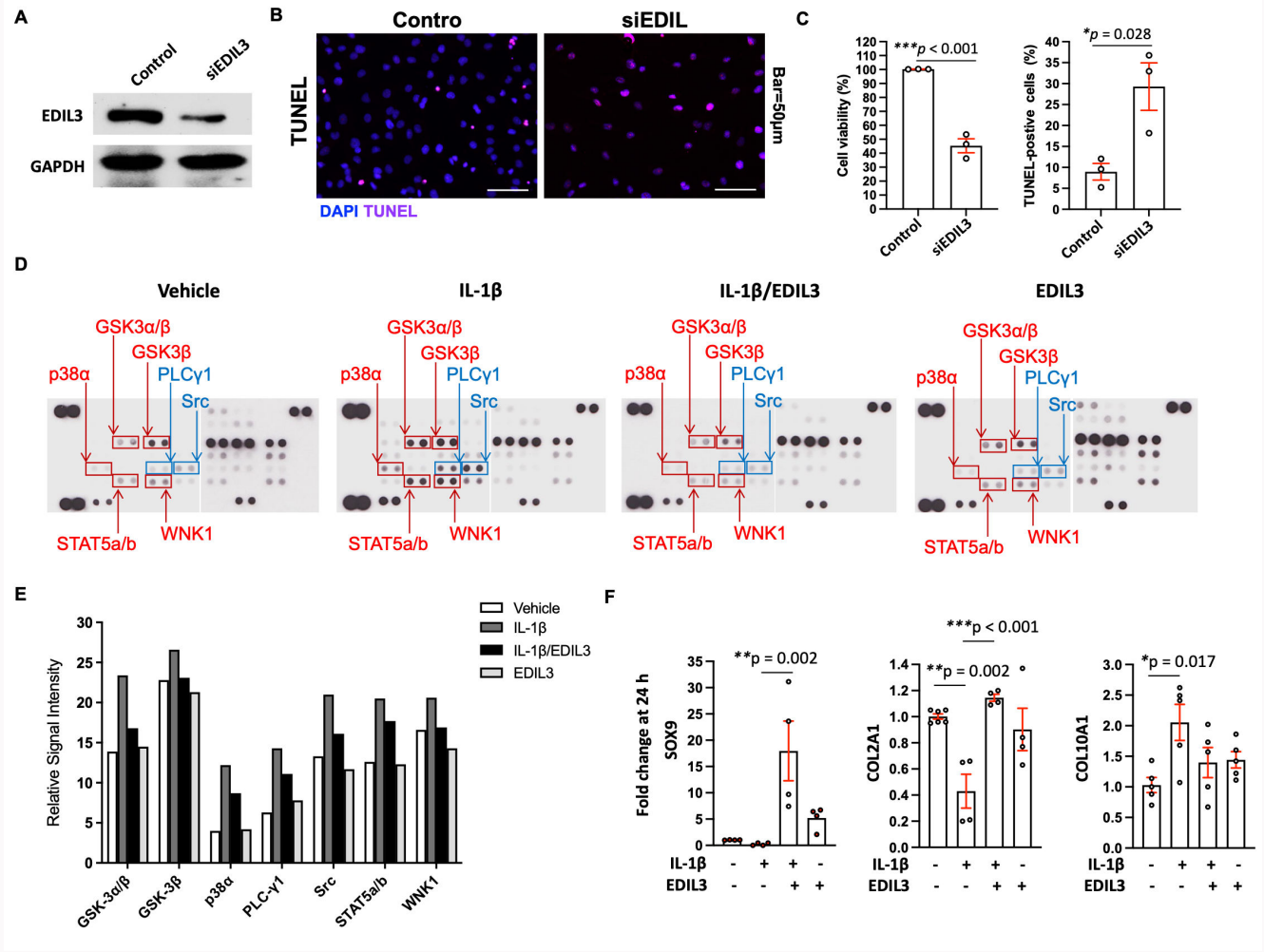

Fig. 6

EGF-like repeats and discoidin I-like domains-containing protein 3 (EDIL3) prevents chondrocyte loss by inhibiting phosphorylation of glycogen synthase kinase 3 alpha/beta (GSK-3α/β) and phospholipase C gamma 1 (PLC-γ1). a) Chondrocytes were transfected with siEDIL3 or scramble small interfering RNA (siRNA). In comparison with scramble siRNA, siEDIL3 successfully inhibited EDIL3 protein expression in chondrocytes. b) and c) The siRNA-mediated EDIL3 knockdown in chondrocytes attenuated cell viability and increased TUNEL signal. d) Intracellular proteins were collected from chondrocytes and subsequently phosphokinase protein arrays were performed to measure the phosphorylation profiles of the kinases. e) Spots with high-intensity changes were measured by Image J software. EDIL3 attenuated the interleukin (IL)-1β-enhanced phosphokinase protein expression pattern in the chondrocytes, including GSK-3α/β, p38α, PLC-γ1, Src, STAT5ab, and WNK1. f) Hypertrophic chondrocyte-related genes were measured in IL-1β-treated chondrocytes, including SOX9, type II procollagen gene (COL2A1), and COL10A1. EDIL3 restored IL-1β-decreased SOX9 and COL2A1 expression. EDIL3 did not prevent IL-1β-increased type X procollagen gene (COL10A1) expression. Data are presented as means and standard deviations. *p < 0.05, **p < 0.01, ***p < 0.001. Data in c) were analyzed using an independent-samples *t*-test. Data presented in f) were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test for selected pairs of groups for multiple comparisons. DAPI, 4′ ,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

confirmed that IL-3,[42] M-CSF,[43] IL-4,[44] and IL-25[45] are involved in chondroprotection and chondrogenesis. Moreover, IL-13,[45,46] IL-10,[47] and IL-19[48] have been demonstrated to slow OA progression. IL-12[49] and IL-27[50] also exert anti-inflammatory effects. Here, compared with the control group, the administration of EDIL3 Ab to STR/ort mice increased many pro-inflammatory cytokines in mouse serum, including IL-17A, IL-9, IL-22, monocyte chemotactic protein-3 (MCP-3), GROα, receptor activator of nuclear factor kappa-B ligand (RANKL), betacellulin (BTC), IL-2RA, IL-1beta, IL-18, MCP-1, IL-15/IL-15R, IL-31, leukaemia inhibitory factor (LIF), IL-7R, IL-23, IL-28, IL-19, and IL-33 (Figure 5b). Moreover, EDIL3 Ab administration increased only a few anti-inflammatory cytokines, including IL-12p70, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, vascular endothelial growth factor (VEGF)-A, and IL-25 (Figure 5b). Overall, 48 cytokines appeared to favour a pro-inflammatory immune response in EDIL3 antibody-treated STR/ort mice.

We observed that the levels of many pro-inflammatory cytokines in the serum increased with age, including MCP-3, RANTES, IL-17A, IL-22, and GRO-alpha (Figure 5c). The EDIL3 antibody significantly promoted the increase of these pro-inflammatory cytokines (Figure 5c).

EDIL3 may inhibit the phosphorylation of GSK-3α/β and PLC-γ1 to prevent the reduction of SOX9 expression and cell death in IL-1β-treated chondrocytes

Specific siRNAs against EDIL3 were used to investigate the effects of EDIL3 on chondrocytes in vitro. Chondrocytes were transfected with siEDIL3 or scrambled siRNA. Compared

with the control group, siEDIL3 successfully inhibited EDIL3 protein expression in chondrocytes (Figure 6a). Moreover, siRNA-mediated EDIL3 knockdown in chondrocytes attenuated cell viability and increased TUNEL signalling (Figures 6b and 6c).

Previous studies have already addressed the involvement of phosphokinase protein in OA chondrocyte events, such as phospholipase C gamma 1 (PLCγ1), glycogen synthase kinase 3 alpha/beta (GSK-3β), STAT, and Src.[51–54] PLCγ1 and GSK-3β inhibition conferred cartilage protection against OA through promotion of ECM synthesis in OA chondrocytes.[51,52] Thus, we collected intracellular proteins from IL-1β-treated chondrocytes and performed phosphokinase protein arrays to measure the phosphorylation profiles of kinases (Figure 6d). The relative signal intensity reflected the expression levels observed in the different groups. EDIL3 attenuated the IL-1β-enhanced phosphokinase protein expression pattern in the chondrocytes, especially GSK-3α/β, p38α, PLC-γ1, Src, STAT5ab, and WNK1 (Figures 6d to 6e).

Hypertrophic chondrocytes are characterized by high expression of the type X procollagen gene (COL10A1) and low expression of cartilaginous-specific markers such as the type II procollagen gene (COL2A1) and SOX9. Therefore, we measured whether EDIL3 changed the expression pattern of hypertrophic chondrocyte-related genes in IL-1β-treated chondrocytes, including SOX9, COL2A1, and COL10A1. We observed that EDIL3 prevented IL-1β-decreased COL2A1 expression and restored IL-1β-decreased SOX9 expression (Figure 6f). However, EDIL3 did not change IL-1β-increased COL10A1 expression (Figure 6f).

Discussion

As the role of EDIL3 in the maintenance of hyaline cartilage is unknown as yet, this study used an ex vivo model of human cartilage plug and an in vivo model of OA in mice to explore the possible effect of EDIL3 on cartilage protection. We confirmed that the expression of EDIL3 in the damaged cartilage of patients with OA was higher than that in the non-injured area. Altered chondrocyte morphology, especially in chondrocyte clusters, is a hallmark of OA.[55,56] Our ex vivo experimental model of cartilage plugs confirmed that EDIL3 protein prevents chondrocyte clustering and maintains chondrocyte number and SOX9 expression. The OA mouse model also confirmed that the administration of EDIL3 protein can prevent the progression of OA, including maintaining the number of chondrocytes and the number of MPCs. It reduces the degradation of aggrecan, the expression of MMP-13, the OARSI score, and bone remodelling, and also increases the porosity of the subchondral bone plate. Administration of the EDIL3 antibody increased the number of MNCs. From these findings, we can confirm that EDIL3 protein has a chondroprotective effect. In contrast, administration of CD98 protein (β1 and β3 integrin agonist) increased the expression of SOX9 in OA mice. Furthermore, we observed that the serum concentrations of OA-associated pro-inflammatory cytokines increased with age in OA mice, including MCP-3, RANTES, IL-17A, IL-22, and GROα. Administration of the EDIL3 antibody increased the serum concentrations of these OA-associated pro-inflammatory cytokines. Finally, our in vitro experiments revealed that EDIL3 may protect cartilage by attenuating the expression of IL-1β-enhanced phosphokinase proteins in chondrocytes, especially GSK-3α/β, p38α, PLC-γ1, Src, STAT5ab, and WNK1.

A few studies have reported that EDIL3 can be used as a beneficial factor for chondrocytes.[28] Deletion of EDIL3 led to decreased amounts of cartilage in the ears and knee joints of mice with otherwise normal skeletal morphology. This destabilization of the knee led to more severe OA compared to that in controls.[28] This effect was mediated primarily through integrin αVβ3.[27] Additionally, ageing-associated periodontitis is accompanied by a lower expression of EDIL3, which is an endogenous inhibitor of neutrophil adhesion dependent on integrin lymphocyte function-associated antigen 1 (LFA-1). There is reciprocal higher expression of interleukin 17 (IL-17).[41] EDIL3 suppresses LFA-1-dependent recruitment of neutrophils and IL-17-triggered inflammatory pathology, and thus may be a promising therapeutic agent for inflammatory diseases.[41] Locally administered human EDIL3 blocks inflammatory periodontal bone loss in non-human primates.[29] EDIL3 reduces osteoclastogenesis and inhibits inflammatory bone loss in non-human primates.[29] Thus, EDIL3 represents a new class of endogenous therapeutics with the potential to treat inflammation-associated bone loss. In summary, regulation of EDIL3 expression is a potential therapeutic target for OA.

Patients with OA present a continuously progressive disease state with partial but incomplete cartilage degeneration and loss. The severity of articular cartilage loss is a crucial indicator of OA progression. Since the male STR/Ort mice develop knee OA spontaneously, as an excellent animal model for OA studies,[57] many studies describe the anatomical and clinical features of the knee joint of STR/ort OA mice as being similar to those of patients with OA; thus, cartilage histological score analyses of STR/ort mice have been confirmed.[32,33] Therefore, we carefully examined the Safranin-O-stained image and measured the OARSI score of knee joints in STR/ort mice with or without EDIL3 treatment.

CD98 is a glycoprotein heterodimer composed of SLC3A2 and SLC7A5, and forms a large neutral amino acid transporter (LAT1). SLC3A2 and SLC7A5 are the heavy and light chains of CD98, respectively. The SLC3A2/cd98hc complex was used in this study. According to our data, CD98 protein treatment reduced PI.Th in STR/ort mice. In addition, we found that EDIL3 may activate β1 and β3 integrin to protect cartilage, and that CD98 is a β1 and β3 integrin agonist. Therefore, we speculate that EDIL3 may partially regulate the activity of αVβ3 integrins to achieve the chondroprotective effect. We also believe that αVβ3 integrin might just be one of the regulatory mechanisms of EDIL3.

Aggrecan fragments (G1-IGD-G2 domains) and MMP-13 are markers of chondrocyte hypertrophy-like changes and cartilage degradation in OA.[58,59] The aggrecan G1-IGD-G2 domain is the site of MMP attack on aggrecan during pathological cartilage degradation; G1-IGD-G2 also appears to be involved in the physiological turnover of aggrecan.[60] MMP-13, also referred to as collagenase-3, is a member of the MMP family of neutral endopeptidases and is highly overexpressed in hypertrophic chondrocytes and synovial cells in OA.[61] Knee OA is characterized by increased subchondral PI.Th and trabecular BV/TV. Subchondral trabecular bone remodelling and microstructural changes in the tibial plateau are associated with cartilage degradation.[62] This

study demonstrated that EDIL3 protein treatment prevents subchondral bone plate thickening and epiphyseal trabecular mineralization. EDIL3 is a potential new factor in OA treatment as we demonstrated that it prevents aggrecan degradation, MMP-13 expression, and subchondral PI.Th in OA mice. We believe that our findings will facilitate the development of novel strategies for the treatment of OA and contribute to the field of chondrocyte biology.

Supplementary material

An ARRIVE checklist is included to show that the ARRIVE guidelines were adhered to in this study.

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

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Ethical review statement

The patient specimens were approved by our institutional review board, and the study was compliant with accepted ethical standards at Chang Gung Memorial Hospital. The written informed consent was obtained from all patients prior to their participation in the study (IRB number 202100948B0C603). This study was carried out in accordance with the ethical standards in the 1964 Declaration of Helsinki. All animal procedures complied with the National Institutes of Health in the United States guidelines, and were reviewed and approved by the local Hospital Animal Care and Use Committee (IACUC approval number 2018060702, approval date: 25/06/2021).

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