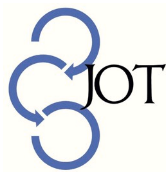
Journal of Orthopaedic Translation 49 (2024) 96–106



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Journal of Orthopaedic Translation

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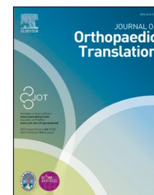

Original Article

CD73 alleviates osteoarthritis by maintaining anabolism and suppressing catabolism of chondrocytes extracellular matrix

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ARTICLE INFO

Keywords:
Adenosine
CD73
Chondrocytes extracellular matrix
Osteoarthritis
Single nucleotide polymorphisms

ABSTRACT

Background: Osteoarthritis (OA) is the most common degenerative joint disease, with articular cartilage degeneration as primary manifestation. Intra-articular injection of exogenous liposomal adenosine in mice knee has been shown to alleviate OA progression. However, the role of CD73, the rate-limiting enzyme of extracellular adenosine synthesis, in OA is still unknown.

Methods: In this work, we explored the expression changes of adenosine-related molecules via bioinformatic analysis. In addition, the expression level of these molecules was detected in OA cartilage. We also conducted a case–control study to investigate the genetic variants of selected SNPs on genes encoded adenosine-related molecules. To further explore the function of CD73 in chondrocytes, we knocked down the expression of CD73 with small interfering RNA and overexpressed CD73 with the use of lentivirus, and detected the expression of markers for anabolism and catabolism in mouse primary chondrocytes with or without IL-1β treatment. We also conducted *in vivo* experiments to explore the role of CD73 in OA.

Results: We found that the expression of CD73 was upregulated in OA, and the variants of SNP rs2229523 (base A to G) on *NT5E* (the encoding gene of CD73) were significantly higher in OA population, which might cause the amino acid encoded by this SNP change from threonine to alanine. The original helix structure in the adjacent region of amino acid encoded by SNP rs2229523 would be deconstructed after its mutation. Furthermore, we found that CD73 promoting the expression of Col2a1 but suppressing the expression of Mmp13 expression in mouse primary chondrocytes under inflammatory environment. The overexpression of CD73 attenuated bone remodeling and alleviated cartilage degeneration in DMM mice. Moreover, the physical activities were also improved in DMM mice overexpressed CD73 with the use of adeno-associated virus.

Conclusions: The variants of SNP rs2229523 (base A to G) on *NT5E* were significantly higher in OA population, and CD73 could alleviate OA by maintaining anabolism and suppressing catabolism of chondrocytes extracellular matrix.

The Translational Potential of this Article: This work showed that CD73 might be one of the biological therapeutic targets of OA, which would provide a reference for future novel treatment strategy of OA.

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https://doi.org/10.1016/j.jot.2024.08.014
Received 11 March 2024; Received in revised form 4 July 2024; Accepted 23 August 2024
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1. Introduction

Osteoarthritis (OA) is the most common degenerative joint disease, with articular cartilage degeneration, synovitis and subchondral bone remodeling as primary manifestations [1]. Current drug treatments for OA are mainly based on anti-inflammatory and analgesic, which cannot precisely target the pathophysiological mechanisms of OA, such as the imbalance of chondrocyte metabolism [2]. Therefore, it is important to investigate genetic variants of genes associated with OA to explore the screening measures before the onset of OA, and reveal the role of these molecules in the pathogenesis of OA.

Adenosine, an endogenous purine nucleoside produced by the catabolism of adenosine triphosphate (ATP), normally binds to its four transmembrane G protein coupled receptors (ADORA1, ADORA2A, ADORA2B, ADORA3) to exert physiological functions such as anti-inflammatory effects [3,4]. In a stressful environment such as inflammation, adenosine could be accumulated extracellularly [5]. CD39 and CD73 are ectonucleotidases on the cell membrane that catalyze the conversion of extracellular ATP to adenosine, and CD73 is the rate-limiting enzyme [6,7]. It has been reported that intra-articular injection of exogenous liposomal adenosine in mice knee can alleviate OA progression by inhibiting cartilage degeneration [8]. However, the extracellular metabolism of adenosine is rapid, which weakens the long-term therapeutic effect of intra-articular injection of exogenous adenosine [9]. Therefore, revealing the association between CD73 and OA progression could provide a basis for novel biological diagnosis and treatment of OA.

In this work, we figured out that there were genetic variants of adenosine-related molecules existed in OA population, and CD73, the rate-limiting enzyme of adenosine synthesis, played a role in alleviating OA by promoting anabolism and suppressing catabolism of chondrocyte extracellular matrix.

2. Materials and methods

2.1. Clinical specimen

The femoral condyles from five patients (58–76 years old) undergoing total knee arthroplasty at the end stage of OA were collected to extract total proteins. Supplementary Table 1 showed basic information of these patients. The study protocol was approved by the Ethical Committee of Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University (2,009,022). All the patients had signed informed consent before surgery.

2.2. Animals

To figure out the expression changes of adenosine-related molecules in OA *in vivo*, Male C57BL/6J mice were subjected to Destabilization of Medial Meniscus (DMM) surgery on the right knees to establish OA model at 10 weeks old, and were sacrificed 8 weeks after surgery to obtain knees specimen [10,11]. The sham group received immediate suture after exposure of right knee meniscus. To investigate the function of CD73 in OA, intra-articular injected adeno-associated virus (AAV) was adopted to overexpress CD73 in mice from the sham group and DMM group. According to the manufacturer's instructions, three weeks before the initial surgery, the mice were randomly treated with a total of 8 μL solution containing AAV negative control virus (AAV-control) or AAV-CD73 (PackGene, China) to ensure the transfection efficiency [12]. The primary mouse chondrocytes were extracted from costal cartilage of suckling mice aged less than 3 days. All of the experiments were authorized and conducted in accordance with the Animal Care and Use Committee of Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University (2020AE01102).

2.3. Open field test (OFT)

Spontaneous activity and exploratory behavior of the animals were assessed using a tracking system (Zhenghua Technology, China). Each mouse was placed sequentially in an open 50 cm × 50 cm square indoor field without lighting. A camera was used to monitor the mouse's activity trajectory in real-time within 3 min and to evaluate its relative activity, active time, distance, and mean speed.

2.4. Footprint experiment

The front and back paws of the mice were dipped in red and blue ink, respectively, and their walking trajectories were recorded using ink blots [13]. The red ink marks represent the two front paws and the blue ink marks represent the two hind paws. The mice were allowed to walk freely through a 70 cm long by 20 cm wide track covered with white paper to assess their physical activities and pain in natural walking conditions. And the mice will be acclimated to the environment for one week before the assay. The process was done in a relatively darkroom environment without noise interference, with each mouse tested at least three times, and the ink is safe and non-toxic.

2.5. Micro-computed tomography (micro-CT) analysis

Evaluation of the mouse knee joint by micro-CT using a Swiss VivaCT 80 scanner (Scanco Medical AG). The scan resolution of the joint was 18.38 μm, and reconstructed three-dimensional (3D) images were constructed by Scanco medical software. The cancellous bone was analyzed to obtain trabecular-related parameters and subchondral bone volume, and the number of bone fragments and subchondral bone thickness was calculated for each knee joint by 3D reconstruction.

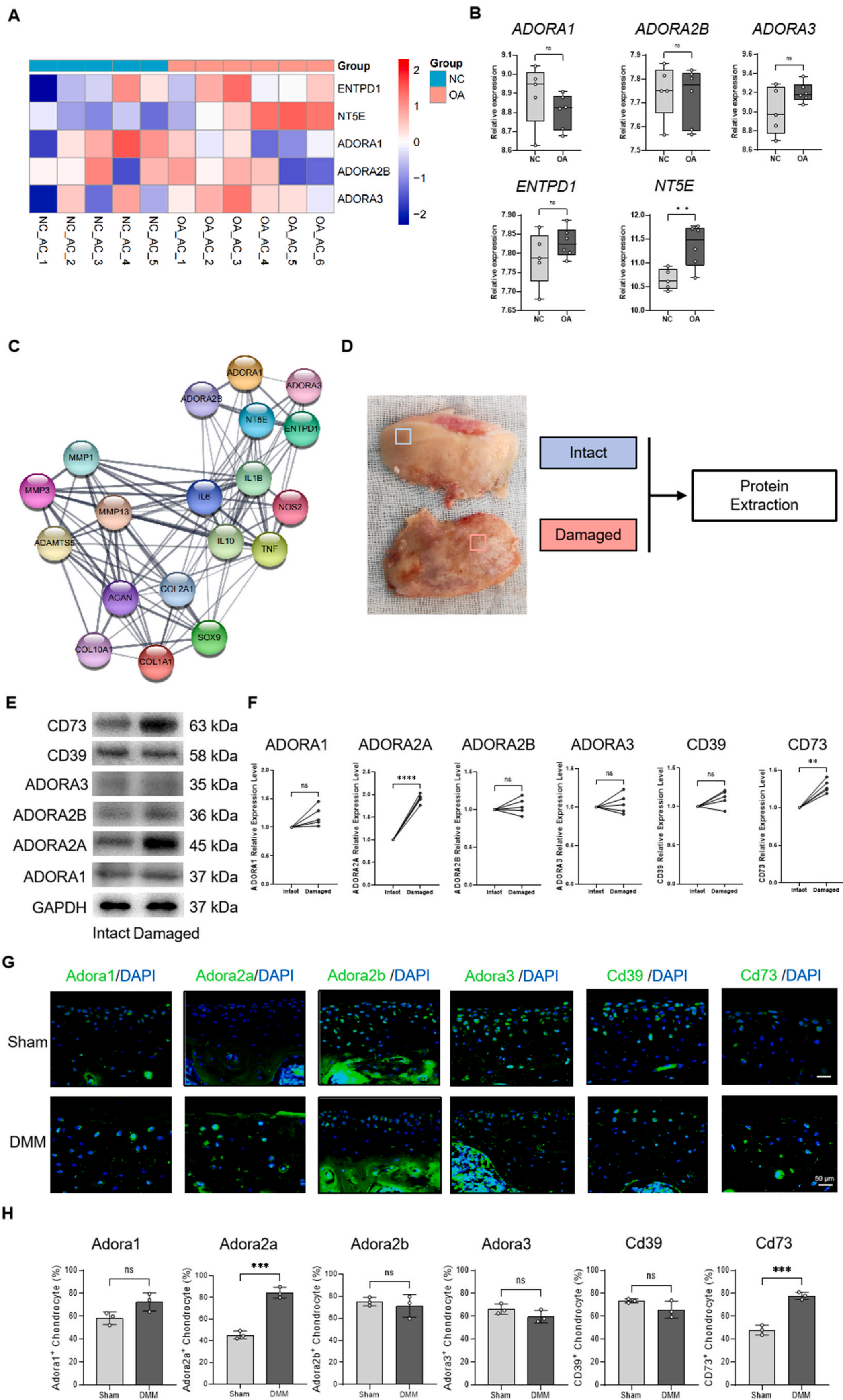
2.6. Histological analysis

After micro-CT analysis, the knee joints of mice were soaked in a 10 % EDTA (#1340, Biofroxx, Germany) solution for decalcification. The knee joint embedded in the paraffin block was cut into continuous coronal slides (5 μm thick) using a microtome (Thermo, Germany). The slices were detected by Safranin-O/fast green (S.O.) (#G1371, Solarbio, China) and haematoxylin (H&E) (#C0105M, Beyotime, China) staining to observe the integrity and thickness of articular cartilage. The degree of synovitis was evaluated according to synovitis score [14]. The severity of OA was assessed using the OARSI scoring system (0–6 scale) by blinded observers. The highest OARSI score for each section was recorded and the average of all scores were calculated.

2.7. Cell culture

The mouse primary chondrocytes were cultured in dulbecco's modified eagle's medium (Gibco, CA) supplemented with 10 % fetal bovine serum (Gibco) and 1 % penicillin and streptomycin (Gibco) at 37 °C and 5 % CO_2 condition. To mimic OA environment in vitro, the mouse recombinant IL-1β (R&D Systems, USA) was added into the medium at 10 ng/mL. The mouse small interfering RNA (siRNA) of *NT5E* were synthesized (Hippo Bio., China) and adopted for knocking down the expression of CD73. Lentivirus was adopted for the overexpression of CD73 in mouse chondrocytes.

Mouse chondrocytes were plated in a six-well plate and amplified to a cell density of 80 %, then a mixture of lentivirus (MOI = 50, hippo bio., China) and polybrene (6 μg/mL, Solarbio, China) was added to the medium. After 48 h, the chondrocytes were screened with puromycin (Beyotime, China). The messenger RNA (mRNA) and total protein were extracted from mouse primary cells knocked down or overexpressed CD73 which treated with or without IL-1β, and analyzed to explore the role of CD73 in chondrocytes.



(caption on next page)

Fig 1. The expression of CD73 was upregulated in OA. A. The heat map that listed adenosine-related molecules. The colors of each rectangle reflected the expression changes of the corresponding genes in normal and OA populations. B. The quantification of the expression changes of genes encoded adenosine-related molecules in normal and OA populations. C. The PPI network showed the connections between different genes. The more connections between these genes suggested there might be more relevant at molecular level. D. Schematic diagram of dividing the cartilage on the human femoral condyle into intact and damaged areas and extracting proteins respectively. E. Western blot results of CD39, CD73, ADORA1, ADORA2A, ADORA2B and ADORA3 in the intact and damaged areas of OA cartilage. F. The quantification of western blot results. G. Immunofluorescence staining of Cd39, Cd73, Adora1, Adora2a, Adora2b and Adora3 in the mice knees of control and OA groups. H. The quantification of immunofluorescence staining. Data (n ≥ 5 of clinical samples and n = 3 of animal samples) were shown as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.8. Extracellular ATP assay

The ATP Content HPLC Assay Kit (#BC0305, Solarbio) was adopted to evaluate extracellular ATP level of mouse chondrocytes. Briefly, mouse chondrocytes were treated with CD73 SiRNA or CD73 lentivirus with or without IL-1β for 24h. Then the cell culture medium was collected to detect ATP level. The following steps were carried out according to the manufacturer's protocol.

2.9. Extracellular adenosine assay

The Adenosine Assay Kit (Fluorometric) (#ab211094, abcam) was adopted to evaluate extracellular adenosine level of mouse chondrocytes. Briefly, mouse chondrocytes were treated with CD73 SiRNA or CD73 lentivirus with or without IL-1β for 24h. Then the cell culture medium was collected to detect adenosine level. The following steps were carried out according to the manufacturer's protocol.

2.10. Western blot

RIPA lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (Solarbio) and 1 mM phosphatase inhibitor cocktail (Bimake, USA) was adopted for protein extraction from human cartilage and mouse primary chondrocytes. The BCA Protein Assay Kit (Thermo Scientific, USA) was used to determine the protein concentrations. The proteins were then separated on 10 % (w/v) sodium dodecyl sulfate-polyacrylamide gel (EpiZyme, China) electrophoresis and were subsequently transferred onto polyvinylidene fluoride membranes (Millipore, USA) according to experiment standard protocol. Later, the membranes were blocked with 5 % (w/v) milk (Biofroxx) for 1 h at room temperature and then incubated with primary antibodies (1:1000 dilution) of GAPDH (#10494-1-AP, Proteintech, China), CD39 (#14211-1-AP, Proteintech), CD73 (#12231-1-AP, Proteintech), ADORA1 (#20332-1-AP, Proteintech), ADORA2A (#53509, CST, China), ADORA2B (#21071-1-AP, Proteintech), ADORA3 (#ab203298, Abcam, UK), COL2A1 (#BA0533, Boster, China) and MMP13 (#69926, CST). After incubated with secondary antibody that was a horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (1:5000, Biosharp, China), the cut mem-branes were placed into the ChemiDocXRS + Imaging System (Tanon, China) to detect signals. The quantitative analysis of protein densitometry was conducted with the use of Image J (version 1.8.1).

2.11. Immunofluorescence staining

The mouse primary chondrocytes were fixed in 4 % PFA and permeated by 0.3 % Triton X-100 for 15 min. After blocking with 5 % BSA, the chondrocytes were incubated with primary antibody of Col2a1 (1:200, Boster) and Mmp13 (1:200, CST) at 4 °C overnight. The chondrocytes in the 24-pore plates were washed with PBST and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h at room temperature. 4',6-diamidino-2-phenylindole was adopted to mark the nuclei.

Mouse knee sections were dewaxed and hydrated in xylene and graded alcohol, and antigen repair was performed using pepsin for 1 h at 37 °C. After washed by phosphate-buffered saline (PBS), the slides were blocked with 5 % bovine serum albumin (BSA) for 1 h at 37 °C. Then, the slices were incubated overnight (4 °C) with primary antibodies of Cd39 (1:200, Proteintech), Cd73 (1:200, Proteintech), Adora1 (1:200, Proteintech), Adora2a (1:200, CST), Adora2b (1:200, Proteintech), Adora3 (1:200, Abcam), Col2a1 (1:200, Boster) and Mmp13 (1:200, CST). After three washes with TBST, the slices were incubated with fluorescein isothiocyanate (FITC)-coupled secondary antibody for 1h at room temperature. At last, 4',6-diamidino-2-phenylindole was adopted to mark the nuclei.

The fluorescence images were randomly selected by a fluorescence micro-scope (Zeiss, Germany) and quantified by Image J (version 1.8.1) [15]. In order to avoid the impact of different staining batches on mean fluorescence intensity, the proportion of positive chondrocytes were adopted to quantify the immunofluorescence staining on knee sections [16]. However, for immunofluorescence staining on cultured chondrocytes, all cells captured for the same marker were plated on the same 24-well plate, and thus mean fluorescence intensity was used to quantify immunofluorescence staining on cultured chondrocytes.

2.12. The case–control study

There was a total of 642 patients participated in this study. All the patients were Chinses Han living in the Jiangsu Province, China, and they had signed informed consent before being in hospital. The study protocol was approved by the Ethical Committee of the Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University (2,009,022).

The patients admitted by the Division of Sports Medicine and Adult Reconstructive Surgery, Department of Orthopedic Surgery, Nanjing Drum Tower Hospital from May 2020 to October 2021 were included in this study. The patients diagnosed as femur neck fractures, femur intertrochanteric fractures or femoral head necrosis, were assigned to the control group (300 patients). While the patients hospitalized during the same period and diagnosed as knee OA were assigned to the OA group (342 patients). Patients with family history of genetic diseases were excluded in this study.

The SNPs on genes encoded adenosine-related molecules in this study were: CD73 (*NT5E*: rs2229523, rs387906620), ADORA1 (ADORA1: rs17852405, rs748346254), ADORA2A (ADORA2A: rs8192446, rs17650937), ADORA2B (ADORA2B: rs2015353), ADORA3 (ADORA3: rs2800889). They were selected with the criteria: 1, Referred to reports in related literature; 2, Located at exon area; 3, The variants of which would cause missense variant; 4. A cut-off of minor allele frequency is at least 10 % in Mongolians (in the Ensemble Genome Browser: https://www.ensembl.org/index.html) (Supplementary Table 2).

The genomic DNA of participated patients was extracted from peripheral whole blood with the use of QIAmp Kit (Qiagen, USA) in accordance with the manufacturer's protocol. The SNP Genotyping Service was provided by Shanghai Blowing Applied Biotechnology (China). In addition, the OncoArray was used for genotyping in this study (Illumina, USA).

2.13. Prediction of CD73 protein structure and function changes

Domains of CD73 were searched and visualized on SMART database (https://smart.embl.de/) and the detailed location of SNP rs229523 in *NT5E* gene and the position of the corresponding amino acid were marked in the figure. The crystal structures of the CD73 were obtained

Table 1
Associations between selected SNPs and OA.

sGenes	SNPs	Genotypes						Allelic Frequency				P value				Odds Ratio (95%CI)		
		Control			OA			Control		OA		Genotype	Allele	AA vs. Any T/G CC vs. Any T/G	GG vs. Any A/C TT vs. Any A/C	Allele	AA vs. Any T/G CC vs. Any T/G	GG vs. Any A/C TT vs. Any A/C
NT5E	**rs2229523**	42 (AA)	186 (AG)	72 (GG)	45 (AA)	163 (AG)	134 (GG)	0.45 (A)	0.55 (G)	0.37 (A)	0.63 (G)	<0.0005	<0.005	0.76	<0.0001	1.39 (1.11–1.74)	1.07 (0.68–1.69)	2.04 (1.45–2.87)
	rs387906620	0 (AA)	0 (AG)	300 (GG)	0 (AA)	0 (AG)	342 (GG)	0 (A)	1 (G)	0 (A)	1 (G)	—	—	—	—	—	—	—
ADORA1	rs17852405	300 (CC)	0 (CT)	0 (TT)	342 (CC)	0 (CT)	0 (TT)	1 (C)	0 (T)	1 (C)	0 (T)	—	—	—	—	—	—	—
	rs748346254	0 (AA)	0 (AG)	300 (GG)	0 (AA)	0 (AG)	342 (GG)	0 (A)	1 (G)	0 (A)	1 (G)	—	—	—	—	—	—	—
ADORA2A	rs8192446	300 (CC)	0 (CT)	0 (TT)	342 (CC)	0 (CT)	0 (TT)	1 (C)	0 (T)	1 (C)	0 (T)	—	—	—	—	—	—	—
	rs17650937	300 (CC)	0 (CG)	0 (GG)	342 (CC)	0 (CG)	0 (GG)	1 (C)	0 (G)	1 (C)	0 (G)	—	—	—	—	—	—	—
ADORA2B	rs2015353	6 (CC)	69 (CT)	225 (TT)	12 (CC)	66 (CT)	264 (TT)	0.14 (C)	0.86 (T)	0.13 (C)	0.87 (T)	0.30	0.86	0.25	0.52	1.03 (0.75–1.42)	0.56 (0.21–1.51)	1.13 (0.78–1.62)
ADORA3	rs2800889	297 (AA)	3 (AT)	0 (TT)	336 (AA)	6 (AT)	0 (TT)	0.99 (A)	0.01 (T)	0.99 (A)	0.01 (T)	—	0.42	0.42	—	0.76 (0.44–7.07)	0.77 (0.44–7.13)	—

from the Protein Data Bank (PDB) with entries ID 7QGO (WT) and 7P9T (MUT), respectively. 3D view was displayed on PDB WebGL, and the detailed structure change caused by SNP rs229523 and the corresponding amino acid were highlighted. The simulated visualization images of CD73 combining with AMP and the assessment of its capacity for combining with AMP before and after SNP rs2229523 mutation were available on the website http://hdock.phys.hust.edu.cn/ [17].

2.14. Quantitative real-time PCR (qPCR)

Total RNAs were extracted from mouse primary chondrocytes via the RNA-quick Purification Kit (ESScience, China) according to the manufacturer's instructions. RNA reverse transcription was performed using the RT Master Mix for qPCR II (gDNA digester plus) (Vazyme, China). After RNA reverse transcription to cDNA, the qPCR experiment was performed by magnifying 20 μL of diluted complementary cDNA with the SYBR Green Q-PCR Kit (Vazyme, China) on Light Cycler 480 PCR System (Roche, Switzerland). Supplementary Table 3 listed the primer sequences used in this research.

2.15. Bioinformatic analysis

To figure out the expression changes of genes encoded adenosine-related molecules in OA population, we explored molecules related to adenosine, inflammatory response, and chondrocyte metabolic homeostasis based on published literature [18–20].

Transcription profiles of articular cartilage were acquired from Gene Expression Omnibus (GEO) database (GSE169077, NC = 5, OA = 6). Raw data were normalized with affy (v1.68.0) R package, and the expression values were extracted and constructed the heatmap with pheatmap (v1.0.12) R package. PPI (protein–protein interaction) network was predicted on STRING database (https://cn.string-db.org/) and visualized on cyto-scape (v3.9.0) software.

2.16. Statistical analysis

In the case–control study, the comparison of baseline patient characteristics between control group and OA group were analyzed using unpaired two-tailed Student's t test and Chi-square test. Chi-square test was also adopted for comparing the genotypes and alleles of selected SNPs between two groups. To compare the differences of gene expression in intact cartilage and damaged cartilage, paired two-tailed Student's t test was used in this research. Generally, unpaired two-tailed Student's t test was adopted for comparing values between two groups, while one-way analysis of variance, followed by Tukey's multiple comparison tests were used to compare the data from more than two groups. Shapiro–Wilk method was used to estimate the normal distribution of data and Levene method was used to test the homogeneity of variance. There were at least 3 independent replicates in all experimental groups in this study. GraphPad Prism software (version 9.4.1) were used for statistical analysis and quantification diagrams. The data were presented as mean values ± SD. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The expression of CD73 was upregulated in OA

Bioinformatic analysis was conducted to investigate the association between genes encoding adenosine-related molecules and OA. Adenosine-related genes were listed and their expression changes were showed via heat map (Fig. 1A). Among them, *ENTPD1* and *NT5E* were genes encoding CD39 and CD73 respectively, which were the enzymes catalyzed adenosine synthesis. Adenosine receptors include: ADORA1, ADORA2B and ADORA3 (Fig. 1A). The quantization of the expression changes of these genes suggested that only *NT5E* was significantly

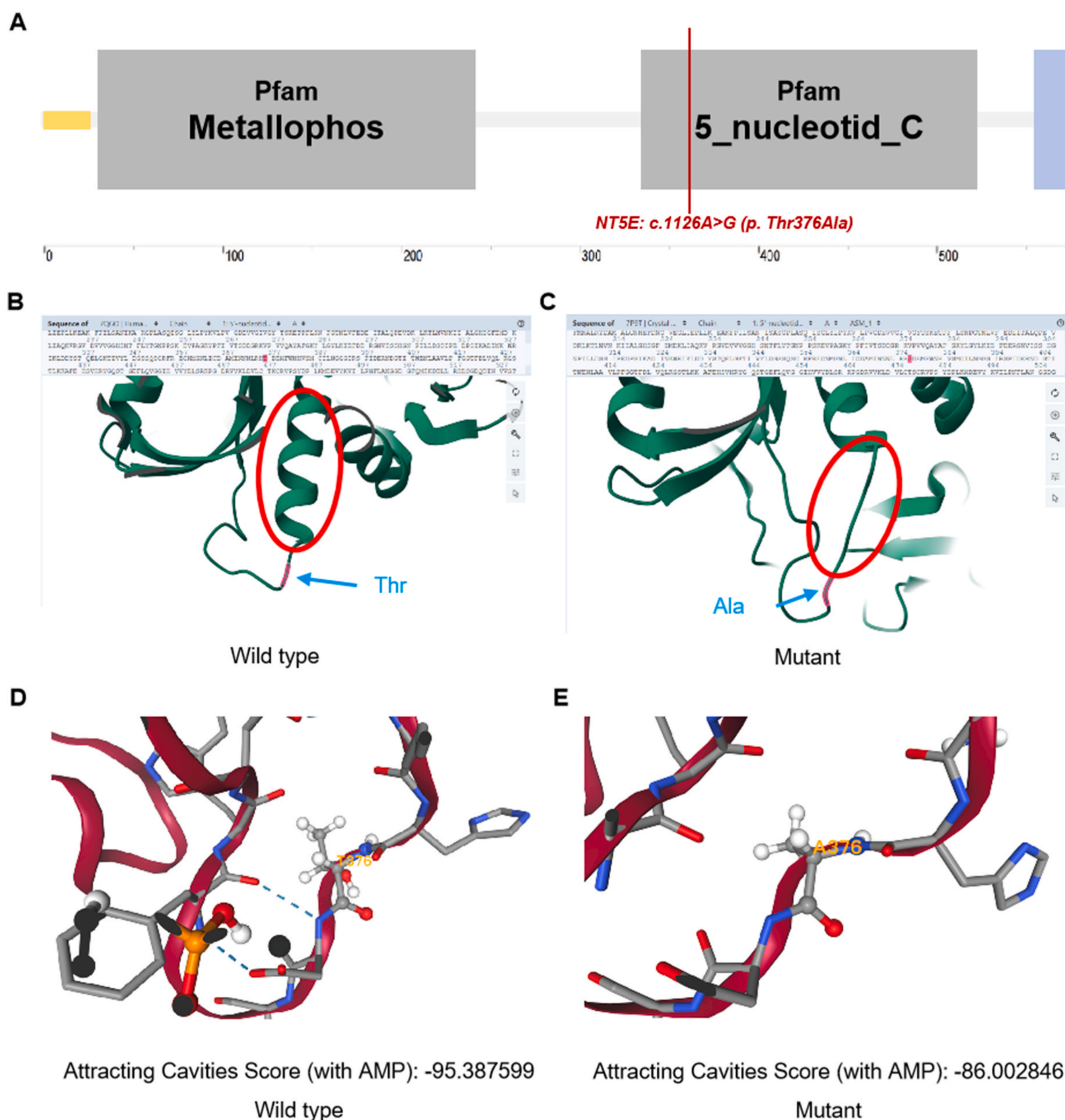


Fig 2. Prediction of CD73 protein structure changes after SNP rs2229523 **mutation.** A. The detailed position of SNP rs2229523 on *NT5E* gene and the amino acid encoded by SNP rs2229523 changes after its mutation on CD73 protein. B. The helix structure at the adjacent region of the amino acid encoded by SNP rs2229523 on CD73 protein. C. After SNP rs2229523 mutation, the helix structure at the adjacent region deconstructed. D. Visualized simulation of the capacity of CD73 for combining with AMP. E. After SNP rs2229523 mutation, the capacity of CD73 for combining with AMP decreased.

upregulated in OA population, while the others showed no significance (Fig. 1B). The PPI network diagram revealed that adenosine-related genes were associated with those related to inflammatory response, especially IL-1β. Chondrocytes metabolic homeostasis related genes interacted with inflammatory response related genes as well (Fig. 1C). These results suggested that CD73 might be involved in OA progression. To further verify the association between adenosine-related molecules and OA, the expression levels of these molecules were detected in OA cartilage from clinical and animal specimen. Cartilage tissue on the human femoral condyles was divided into damaged area and intact area, and the proteins were extracted from each area (Fig. 1D). CD73 and ADORA2A were upregulated in damaged area at protein level while other adenosine-related molecules showed no significance in the two areas (Fig. 1E and F). Immunofluorescence staining on knees from mice showed consistent results. Compared with sham group, Cd73 and Adora2a were upregulated in OA model mice while others showed no significant differences (Fig. 1G and H). These results further verified the association between adenosine-related molecules, especially CD73, and OA.

3.2. The variants of SNP rs2229523 on NT5E were significantly higher in OA population

To figure out genetic variants of adenosine-related molecules (CD39, CD73, ADORA1, ADORA2A, ADORA2B, ADORA3) in OA population, we conducted a retrospective case–control study. There were 300 patients included in the control group and 342 patients in OA group. The baseline characteristics of patients were showed in Supplementary Table 4. The sex ratio, age, BMI of patients in the two groups showed significant differences. SNPs selected in this work and their main characteristics were listed in Supplementary Table 2. More detailed locations of selected SNPs were shown in Supplementary Fig. 1. Among these SNPs,

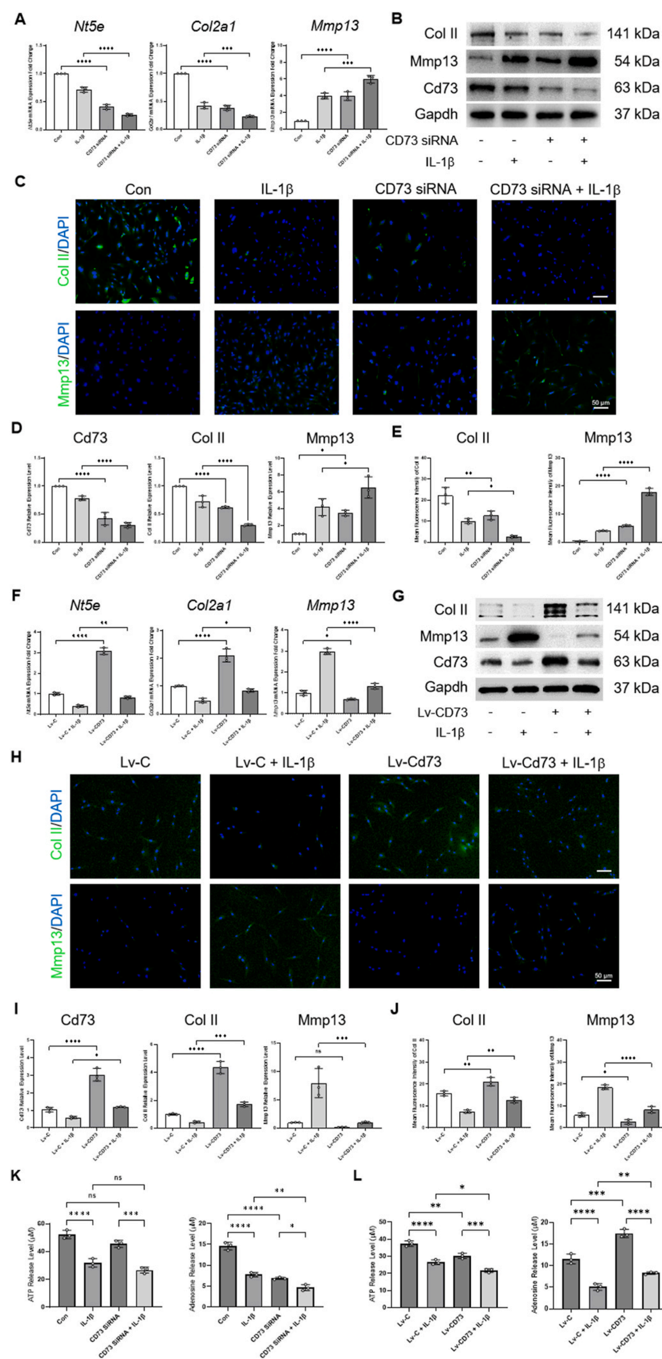


Fig 3. The function of maintaining anabolism and suppressing catabolism of CD73 in chondrocytes with IL-1β treatment. A. qPCR results of mRNA expression levels of Nt5e, Col2a1, and Mmp13 in mouse chondrocytes received siRNA treatment for knocking down Cd73 expression with or without IL-1β. B. Western blot results of Cd73, Col II and Mmp13 in the mouse chondrocytes. C. Immunofluorescence staining of Col II and Mmp13 in the mouse chondrocytes. D. The quantification of western blot in the mouse chondrocytes. E. The quantification of immunofluorescence staining in the mouse chondrocytes. F. qPCR results of mRNA expression levels of Nt5e, Col2a1, and Mmp13 in mouse chondrocytes received lentivirus for overexpressing Cd73 with or without IL-1β. G. Western blot results of Cd73, Col II and Mmp13 in the mouse chondrocytes. H. Immunofluorescence staining of Col II and Mmp13 in the mouse chondrocytes. I. The quantification of western blot in the mouse chondrocytes. J. The quantification of immunofluorescence staining in the mouse chondrocytes. K & L. The extracellular ATP and adenosine levels of the mouse chondrocytes, respectively. Data (n = 3) were shown as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

the SNP rs2229523 on *NT5E* was significantly associated with OA, while other SNPs showed no significance (Table 1). More frequent mutation of rs2229523 (base A to G) in OA population was discovered (Table 1). Considering rs2229523 was located at exon area and its mutation might lead to missense variant, we conjectured that the variant of rs2229523 might result in the change of structure or function of CD73 protein.

3.3. Prediction of structure and function changes of CD73 after SNP rs2229523 mutation

To figure out whether the structures of CD73 protein would change after the mutation of SNP rs2229523, we searched and visualized the domains of CD73 on SMART database. SNP rs2229523 was located at 5_nucleotid_C area in *NT5E* gene. It was the 1126th base pair from the C-terminus in the gene and encoded the 376th amino acid in CD73 protein, and the mutation of which might cause the normally encoded threonine be changed to alanine (Fig. 2A). The crystal structures of CD73 showed that the original helix structure in the adjacent region of the encoded amino acid would be deconstructed after SNP rs2229523 mutation, which suggested that the mutation of SNP rs2229523 might cause the change of function of CD73 (Fig. 2B and C). The human SNP rs2229523 showed poor conservation across different species in our study, indicating less selection pressure during the evolutionary process (Supplementary Fig. 2). In addition, CD73 is the membrane protein responsible for directly converting extracellular AMP into adenosine [7]. Therefore, to investigate the potential function changes of CD73 after SNP rs2229523 mutation, we simulated the visualization images of CD73 combining with AMP before and after the mutation at this site. After SNP rs2229523 mutation, the capacity of CD73 for combining with AMP decreased (Fig. 2D and E). These results suggested that the mutation of SNP rs2229523 might cause changes of CD73 protein structures and functions.

3.4. CD73 maintained anabolism and suppressed catabolism of chondrocytes extracellular matrix

To explore the role of CD73 in chondrocytes, we knocked down the expression of CD73 through CD73 small-interfering RNA (CD73 siRNA) in mouse primary chondrocytes (Fig. 3A). IL-1β was a common inflammatory mediator and had been widely adopted to mimic inflammatory environments in vitro [21–23]. Considering the association between inflammatory response and chondrocyte metabolism homeostasis, we chose IL-1β to mimic inflammatory environment (Fig. 1C). Col2a1 and Mmp13 were chosen as markers of chondrocyte anabolism and catabolism respectively. Under the stimulation of IL-1β, the expression of Col2a1 was downregulated in mouse chondrocytes while Mmp13 was upregulated, which revealed that the metabolic homeostasis of chondrocytes was impaired both in mRNA and protein levels (Fig. 3A, B, D). When CD73 was knocked down, the anabolism was impaired and the catabolism was promoted in mouse chondrocytes as well. Furthermore, after CD73 was knocked down in chondrocytes treated with IL-1β, the metabolic homeostasis was impaired more severely than that of chondrocytes only received IL-1β treatment (Fig. 3A, B, D). The immunofluorescence staining showed similar results (Fig. 3C–E). To further clarify the role of CD73 in maintaining chondrocyte metabolic homeostasis, we adopted lentivirus to overexpress CD73 in mouse chondrocytes, and detect the expression of Col2a1 and Mmp13 when the chondrocytes received IL-1β treatment or not. When CD73 was overexpressed, the anabolism was enhanced and the catabolism was suppressed in mouse chondrocytes. In addition, after CD73-overexpressed chondrocytes were treated with IL-1β, the metabolic homeostasis was maintained (Fig. 3F, G, I). The trend of immunofluorescence staining results was consistent (Fig. 3H–J). CD73 is the rate-limiting enzyme that catalyzes the conversion of extracellular ATP to adenosine [6]. In order to verify whether CD73 mediated the conversion of extracellular ATP to adenosine in chondrocytes, we detected extracellular ATP and

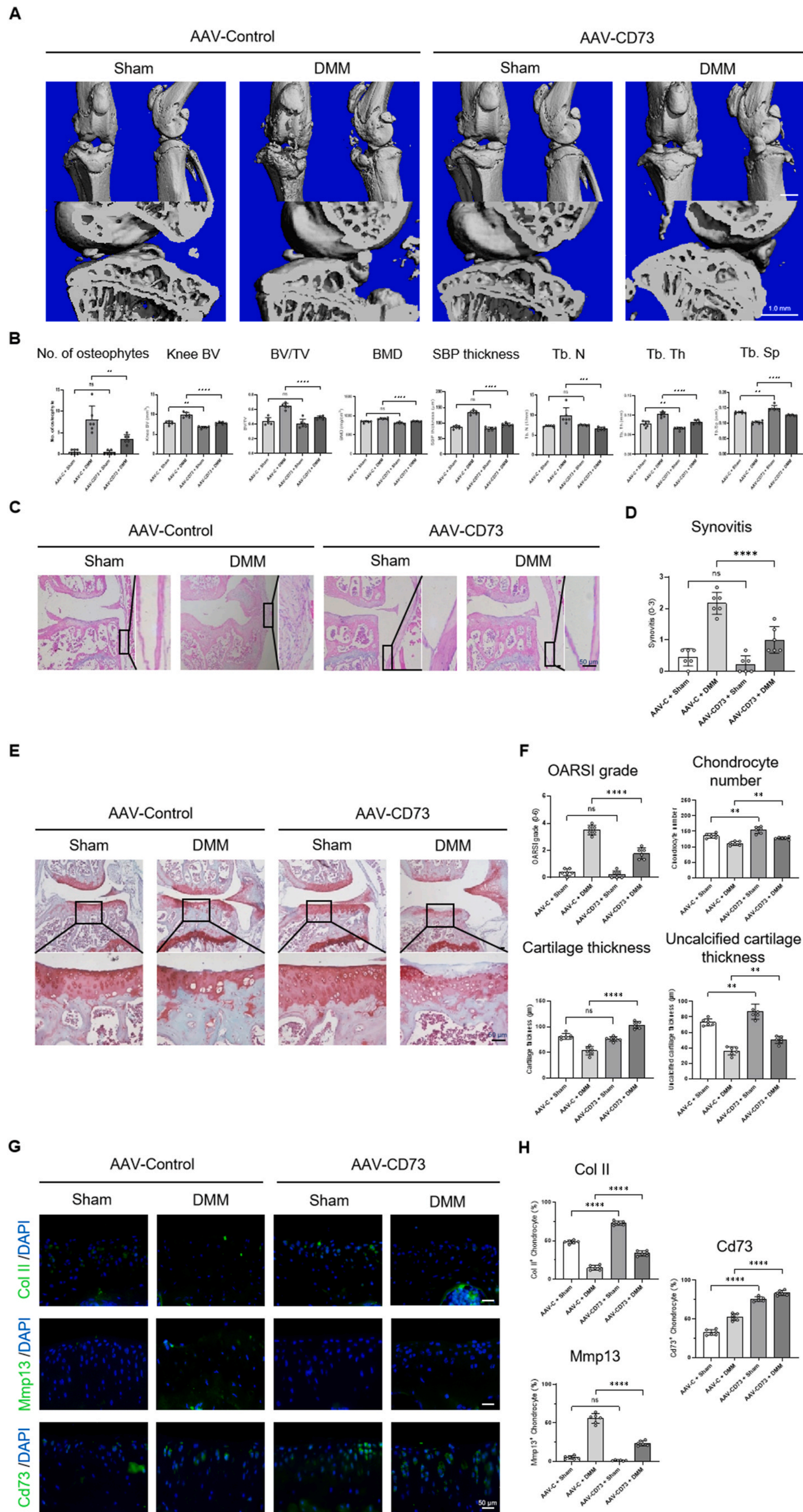


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Fig 4. The overexpression of CD73 attenuates bone remodeling and protects cartilage in DMM mice. A. Three-dimensional images of the mouse knee joint were reconstructed by micro-computed tomography (micro-CT) to highlight changes in the femoral and tibial surfaces. The sagittal images of the medial joint compartment show changes in the thickness of the subchondral bone plate (SBP). B. The quantification of changes in knee bone volume (Knee BV), the ratio of bone volume to tissue volume (BV/TV), bone mineral density (BMD), trabecular number (Tb.N), trabecular thickness (Tb. Th), trabecular separation (Tb.Sp), number of osteophytes and SBP thickness. C. Hematoxylin & Eosin (H&E) staining of mouse knee. D. The quantification of H&E staining (synovitis grade). E. Safranin-O/fast green (S.O.) staining. F. The quantification of S.O. staining, including OARSI grade, chondrocyte number, cartilage thickness and uncalcified cartilage thickness. G. Immunofluorescence staining of the mice knees. H. The quantification of immunofluorescence staining. Data ($n = 6$) were shown as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

adenosine level of mouse chondrocytes knocked down CD73 or overexpressed CD73 treated with or without IL-1β. The extracellular ATP level significantly decrease in mouse chondrocytes treated with IL-1β alone but not in chondrocytes treated with CD73 siRNA alone (Fig. 3K). Under inflammatory environment induced by IL-1β, knocking down CD73 in chondrocytes could not significantly alter extracellular ATP level as well (Fig. 3K). However, the extracellular adenosine level showed remarkable decrease in chondrocytes treated with IL-1β alone or CD73 siRNA alone (Fig. 3K). Besides, when chondrocytes knocked down CD73 were treated with IL-1β, the extracellular adenosine level was further reduced (Fig. 3K). On the other hand, overexpressing CD73 in mouse chondrocytes could decrease extracellular ATP level but restore extracellular adenosine level under inflammatory environment (Fig. 3L). These results suggested that CD73 could maintain anabolism and suppress catabolism in chondrocytes under inflammatory environment, and mediate the conversion of extracellular ATP to adenosine, which indicated that CD73 might be one of the therapeutic targets for OA progression.

3.5. The overexpression of CD73 attenuated subchondral bone remodeling and cartilage degeneration in DMM mice

The *in vivo* experiments were conducted to investigate the role of CD73 in OA. Intra-articular injected adeno-associated virus was adopted to overexpress CD73 in knees of DMM mice. The pathological characteristics of OA also include bone remodeling [24]. To evaluate the function of CD73 in OA, we analyzed the bone status of the knee joint in mice through micro-computed tomography (micro-CT) analysis. Three-dimensional (3D) reconstruction of the mouse knee joint showed increased number of osteophytes and subchondral bone sclerosis in DMM mice, with unsmooth bone surfaces of the tibia and femur and increased knee bone volume (Fig. 4A and B). Compared with mice in the sham group, micro-CT analysis of the subchondral bone region revealed that DMM mice exhibited increased ratio of bone volume to tissue volume, bone mineral density (BMD), subchondral bone thickness (SBP thickness), trabecular number (Tb. N), trabecular thickness (Tb. Th) and decreased trabecular separation (Tb. Sp). These OA related pathological changes were ameliorated in DMM mice with the overexpression of CD73 (Fig. 4A and B). We also assessed the grade of synovitis and the integrity of articular cartilage by hematoxylin & eosin (H&E) staining and Safranin-O/fast green (S.O.) staining. The results showed severe synovitis, evident cartilage degradation and cartilage thickness reduction in DMM mice, while the degree of synovitis and degeneration of cartilage in DMM mice overexpressed CD73 were alleviated obviously as revealed by decreased synovitis grade, Osteoarthritis Research Society International (OARSI) score, increased chondrocyte count, and improved cartilage thickness (Fig. 4C–F). In order to figure out whether the overexpression of CD73 maintained anabolism and suppressed catabolism of chondrocytes extracellular matrix in DMM mice, we determined the expression of Col II and Mmp13 in the mice knee sections by immunofluorescence staining. Compared with sham group, the homeostasis between anabolism and catabolism of chondrocytes extracellular matrix was impaired in DMM mice indicated by downregulated Col II and upregulated Mmp13. However, this disruption of chondrocytes extracellular matrix anabolism-catabolism homeostasis was alleviated in DMM mice overexpressed CD73 (Fig. 4G and H).

3.6. The overexpression of CD73 improved physical activities of DMM mice

We further analyzed the physical activities of the mice by behavioral experiments to evaluate mice knee function. Mice were subjected to an open field test (OFT) to record their spontaneous activity over 3 min (Fig. 5A). The results showed that the relative activity, active time, activity distance, and the mean speed significantly declined after DMM surgery relative to sham, which was remarkably reversed by the overexpression of CD73 (Fig. 5B). Furthermore, we assessed the pain and gait conditions by footprint experiments [25]. We observed that the overexpression of CD73 significantly reduced the pain of DMM mice, which was indicated by the improvement of the stride length and step length and the shorten of the front/rear print length (Fig. 5C and D). Therefore, the overexpression of CD73 improved physical activities of DMM mice.

4. Discussion

It has been reported that adenosine played an important role in OA [26,27]. In this study, we mainly focused on CD73, the rate-limiting enzyme in adenosine extracellular synthesis. We revealed the association between CD73 and OA, and firstly reported the variants of SNP rs2229523 on *NT5E* (the gene encoded CD73) significantly associated with OA population. The mutation of SNP rs2229523 (base A to G) might cause the changes of CD73 protein structures and functions of combining with AMP, which raised our concerns about the function of CD73 in OA. At last, we discovered that CD73 played the role of relieving OA by maintaining anabolism and suppressing catabolism of chondrocytes extracellular matrix, which indicated that CD73 might be a novel therapeutic target for alleviating OA progression.

Interestingly, considering CD73 was upregulated in OA population and catalyzed the conversion of ATP to adenosine, intra-articular injection of adenosine could still alleviate OA [8,9]. We hypothesized that the upregulation of CD73 *in vivo* was a self-regulatory mechanism in OA conditions, but the adenosine produced by upregulated CD73 is still not sufficient to prevent OA progression [9]. The molecular mechanisms leading to this phenotype are currently unknown. Another interesting finding of this research was that adenosine receptor ADORA2A showed significant upregulation in OA cartilage, which was consistent with published data as well [9]. CD73 mediated the synthesis of extracellular adenosine, and adenosine exerted physiological effects through adenosine receptors, which indicated that ADORA2A might be the downstream molecule of CD73 in OA.

There were still some limitations in this research. Firstly, despite the findings from OA cartilage, ADORA2A was not included in the database for bioinformatics analysis. Secondly, the baseline characteristics of patients in the two groups in the case–control study showed significant differences. Considering the DNA sequence would not be changed by different baseline characteristics, the validation of SNPs data in this study would not be severely affected. Thirdly, we had tried to search for the eligible SNPs on the ENTPD1 gene under the SNPs screening criteria in this research but failed, although the expression CD39 (encoded by ENTPD1) showed no significant changes in OA cartilage in this study. In addition, despite we reported the mutation of SNP rs2229523 in OA population, targeted intervention for SNP rs2229523 was not conducted in this study. Last but not least, despite of findings from Open Filed Test and footprint experiments, we did not adopt Von Frey fiber test to

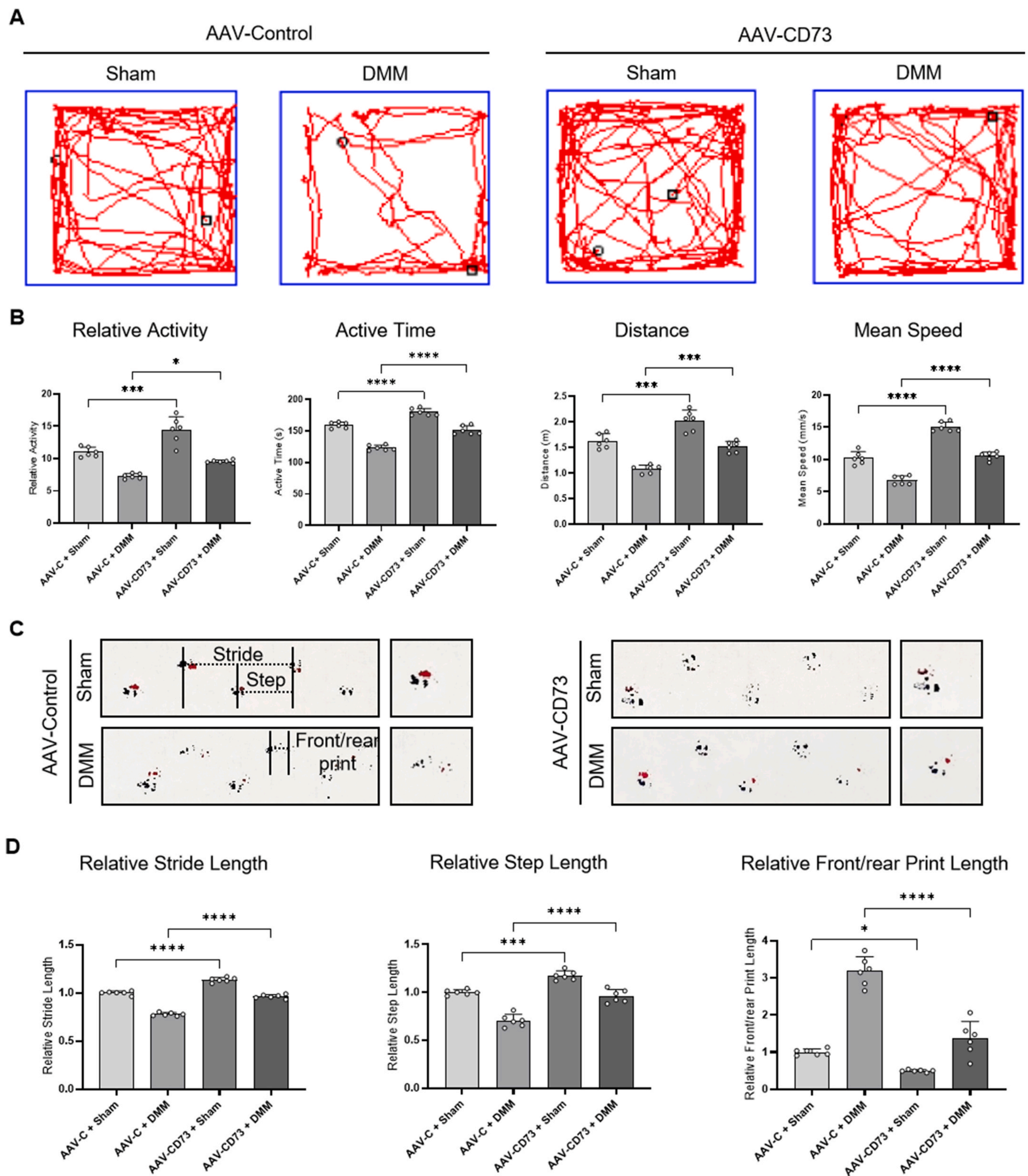

Fig 5. The overexpression of CD73 improves physical activities in DMM mice. A. Representative trajectory plots show that the spontaneous activity of mice after DMM surgery decreases in the open field test. B. The quantification of changes in spontaneous activity, including relative activity, active time, distance, and mean speed. C. The footprints of the two front paws of the manipulated mice were marked with red ink and the footprints of the two hind paws were marked with blue ink. Representative pictures of the footprints of each group. D. The quantification of relative step length, relative stride length, and relative front/rear print length of each group. Data (n = 6) were shown as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

further assess the pain sensitivity of the mice. Further research on deeper molecular mechanisms behind findings in this study was still needed.

5. Conclusions

In this study, we revealed the association between CD73 and OA, and the variants of SNP rs2229523 (base A to G) on *NT5E* were significantly higher in OA population. Furthermore, we discovered the function of maintaining chondrocytes anabolism and suppressing catabolism of CD73 under inflammatory environment, which indicated that CD73 might be a novel therapeutic target for alleviating OA progression.

Conflicts of interest

The authors declare that they have no competing interests.

Funding

This work was supported by National Science Foundation of China (82325035, 82172481, 32271409), Six Talent Peaks Project of Jiangsu Province (WSW-079), Innovation Project of National Orthopedics and Sports Medicine Rehabilitation Clinical Medical Research Center (2021-NCRC-CXJJ-ZH-16).

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethical Committee of the Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University (2,009,022). The animal study protocol was approved by the Animal Care and Use Committee of Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University (2020AE01102).

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2024.08.014.

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