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LncRNA PMS2L2 protects ATDC5 chondrocytes against lipopolysaccharide-induced inflammatory injury by sponging miR-203

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ABSTRACT

Aims: PMS1 Homolog 2, Mismatch Repair System Component Pseudogene 2 (PMS2L2) has been reported as an up-regulated long non-coding RNA (lncRNA) in osteoarthritis (OA) tissues. The purpose of the present work is to explore whether the differently expressed PMS2L2 is associated with the pathogenesis of OA.

Main methods: Chondrogenic ATDC5 cells were exposed to various doses of lipopolysaccharide (LPS). The expression of PMS2L2, miR-203, and MCL-1 in cell was altered by transfection. Thereafter, cell viability, apoptosis, the expression changes of apoptosis-related factors and the release of pro-inflammatory factors were respectively assessed. Moreover, the regulatory relationship between PMS2L2 and miR-203, as well as between miR-203 and MCL-1 were studied.

Key findings: PMS2L2 expression was down-regulated following LPS stimulation. PMS2L2 protected ATDC5 cells against LPS-induced injury by increasing cell viability, decreasing apoptosis, and repressing the release of pro-inflammatory factors. Meanwhile, PMS2L2 increased the expression levels of COL2A1 and ACAN, while down-regulated the expression levels of MMP13 and ADAMTS-5. PMS2L2 worked as a molecular sponge for miR-203. Besides, miR-203 overexpression partially abolished the chondroprotective effects of PMS2L2. MCL-1 was a direct target of miR-203, and it exerted the similarly chondroprotective effects as PMS2L2. Furthermore, PMS2L2 and MCL-1 blocked Wnt/β-Catenin and JAK/STAT signaling pathways also via a miR-203-dependent manner.

Significance: Our study reveals a protective role of PMS2L2 in LPS-induced inflammatory injury in chondrocytes. PMS2L2/miR-203/MCL-1 axis may serve as a new gene therapy strategy for the treatment of OA.

1. Introduction

Osteoarthritis (OA) is a common chronic condition which may lead to local inflammatory responses, tissue damages and cartilage structure abnormality [1]. Many factors are associated with an increased risk of OA, including genetic, mechanical, and environmental factors [2,3]. Despite every age can be affected with this disease, prevalence increases dramatically with age with a greater incidence in subjects between 40 and 50 years old [4]. Early in 1998, significant correlations between chondrocyte apoptosis and OA grade has been reported [4]. Besides, recent literatures demonstrated that pro-inflammatory factors such as tumor necrosis factor (TNF) and interleukins (ILs) produced by chondrocytes will further promote the apoptosis of chondrocyte [5,6]. Thus, inhibition of chondrocyte apoptosis and inflammatory response has been considered as a promising strategy for prevention and treatment of OA.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs that are longer than 200 nucleotides. They have crucial functions in translation, RNA splicing, and gene regulation [7,8]. Meanwhile, lncRNAs are central regulators of multiple biological processes, including cell growth, proliferation, apoptosis [9], as well as the inflammatory response [10]. Accumulating evidence has suggested that a number of lncRNAs are implicated in the onset and progression of OA. For example, CIR contributes to the degradation of extracellular matrix (ECM) degradation and may serve as a potential target for OA therapy [11]. PCGEM1 may represent a novel diagnostic indicator in OA synoviocytes, and it plays a significantly role in regulating synoviocyte apoptosis, autophagy and proliferation [12]. PMS1 Homolog 2, Mismatch Repair System Component Pseudogene 2 (PMS2L2) is an lncRNA locates on human chromosome 7 with the coding length of 2401 bp. By

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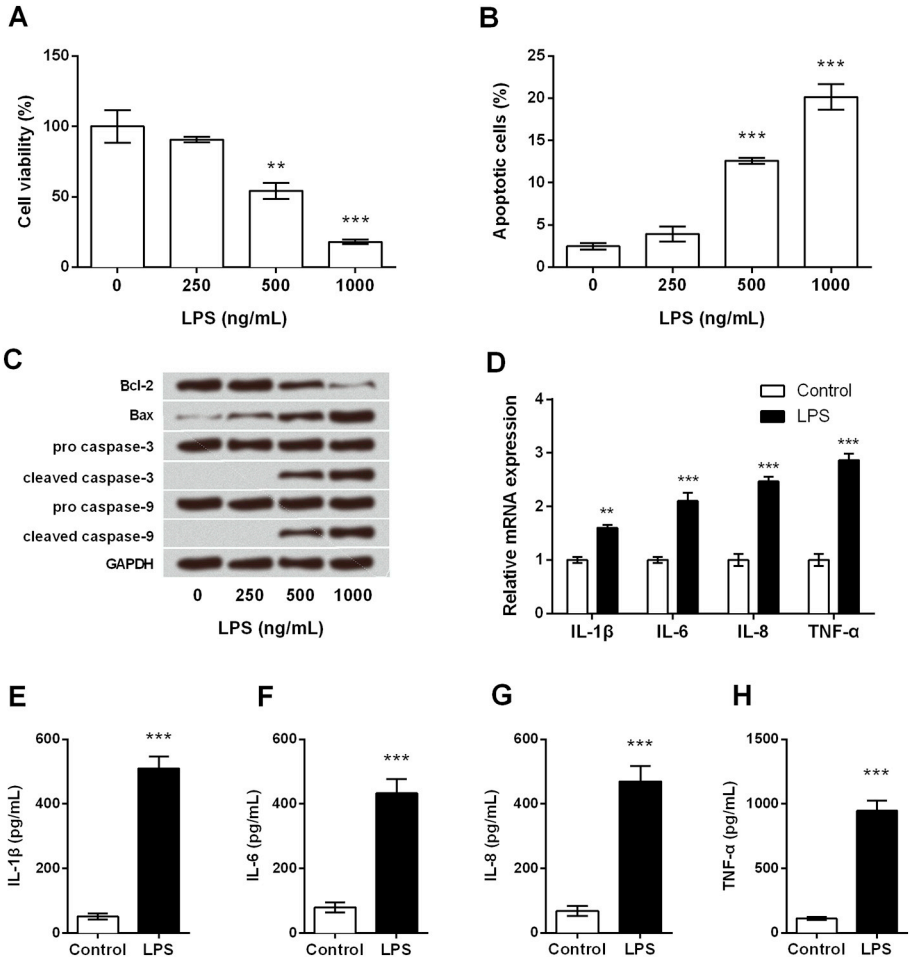

Fig. 1. LPS-induced inflammatory injury in ATDC5 cells. Various concentrations of LPS were used for stimulating ATDC5 cells for 12 h. (A) Cell viability, (B) apoptotic cells, and (C) the expression of apoptosis-related proteins were respectively measured. After treatment with 500 ng/mL LPS for 12 h, (D) mRNA levels of pro-inflammatory factors, as well as (E–H) concentrations of these factors in the culture supernatant were detected. Data are presented as the mean ± SD (n = 3 each group). $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA).

using RT-PCR after microarray analysis, up-regulation of PMS2L2 has been found in OA compared with normal tissue [13]. However, whether the differently expressed PMS2L2 is associated with the initiation and progression of OA is still unclear. In addition to lncRNAs, microRNAs (miRNAs) is another kind of non-coding RNAs that regulate the expression of target genes by interaction with the 3′-untranslated region (3′-UTR) of mRNAs [14]. Interestingly, lncRNA can crosstalk with miRNA via complex gene regulatory networks. Recently, lncRNAs have been reported as competitive endogenous RNAs (ceRNAs) or molecular sponges in regulating the biological functions of miRNAs [15,16]. LncRNAs work like sponges in having miRNAs exhausted, and thus leaving mRNAs alone without being decayed by miRNAs. miR-203 was found to be highly expressed in several cancers, and it was mainly identified as a tumor suppressor by controlling tumor cells growth and metastasis [17,18]. In the regard of OA, miR-203 was low expressed in OA synovial fibroblasts compared to rheumatoid arthritis synovial fibroblasts [19]. Besides, the elevated expression of miR-203 in endothelial cells could diminish an anti-inflammatory activity and increase the endothelial inflammatory response in preeclampsia [20]. Primo et al., indicated that miR-203 serves to fine-tune cytokine signaling and may dampen skin immune responses by repressing key pro-inflammatory cytokines [21]. Likewise, miR-203 has been reported to be an important regulator in macrophages and chondrocyte against lipopolysaccharide (LPS) [22,23]. These authors demonstrated miR-203 as an inflammatory response miRNA.

The purpose of the present work was to investigate the detailed functions of PMS2L2 on LPS-stimulated chondrocyte, in order to reveal the role of PMS2L2 in the onset and progression of OA. Moreover, molecular mechanisms via which PMS2L2 impacted chondrocyte were discovered by focusing on the cross-regulations between PMS2L2 and miR-203.

2. Materials and methods

2.1. Cell culture and LPS treatment

Human cartilage ATDC5 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in complete RPMI-1640 (Gibco, Grand Island, NY) medium supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified incubator containing 5% CO_2 at 37 °C.

LPS (Sigma-Aldrich, St. Louis, MO) were dissolved in cell culture medium yielding solutions with final concentrations of 0, 250, 500, and 1000 ng/mL. For LPS treatment, ATDC5 cells were exposed to these different concentrations of LPS for 12 h.

2.2. Cell transfection

For detection of PMS2L2 and MCL-1, short-hairpin RNA (shRNA) directed against human PMS2L2 and MCL-1 were respectively ligated into the pGPU6 plasmid (GenePharma, Shanghai, China) and were referred as to sh-PMS2L2 and sh-MCL-1. The full-length of PMS2L2 and

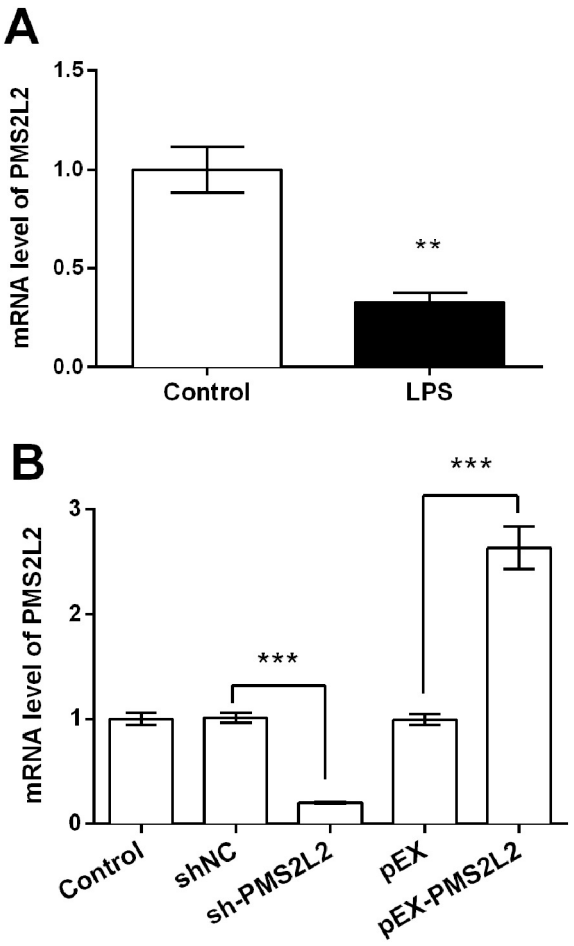

Fig. 2. PMS2L2 expression level was decreased after LPS stimulation. (A) Following 500 ng/mL of LPS treatment for 12 h, the expression level of PMS2L2 in ATDC5 cells were analyzed. (B) ATDC5 cells were transfected with sh-PMS2L2, pEX-PMS2L2 or the negative controls, after which the expression changes of PMS2L2 were tested. Data are presented as the mean ± SD (n = 3 each group). $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA).

MCL-1 sequences were respectively constructed in pEX-2 (GenePharma), and were referred as to pEX-PMS2L2 and pEX-MCL-1. The scrambled shRNA and the empty pEX-2 plasmid were respectively transfected as negative controls (NCs). For detection of miR-203, miR-203 mimic, miR-203 inhibitor, and their NCs, namely mimic control and inhibitor control, were all provided by GenePharma. All transfections were performed by using the Lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) for 48 h, according to the manufacturer's instructions. After selecting with 0.5 mg/mL of G418 (Sigma-Aldrich) for approximately 4 weeks, stable transfected cell line was gotten.

2.3. Cell viability assay

Cells were seeded in 96-well plate with a density of 5×10^3 cells/well, and were stimulated with LPS for 12 h. After another 48 h of incubation in normal medium, 20 μL Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD) was added and the plates were incubated for 3 h at 37 °C. The absorbance of each well was measured using a Microplate Reader (Bio-Rad, Hercules, CA) at 450 nm.

2.4. Apoptosis assay

Cell apoptosis was analyzed using FITC-Annexin V/PI detection kit (Beijing Biosea Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions. In brief, after LPS exposure, 1×10^5 cells in each sample were collected and resuspended in 200 μL binding buffer containing 10 μL FITC-Annexin V. After staining for 20 min in the dark at 37 °C, 5 μL PI was added, and the percentage of intact, necrotic and apoptotic cells were analyzed by the cytometry within 30 min.

2.5. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation) according to the manufacturer's instructions. To test the expression of PMS2L2 and MCL-1, cDNA was synthetized from 5 μg of total RNA by using mRNA Selective PCR kit (TaKaRa, Dalian, China). One Step SYBR® PrimeScript®PLUS RT-RNA PCR Kit (TaKaRa) was utilized in the qRT-PCR analysis. To test the expression of miR-203, 5 μg of total RNA from each sample was subjected to reverse transcription using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed by using the Taqman Universal Master Mix II (Applied Biosystems). PMS2L2, MCL-1 and miR-203 levels were normalized to GAPDH or U6. Fold changes were calculated according to the $2^{-\Delta\Delta Ct}$ method [24]. The primer sequences for testing the expression of miR-203 were listed as follows: stem-loop RT-primer for miR-203: 5′-GTC GTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCUA GUG-3′; miR-203, forward 5′-GGGGTGAAATGTTTAGGAC-3′ and reverse 5′CAGTGCGTGTCGTGGAGT-3′.

2.6. Luciferase reporter assay

The binding sequences in MCL-1 3′UTR were amplified by PCR. The PCR products were inserted into pmirGlO dual-luciferase miRNA target expression vector (Promega, Madison, WI) for constructing the reporter vector MCL-1-wt. The mutant type of MCL-1 was inserted as a negative control (MCL-1-mt). The constructed reporter vectors were co-transfected with miR-203 mimic or mimic NC into cells by using the Lipofectamine 3000 reagent. At 48 h of transfection, cells were collected and relative luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.7. Pull-down assay

Pull-down assay was performed as previously described [25]. The biotinylated (Bio) PMS2L2 or the negative control (Boi-NC) (GenePharma) was incubated with Dynabeads M-280 Streptavidin (Invitrogen, CA, USA) at room temperature for 10 min. Then the same was incubated with equal volume of cellular lysates. The bound RNAs were quantified by using qRT-PCR.

2.8. RNA immunoprecipitation

ATDC5 cells were lysed by using RIPA lysis buffer (Beyotime, Shanghai, China). Beads were washed by 500 μL RIP Wash Buffer, and were incubated with human Argonaute2 (Ago2) antibody or the mouse IgG. Ago2, an essential component of RNA-induced silencing complex (RISC), is capable of loading miRNAs. After 30 min of incubation at room temperature, the antibody-coated beads were incubated with cellular extracts for 4 h at 4 °C. Then the purified RNA was subjected to qRT-PCR to demonstrate the present of the binding targets.

2.9. Enzyme-linked immunosorbent assay (ELISA) for cytokine analysis

The content of cytokines including IL-1β, IL-6, IL-8 and TNF-α in the cell culture supernatants were detected by using the specific ELISA kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instruction. Catalogue numbers of these four kits are E-EL-H0149c, E-ELN-H0102c, E-EL-H0048c and E-EL-H0109c.

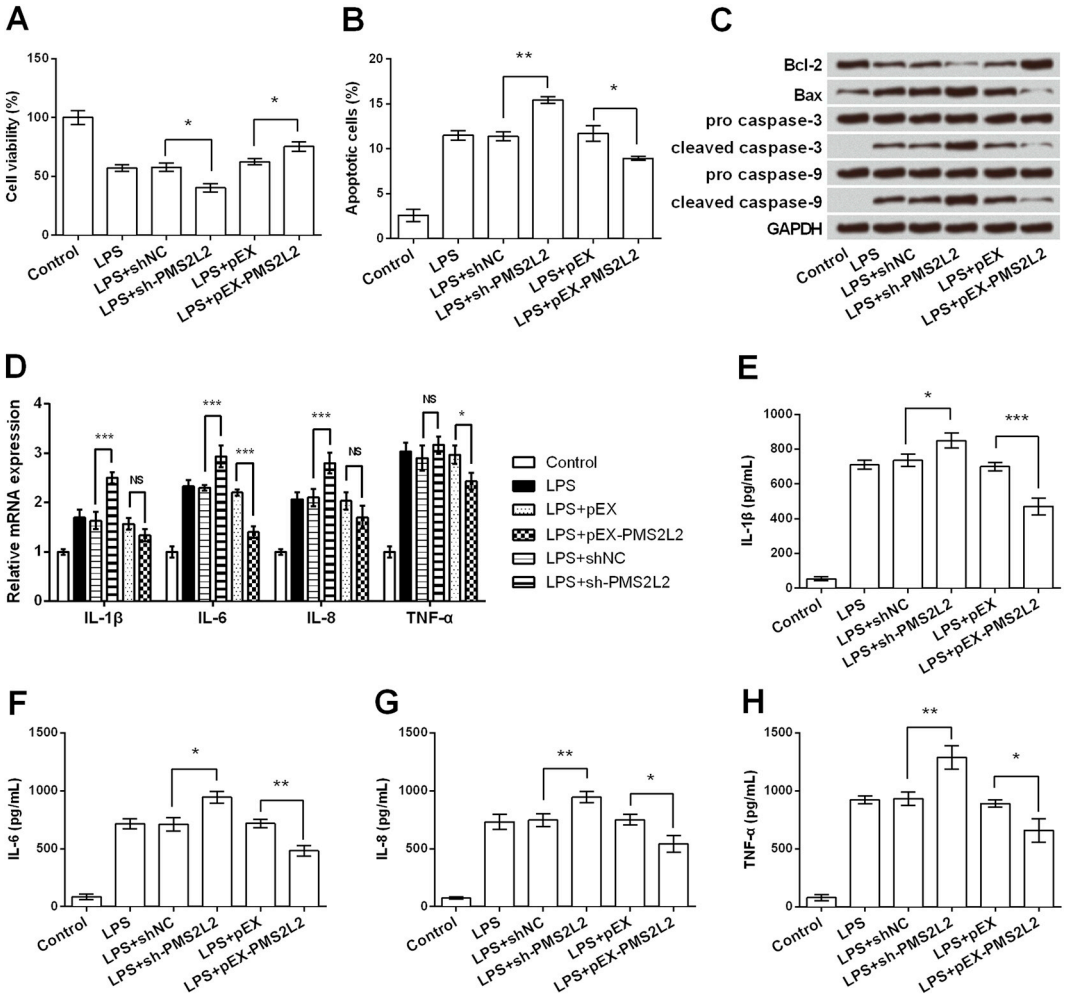

Fig. 3. PMS2L2 alleviated LPS-induced inflammatory injury in ATDC5 cells. ATDC5 cells were transfected with sh-PMS2L2, pEX-PMS2L2 or the negative controls, and were exposed to 500 ng/mL LPS for 12 h. (A) Cell viability, (B) apoptotic cells, (C) the expression changes of apoptosis-related proteins, (D) mRNA levels of pro-inflammatory factors, as well as (E–H) concentrations of these factors in the culture supernatant were respectively measured. Data are presented as the mean ± SD (n = 3 each group). NS, no significance, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA).

2.10. Western blot

Cellular protein used for Western blot analysis was extract by using RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration was determined by BCA Protein Assay Kit (Beyotime). Equal amounts of protein (0.1 mg) from each sample was resolved over SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat dry milk for 1 h at room temperature, and were probed with appropriate primary antibodies at 4 °C overnight. Anti-Bcl-2 (#4223), anti-Bax (#5023), anti-caspase-3 (#14220), anti-cleaved caspase-3 (#9654), anti-caspase-9 (#9508), anti-cleaved caspase-9 (#7232), anti-MCL-1 (#39224), anti-Wnt3a (#2721), anti-β-Catenin (#13727), anti-p-JAK1 (#74129), anti-JAK1 (#3344), anti-p-TYK2 (#68790), anti-TYK2 (#14193), anti-p-STAT1 (#5375), anti-STAT1 (#14994), anti-p-STAT2 (#88410), and anti-STAT2 (#72604) were purchased from Cell Signaling Technology (Inc., Danvers, MA); and anti-Wnt5a (sc-365370), COL2A1 (sc-52658), ACAN (sc-70332), MMP13 (sc-101564), and anti-GAPDH (sc-293335) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA; ADAMTS-5 (ab41037) was purchased from Abcam (Cambridge, MA). Following by incubation with anti-rabbit IgG (#7074) and anti-mouse IgG second antibodies (#7076, Santa Cruz Biotechnology) for 1 h at room temperature, the positive blots were visualized by enhanced chemiluminescence (ECL) method.

2.11. Statistical analysis

Data were form three repeated experiments and were repressed as the mean ± SD. Statistically differences between groups were analyzed using a one-way analysis of variance (ANOVA) in the SPSS version 13.0 software (SPSS Inc., Chicago, IL). A *P*-value of < 0.05 was indicated statistically significant results.

3. Results

3.1. LPS induced inflammatory injury in ATDC5 cells

Various concentrations of LPS (0, 250, 500, and 1000 ng/mL) were used for stimulating ATDC5 cells for 12 h. As a result, cell viability was remarkably reduced after treatment with 500 and 1000 ng/mL of LPS, resulting viability decreases of 54.3 ± 9.7% ($P < 0.01$) and 18.0 ± 3.0% ($P < 0.001$, Fig. 1A). In addition, it seemed that apoptosis was induced by 500 and 1000 ng/mL of LPS treatment, that they respectively increased apoptotic cells rate from 2.5 ± 0.7% to 12.6 ± 0.7% and to 20.2 ± 2.7% (both $P < 0.001$, Fig. 1B and Supplementary Fig. 1). Likewise, LPS stimulation remarkably down-regulated Bcl-2, up-regulated Bax, and activated caspase-3 and caspase-9 (Fig. 1C). No statistical or visible impacts of 250 ng/mL LPS on cell viability and apoptosis were observed. LPS with concentration of

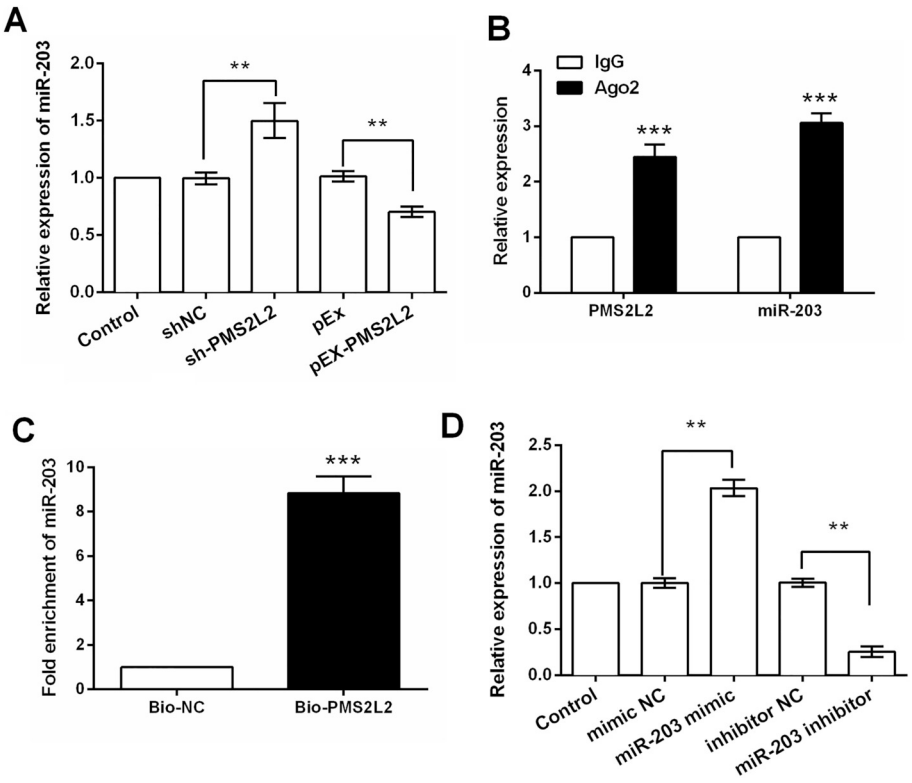

Fig. 4. PMS2L2 worked as a sponge for miR-203. (A) ATDC5 cells were transfected with sh-PMS2L2, pEX-PMS2L2 or the negative controls, and then the expression levels of miR-203 were tested. (B) Detection of miR-203 and PMS2L2 using qRT-PCR in the sample with antibody against IgG or Ago2. (C) Detection of miR-203 using qRT-PCR in the sample pulled down by biotinylated (Bio) PMS2L2 probe. (D) ATDC5 cells were transfected with miR-203 mimic, miR-203 inhibitor, or the negative controls, and then the expression levels of miR-203 were assessed. Data are presented as the mean ± SD (n = 3 each group). $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA).

500 ng/mL was selected for the subsequent experiments.

The impacts of LPS on the release of pro-inflammatory cytokines were then detected. As results shown in Fig. 1D, mRNA levels of IL-1β, IL-6, IL-8 and TNF-α were all greatly up-regulated by 500 ng/mL of LPS ($P < 0.01$ or $P < 0.001$). Besides, much higher concentrations of these four factors were found in the culture supernatant of LPS-treated cells, when compared to those in control cells (all $P < 0.001$, Fig. 1E–H).

3.2. PMS2L2 alleviated LPS-induced inflammatory injury in ATDC5 cells

By using qRT-PCR analysis, we found that LPS could down-regulate PMS2L2 expression, as the RNA level of PMS2L2 was reduced to 0.33 ± 0.09 folds after LPS treatment ($P < 0.01$, Fig. 2A). Based on this finding, we inferred PMS2L2 might be implicated in LPS-induced injury. To confirm this hypothesis, the expression of PMS2L2 in ATDC5 cells was overexpressed or suppressed by transfection with the expressing vector and shRNA specific for PMS2L2.

As shown in Fig. 2B, the expression of PMS2L2 was notably reduced after transfection with sh-PMS2L2, while was increased after transfection with pEX-PMS2L2 (both $P < 0.001$). Results from Fig. 3A–C and Supplementary Fig. 2 showed that the LPS-induced decrease of cell viability and increase of apoptosis were both aggravated by sh-PMS2L2, while were alleviated by pEX-PMS2L2 ($P < 0.05$ or $P < 0.01$). Meanwhile, the mRNA expression and release of IL-1β, IL-6, IL-8 and TNF-α induced by LPS were further increased by sh-PMS2L2 while were suppressed by pEX-PMS2L2 (Fig. 3D–H).

3.3. PMS2L2 alleviated LPS-induced inflammatory injury in ATDC5 cells via down-regulation of miR-203

By performing qRT-PCR analysis, we found that miR-203 level was down-regulated in PMS2L2 overexpressing-cells ($P < 0.01$), while was up-regulated after PMS2L2 was silenced by shRNA transfection ($P < 0.01$, Fig. 4A). RNA pull-down and RNA immunoprecipitation were then performed to test the regulatory relationship between PMS2L2 and miR-203. Fig. 4B showed that when compared to the IgG antibody, both PMS2L2 and miR-203 were pulled down in Ago2 pellet ($P < 0.001$). Data in Fig. 4C showed that, miR-203 was pulled down by a biotin-labeled specific PMS2L2 probe ($P < 0.001$). These indicated that miR-203 could directly bind to PMS2L2.

Next, we altered the expression levels of miR-203 in ATDC5 cells, in order to further detect the role of PMS2L2 in LPS-stimulated cells. qRT-PCR data in Fig. 4D showed that, the expression of miR-203 was successfully overexpressed and suppressed by transfection with the mimic or inhibitor specific for miR-203 (both $P < 0.01$). Data in Fig. 5A–H and Supplementary Fig. 3 showed that miR-203 overexpression significantly abolished the protective effects of PMS2L2 on LPS-induced injury ($P < 0.001$), as PMS2L2 overexpression could not increase cell viability, decrease apoptosis, and repress the release of pro-inflammatory factors when miR-203 was overexpressed.

3.4. MCL-1 alleviated LPS-induced inflammatory injury in ATDC5 cells

MCL-1 is an antiapoptotic member of the BCL-2 protein family whose depletion causes apoptosis [26]. Besides, MCL-1 has been indicated as a key regulator in chondrocyte death [27]. The present work explored the regulation between miR-203 and MCL-1 in ATDC5 cells. Results of qRT-PCR and Western blot analyses revealed that both the mRNA and protein levels of MCL-1 were down-regulated by miR-203 overexpression ($P < 0.05$) while were up-regulated by miR-203 suppression ($P < 0.01$, Fig. 6A and B). The results of dual-luciferase reporter assay showed that miR-203 mimic reduced the activity of the luciferase reporter fused to the 3′-UTR-wt of MCL-1 ($P < 0.01$), rather than the reporter fused to the mt version ($P > 0.05$, Fig. 6C). Altogether, these data suggested MCL-1 was a direct target of miR-203, and it was negatively regulated by miR-203.

Then, the expression of MLC-1 in ATDC5 cells was altered by transfection with the vector or shRNA specific for MCL-1, in order to reveal the functional impacts MCL-1 on LPS-stimulated cells. Transfection efficiency was detected by qRT-PCR and Western blot. As results shown in Fig. 6D and E, the expression of MCL-1 was largely

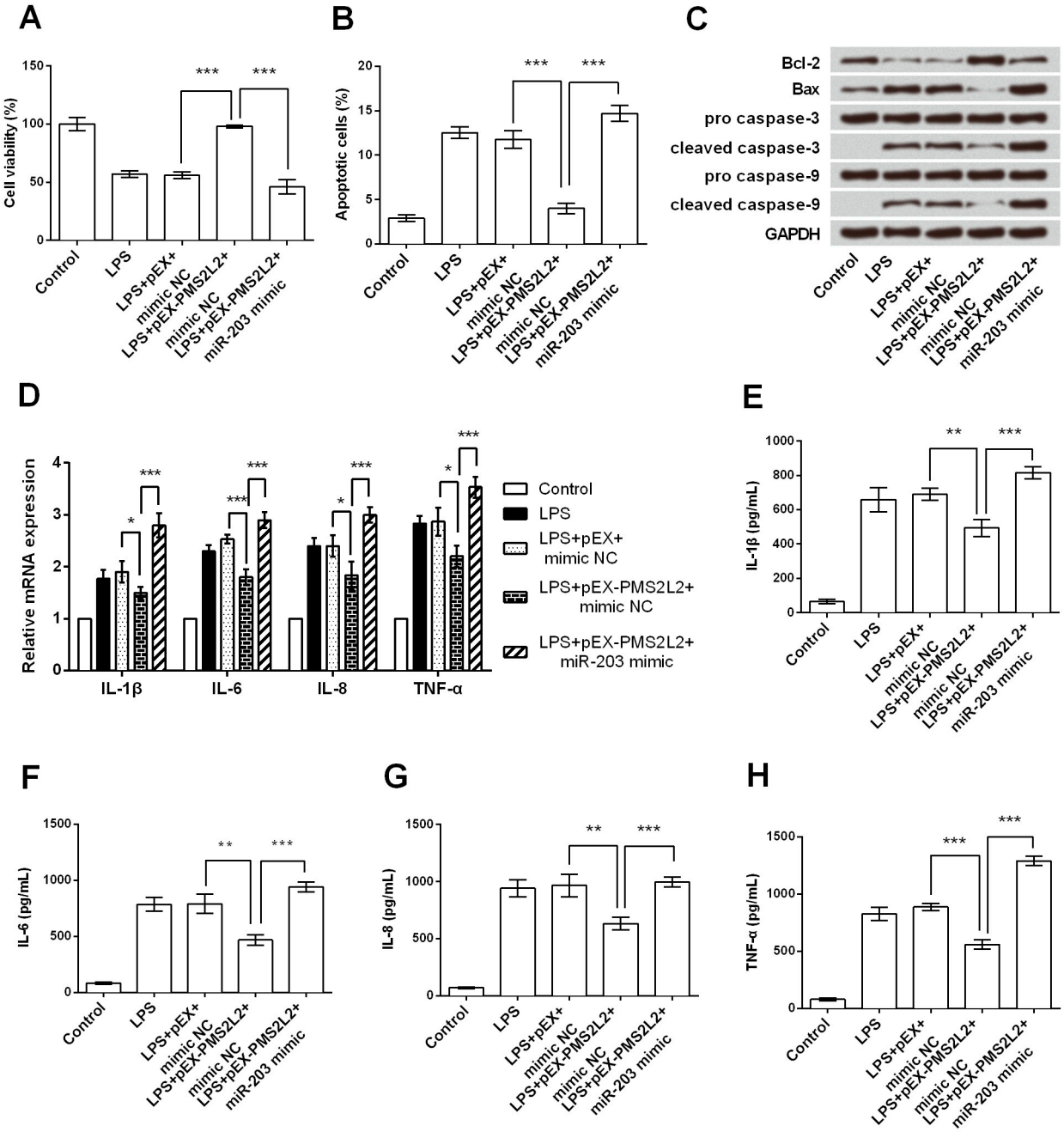

Fig. 5. PMS2L2 alleviated LPS-induced inflammatory injury in ATDC5 cells via down-regulation of miR-203. ATDC5 cells were transfected with pEX-PMS2L2, miR-203 mimic, or the negative controls, and were then exposed to 500 ng/mL LPS for 12 h. (A) Cell viability, (B) apoptotic cells, (C) the expression changes of apoptosis-related proteins, (D) mRNA levels of pro-inflammatory factors, as well as (*E*-H) concentrations of these factors in the culture supernatant were respectively measured. Data are presented as the mean ± SD (n = 3 each group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA).

overexpressed and suppressed after transfection (both $P < 0.001$). More important, we found that MCL-1 exhibited similarly protective functions as PMS2L2 on LPS-stimulated ATDC5 cells, as evidenced by the increase of cell viability ($P < 0.01$), the decrease of apoptotic cells rate ($P < 0.05$), the up-regulation of Bcl-2, the down-regulation of Bax, the deactivation of caspase-3 and caspase-9, and the repression of pro-inflammatory cytokines ($P < 0.01$ or $P < 0.001$, Fig. 7A–H and Supplementary Fig. 4).

3.5. PMS2L2 alleviated the degradation of ECM

Next, the expression changes of ECM-related proteins were detected by using Western blot. Fig. 8A showed that, COL2A1 and ACAN were down-regulated, while MMP13 and ADAMTS-5 were up-regulated by sh-PMS2L2. pEX-PMS2L2 affected the expression of these four proteins resulted in an opposite impact. Fig. 8B showed that, miR-203 mimic could reverse the protein expression changes induced by pEX-PMS2L2, as COL2A1 and ACAN were down-regulated, and MMP13 and ADAMTS-5 were up-regulated when miR-203 mimic was transfected into cell. Results from Fig. 8C, indicated that pEX-MCL-1 remarkably up-regulated COL2A1 and ACAN, while down-regulated MMP13 and ADAMTS-5. Also, sh-MCL-1 affected the expression of these four proteins resulted in an opposite impact.

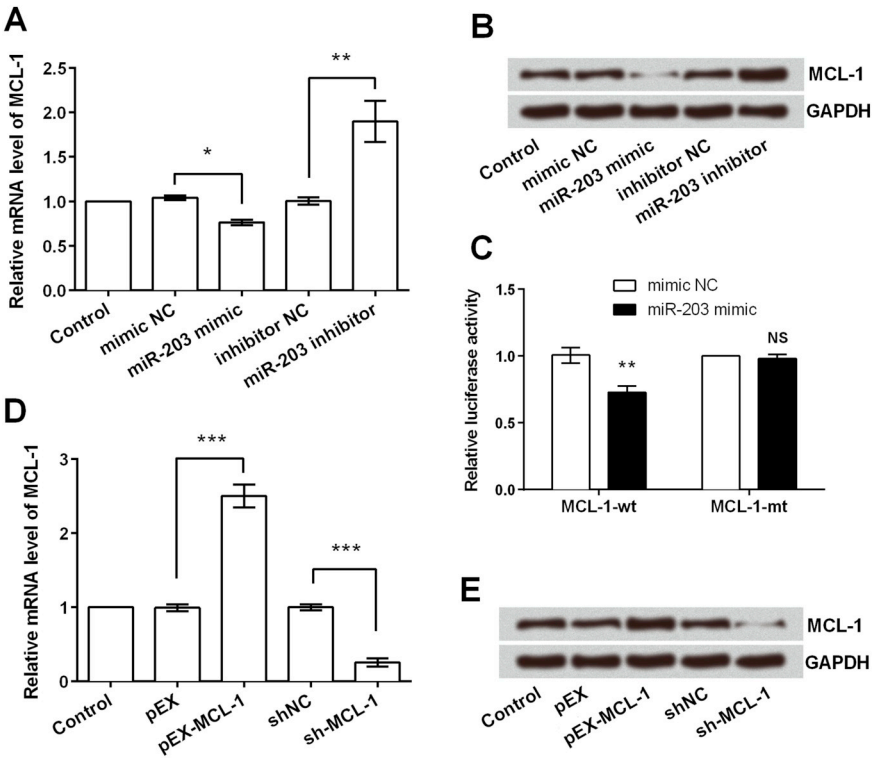

Fig. 6. MCL-1 was a direct target of miR-203. ATDC5 cells were transfected with miR-203 mimic, miR-203 inhibitor or the negative controls, and then the (A) mRNA and (B) protein levels of MCL-1 were tested. (C) Luciferase reporter assay was performed to detect whether miR-203 could bind to the wild type (wt) of MCL-1 3′-UTR. ATDC5 cells were transfected with sh-MCL-1, pEX-MCL-1 or the negative controls, and then the (D) mRNA and (E) protein levels of MCL-1 were tested. Data are presented as the mean ± SD (n = 3 each group). NS, no significance, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA).

3.6. PMS2L2 alleviated LPS challenged ATDC5 cells via Wnt/β-Catenin and JAK/STAT signaling pathways

To further explore the mechanisms via which MCL-1 modulated LPS-stimulated ATDC5 cells, we focused on Wnt/β-Catenin and JAK/STAT signaling pathways. Western blotting results showed that the protein levels of Wnt3a, Wnt5a, β-Catenin, p-JAK1, p-TYK2, p-STAT1, and p-STAT2 were all notably activated by sh-MCL-1, while these proteins were suppressed by pEX-MCL-1 (Fig. 9A and B). Besides, sh-PMS2L2 remarkably up-regulated these protein levels, and the up-regulations induced by sh-PMS2L2 were abolished when miR-203 inhibitor was transfected into cell. Finally, sh-MCL-1 could further up-regulated these protein levels (Fig. 9C and D).

4. Discussion

OA has long been considered as a non-inflammatory disease. However, more recent studies have suggested that low-grade inflammation is linked to disease progression [28,29]. LPS is now known to contribute to low-grade inflammation, which is correlated with OA severity. More importantly, LPS is the main agonist of Toll-like receptor (TLR) 4, which is capable of inducing catabolic responses. Activation of TLR4 by multiple damage-associated molecular-pattern molecules (DAMPs) in joint plays a key role in the onset and progression of OA. In this study, LPS, a potent trigger of inflammation, was used for stimulating chondrogenic ATDC5 cells to mimic an in vitro model of OA. We found that, 500 ng/mL LPS could notably reduce ATDC5 cells viability, induce apoptosis and promote the release of pro-inflammatory cytokines. More importantly, we observed that PMS2L2 protected ATDC5 cells against LPS-induced injury, as evidenced by the increase of cell viability, the decrease of apoptosis, and the repression of inflammatory response and ECM degradation. PMS2L2 worked as a sponge for miR-203, and miR-203 overexpression abolished the chondroprotective functions of PMS2L2. MCL-1 was a direct target of miR-203, and MCL-1 exerted the similarly chondroprotective actions as PMS2L2. Furthermore, it seemed that PMS2L2 and MCL-1 could block the activation of Wnt/β-Catenin and JAK/STAT signaling pathways, while miR-203 could activate these two signaling pathways.

Nowadays, the interest in the regulation of lncRNAs is increasingly growing. LncRNAs are important molecules in regulating diverse biologic functions, including inflammation [30]. A number of lncRNAs with altered expression in response to LPS stimulation has been reported [31]. PMS2L2 was up-regulated in OA cartilage and the elevated expression of PMS2L2 was associated with the pathogenesis of OA by modulation of the expression of MMP-9, MMP13, BMP-2 and ADAMTS5 [13]. In line with this previous study, we found that PMS2L2 up-regulated the protein levels of COL2A1 and ACAN, while down-regulated the protein levels of MMP13 and ADAMTS5. We additionally found that PMS2L2 expression was decreased by 0.33 folds in LPS-treated ATDC5 cells, and our data provided the first in vitro evidences that PMS2L2 could protect LPS-challenged ATDC5 cells. These data suggested PMS2L2 might be implicated in the regulation of LPS-induced inflammatory injury.

miR-203 has been identified as an inflammation-associated miRNA [32]. Elevated levels of miR-203 lead to increased secretion of pro-inflammatory cytokines, such as IL-1α, IL-17A, IL-6, and TNF-α [19,33]. miR-203 also participates in regulating cell proliferation and apoptosis, and it has been identified as a tumor suppressor in several cancers [34]. Consistent with the role of miR-203 in inducing cell apoptosis and inflammation, our results demonstrated that miR-203 could abolish the chondroprotective functions of PMS2L2. Moreover, our data indicated that miR-203 could bind with PMS2LS, suggesting PMS2LS worked as a sponge for miR-203.

Take a step further, we focused on MCL-1 and found it was a direct target of miR-203. This was consistence with a later study, in which MCL-1 was reported to be a target of miR-203, and knockdown of miR-203 alleviated LPS-induced injury by targeting MCL-1 in C28/I2 chondrocytes [23]. MCL-1 is an anti-apoptotic member of the Bcl-2 family that was originally identified as a prosurvival protein [35]. MCL-1 blocks the progression of apoptosis by binding and sequestering the pro-apoptotic proteins (Bak and Bax), which are capable of forming pores in the mitochondrial membrane, allowing the release of cytochrome *c* into the cytoplasm [36]. Previous studies of MCL-1 indicated its expression participate in inflammation response [37]. Besides,

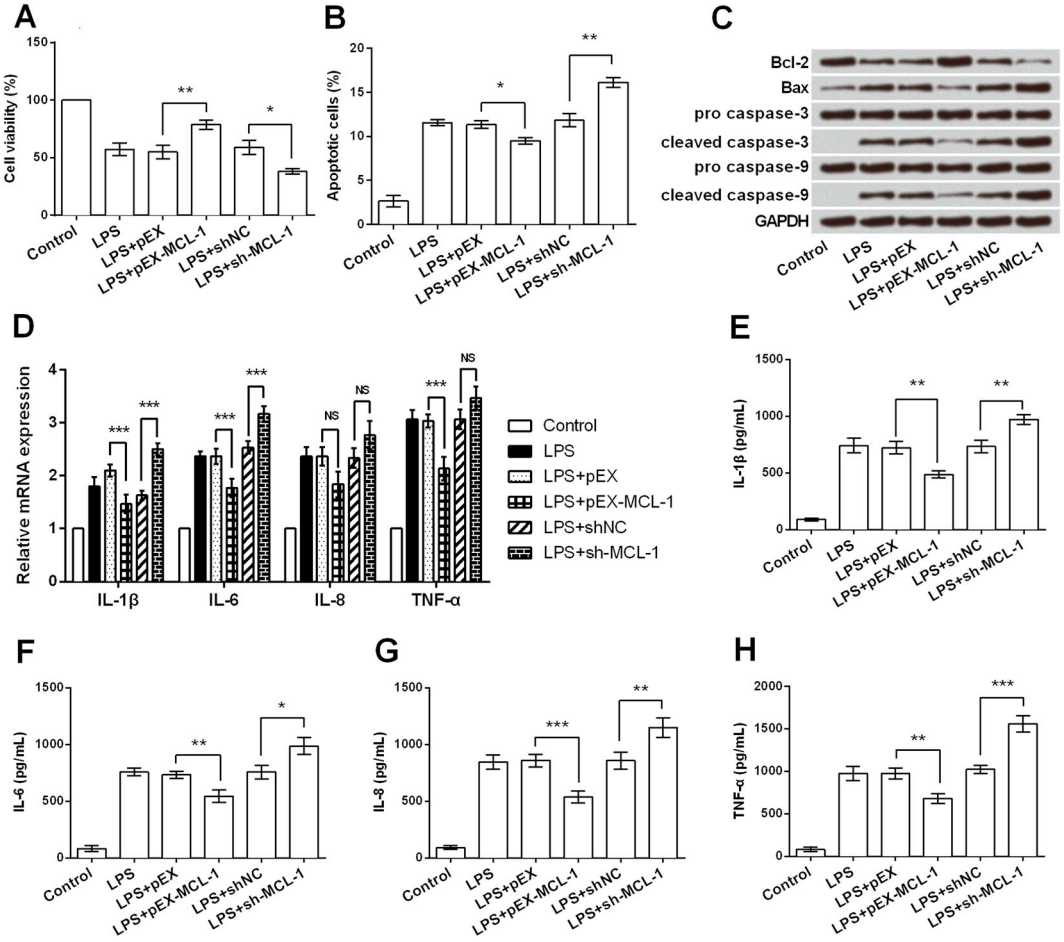

Fig. 7. MCL-1 alleviated LPS-induced inflammatory injury in ATDC5 cells. ATDC5 cells were transfected with pEX-MCL-1, sh-MCL-1 or the negative controls, and then were exposed to 500 ng/mL LPS for 12 h. (A) Cell viability, (B) apoptotic cells, (C) the expression changes of apoptosis-related proteins, (D) mRNA levels of pro-inflammatory factors, as well as (E–H) concentrations of these factors in the culture supernatant were respectively measured. Data are presented as the mean ± SD (n = 3 each group). NS, no significance, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA).

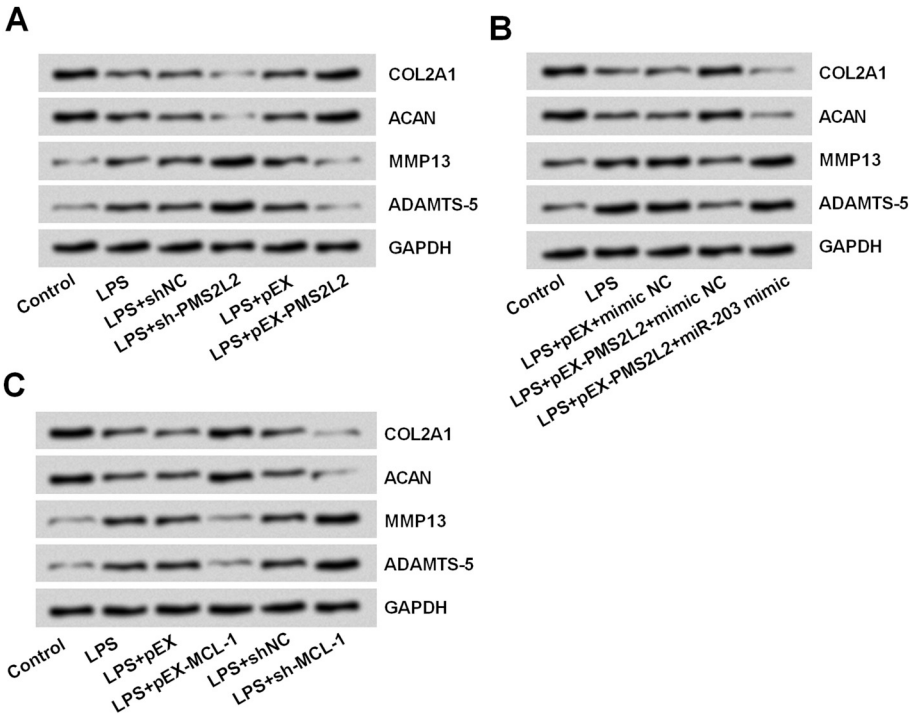

Fig. 8. PMS2L2 alleviated the degradation of extracellular matrix (ECM). The protein levels of COL2A1, ACAN, MMP13 and ADAMTS-5 were assessed after ATDC5 cells were transfected with (A) sh-PMS2L2, pEX-PMS2L2, (B) pEX-PMS2L2, pEX-PMS2L2 plus miR-203, (C) pEX-MCL-1, sh-MCL-1, or the NCs following LPS stimulation for 12 h.

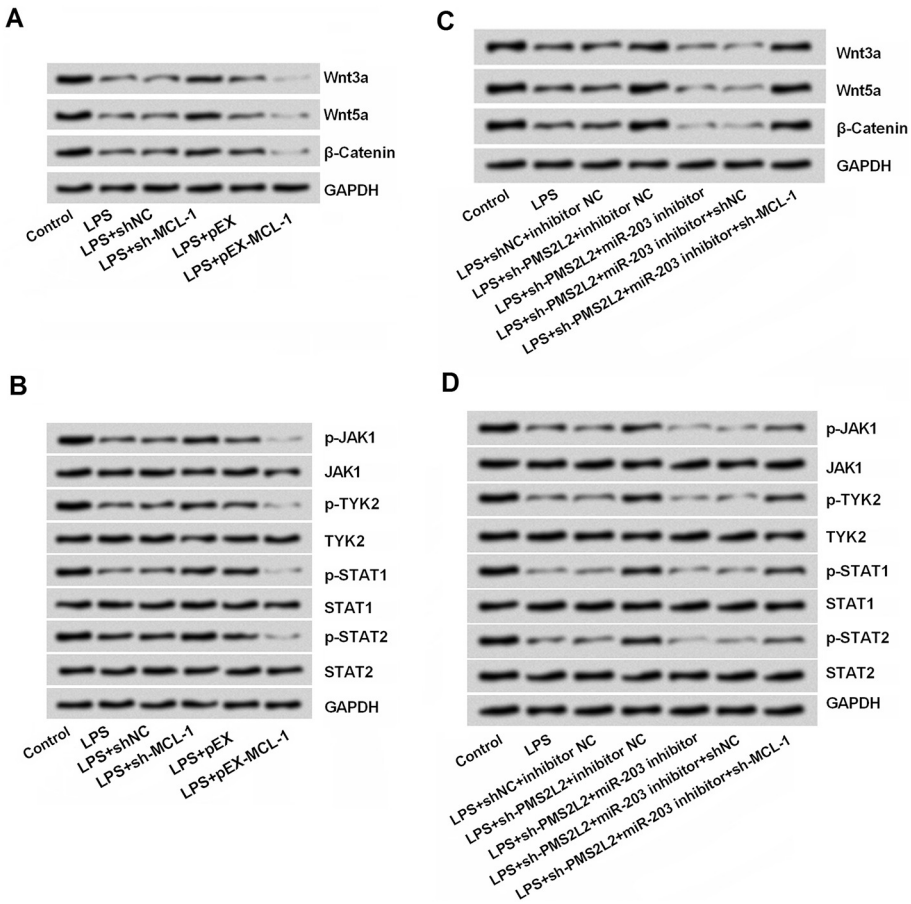

Fig. 9. PMS2L2 deactivated Wnt/β-Catenin and JAK/STAT signaling pathways. ATDC5 cells were transfected with sh-MCL-1, pEX-MCL-1 or the negative controls, and then were exposed to 500 ng/mL LPS for 12 h. The protein levels of main factors in (A) Wnt/β-Catenin and (B) JAK/STAT signaling pathways were measured. ATDC5 cells were transfected with sh-PMS2L2, miR-203 inhibitor, sh-MCL-1 or the NCs, and then were exposed to 500 ng/mL LPS for 12 h. The protein levels of main factors in (C) Wnt/β-Catenin and (D) JAK/STAT signaling pathways were measured.

transcriptional up-regulation of MCL-1 can be induced by a list of inflammatory cytokines, including IL-3 [38], IL-5 [39], IL-6 [40] and etc. In the current study, our data implied a protective role of MCL-1 in LPS-stimulated ATDC5 cells. Combined with the context, we speculated that PMS2L2/miR-203/MCL-1 axis might be a functional targeting of LPS-induced inflammatory injury in chondrocytes.

It is well-established that Wnt/β-Catenin pathway is associated with mechanical force application in joints, and this signaling is activated after mechanical damage to cartilage [41]. In articular chondrocytes, β-Catenin-dependent canonical Wnt signaling is required for progression of endochondral ossification and growth of axial and appendicular skeletons [42], while excessive activation of this signaling causes chondrocyte hypertrophy and ECM degradation, and ultimately leads to OA [43]. In addition to Wnt/β-Catenin, JAK/STAT is considered to be of major importance in driving chronic inflammation in OA [44]. JAK/STAT is involved in MMP13 induction of IL-1β-treated human chondrocytes. Blocking this signaling pathway may have chondroprotective effects in cartilage degeneration [45]. The JAK family contains four members, namely JAK1, JAK2, JAK3 and Tyk2. Upon ligand binding, JAKs phosphorylate the cytokine receptors and induce the recruitment of various signaling proteins, including the STAT family, which directly modulate gene expression as transcription factors [46]. TYK-2 has been known to be involved in ILs and IFNs signal transduction [47]. It has been point out that, Wnt/β-Catenin and JAK/STAT pathways were involved in the protective actions of MCL-1 against LPS induced injury in C28/I2 chondrocytes [23]. In this study, by using Western blot analysis we preliminarily revealed that PMS2L2 and MCL-1 has suppressive impacts on the activation of Wnt/β-Catenin and JAK/STAT pathways, while miR-203 could activate these two signaling. This finding further confirmed the protective role of PMS2L2 in chondrocytes, and provided the evidence that PMS2L2 alleviated LPS challenged chondrocytes might be via Wnt/β-Catenin and JAK/STAT signaling pathways. However, additional experiments are required to reveal whether PMS2L2/miR-203/MCL-1 axis can modulate Wnt/β-Catenin signaling in healthy cartilage, which will largely improve our understanding of this axis in the fine-tuning of Wnt/β-Catenin signaling in the cartilage.

5. Conclusion

To conclude, our study reveals a protective role of PMS2L2 in LPS-induced inflammatory injury in chondrocytes. PMS2L2 may function as a ceRNA for miR-203, preventing MCL-1 from degradation by miR-203. PMS2L2/miR-203/MCL-1 axis may serve as a new gene therapy strategy for the treatment of OA.

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.12.020.

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