Molecules and Cells

Adipose-Derived Stem Cells Suppress Inflammation Induced by IL-1β through Down-Regulation of P2X7R Mediated by miR-373 in Chondrocytes of Osteoarthritis

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Adipose-derived stem cells (ADSCs) were previously considered to have an anti-inflammatory effect, and Interleukin-1β (IL-1β) was found to be a pro-inflammatory factor in chondrocytes, but the mechanism underlying ADSCs and IL-1β is unclear. In this study, we investigate whether P2X7 receptor (P2X7R) signalling, regulated by microRNA 373 (miR-373), was involved in the ADSCs and IL-1β mediated inflammation in osteoarthritis (OA). Chondrocytes were collected from 20 OA patients and 20 control participants, and ADSCs were collected from patients who had undergone abdominal surgery. The typical surface molecules of ASDCs were detected by flow cytometry. The level of nitric oxide (NO) was determined by Griess reagent. Concentrations of prostaglandin E2 (PGE2), interleukin 6 (IL-6), matrix metallopeptidase 3 (MMP-3) were detected by enzyme-linked immunosorbent assay (ELISA). The expressions of IL-6, MMP-3, miR-373 and P2X7R were determined by real-time polymerase chain reaction (PCR), and Western blot was used to detect the protein expression of P2X7R. The typical potential characters of ADSCs were verified. In chondrocytes or OA tissues, the miR-373 expression level was decreased, but the P2X7R expression was increased. IL-1β stimulation increased the level of inflammatory factors in OA chondrocytes, and ADSCs co-cultured with IL-1β-stimulated chondrocytes decreased the inflammation. OA chondrocytes transfected with the miR-373 inhibitor increased the inflammation level. The miR-373 mimic suppressed the inflammation by targeting P2X7R and regulated its expression, while its effect was reversed by overexpression of P2X7R. IL-1β induced inflammation in OA chondrocytes, while ADSCs seemed to inhibit the expression of P2X7R that was regulated by miR-373 and involved in the anti-inflammatory process in OA.

Keywords: adipose-derived stem cells, chondrocytes, miR-373, osteoarthritis, P2X7R

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease and remains the leading cause of disability worldwide (Johnson and Hunter, 2014). OA has long been considered the consequence of many processes resulting in the increase of pressure on a particular joint or cartilage, finally leading to the loss of cartilage (Berenbaum, 2013). Although several research studies have focused on the mechanisms involved in cartilage degeneration, the exact process is still unclear. Clinically, the inflamed synovium contributes to the tissue degeneration of OA (Lambert et al., 2014), and pro-inflammatory cytokine, such as interleukin-1β (IL-1β) and interleukin-6 (IL-6) is always

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elevated in the synovitis (Makki and Haqqi, 2016). Chondrocytes are the resident cells of cartilage in OA, and previous studies have shown that chondrocytes, stimulated by IL-1β, could release NO (Nitric Oxide) and prostaglandin E2 (PEG2), which are inflammatory mediators (Dave and Amin, 2013). Moreover, IL-1β stimulation also increases the production of matrix metalloproteinases (MMPs) in chondrocytes (Fu et al., 2016). Taken together, the chondrocytes' status is important in determining cartilage degeneration, while the related factors such as IL-1β, IL-6, NO, PEG2, MMPs could play an important role in chondrocytes.

Adipose-derived stem cells (ADSCs) have been widely used in several studies because of their convenient isolation and multi-differentiation potential (Kim and Heo, 2014; Shi et al., 2014). Recent studies have verified that ADSCs are related to cell apoptosis, inflammation and immunity (Jiang et al., 2016; van Lent and van den Berg, 2013; Yu et al., 2015). In addition, Jiang et al. reported that the levels of several inflammatory mediators in chondrocytes were interfered with by ADSCs, and the co-cultured ADSCs in chondrocytes manifested an anti-inflammatory effect (2016), and Lai et al. (2013) had found that the co-cultured ADCSs also reduced chondrocytes' differentiation and apoptosis. However, the mechanism of ADSCs mediating the inflammation in chondrocytes remains obscure.

MicroRNA (miRNA) is a class of non-coding RNA with a length of 18-23 nt that functions in the post transcription period in gene expression (Lohse et al., 1975). Numerous studies have supported the theory that miRNA regulation is related to the onset and progression of various human diseases. Conversely, miRNAs also act as crucial regulators in the biological processes in human diseases (Aguero et al., 2017; Fechner et al., 2017). Up until now, hundreds of miRNAs were found to be selectively expressed in degenerative diseases, and other studies also reported miRNA in osteoarthritis regulation (Karlsen et al., 2016; Papanagnou et al., 2016). In 2008, one study found that miR-373 induces gene expression by targeting a complementary sequence in the E-cadherin promoter (Place et al., 2008). Now, studies have revealed that miR-373 expresses specifically in a variety of human diseases, such as human esophageal cancer (Lee et al., 2009), epithelial ovarian cancer (Meng et al., 2016) and breast cancer (Eichelser et al., 2014). And another study discoveredthat miR-373 is down-regulated in chondrocytes (Song et al., 2015). Moreover, the MiRDB database predicted that miR-373 would bind with P2X7R 3'UTR, but the relationship between them remains unconfirmed.

In this study, we examined 20 osteoarthritis patients and 20 normal people, and co-cultured ADSCs with chondrocytes, which were stimulated by IL-1β, to investigate whether ADSCs mediated the expression of P2X7R that targeted by miR-373 was involved in the IL-1β induced inflammation in chondrocytes of OA.

MATERIALS AND METHODS

Isolation of human ADSCs and chondrocytes

Adipose tissues were collected from the patients who had undergone abdominal surgery. Briefly, the bilateral groin fat pads were gathered without the blood vessels and fascia in a sterile environment. Collagenase I was used for the digestion, and then the ADSCs were collected after being filtrated.

We then recruited 20 OA patients and 20 control participants to obtain chondrocytes. The knee cartilage was collected and digested with 0.25% Trypsin-EDTA and 0.25% Trypsin-EDTA. After being centrifuged, the chondrocytes were collected.

All participants in this research provided written, informed consent, and this study was approved by the ethics committee at First Affiliated, College of Medical Zhejiang University.

Cell culture

ADSCs and chondrocytes were cultured with Dulbecco's modified Eagle's medium (DMEM), supplemented with a specific concentration of glucose, 1% penicillin and 10% fetal bovine serum at 37℃, containing 5% CO_2 in a humid atmosphere, which served as a control. The chondrocytes were first pre-treated with 10 ng/ml of IL-1β for 2 h. Then, for the co-culture, ADSCs were cultured in the upper layer of a Transwell with a density of 7×10^4/well, while the chondrocytes were cultured in the bottom layer of the transwell with a density of 5×10^5/well (the density ratio of ADSCs to chondrocytes was 1:7). In the transwell, all cells were cultured with DMEM, containing specific concentrations of glucose, moderate proline, ascorbic acid and sodium pyruvate. After 24 h, the Griess reagent assay experiments, ELISA and real-time PCR were performed.

Flow cytometric analysis

ADSCs cells were collected and the immunophenotypes were detected with a flow cytometer (Becton, Dickinson and Company, US). Briefly, about 1×10^6 cells and 100 μl flow cytometric staining buffer were added to each tube and incubated with the 5 μl antibodies of CD29, CD90, CD49, CD45, CD34 and CD14 at 4℃ for 30 min. Then the cells were centrifuged and resuspended in PBA. Finally, BD FACSCalibur™ was used for the analysis.

Measurement of NO, PGE2,IL-6 and MMP-3

After the co-culture, the level of NO in chondrocytes was measured using Griess reagent assay (Enzo, Switzerland), per the manufacturer's instructions. The concentration of PGE2, IL-6 and MMP-3 in chondrocytes was detected with an ELISA kit (Takara, USA), per the manufacturer's instructions.

Real-time PCR

Total RNA was isolated from cells and tissues using a TRIzol reagent (Invitrogen, USA), per the manufacturer's instructions. The RNA quality was quantified with a NanoDrop (USA). Then 1 μl RNA was used for the reverse transfection, per instructions from a reverse transcription kit (Takara, USA). A PCR assay was performed with the ABI 7900HT Fast Real-time PCR system to detect the mRNA expression of IL-6, MMP-3, miR-373 and P2X7R. The primers used in the experiment were synthesized by BGI Technologies Co., Ltd. (Shenzhen, China).

Western blot

The protein was isolated for ADSCs and chondrocytes. Briefly, cells were rinsed in a phosphate buffer and lysed in RIPA lysis buffer. Then a centrifuge was used for the protein collection. The protein was quantified by a BCA Protein Assay Kit (Thermo Fisher). SDS- polyacrylamide gel electrophoresis (PAGE) was used to separate the protein extracts, and the protein was then transferred into polyvinylidenedifluoride (PVDF) membranes. The primary antibody of P2X7R was diluted to a certain concentration and incubated with membranes at 4℃ for 24 h. Next, the secondary antibodies were incubated with the membranes for another 2 h. Finally, the ECL was used to visualize the bands. The β-actin was selected as the internal control.

Cell transfection

Chondrocytes were isolated and cultured in a 96-well plate. The miR-373 mimic, the miR-373 inhibitor and their negative control (NC) were transfected into chondrocytes by Lipofectamine 2000 reagent (Invitrogen, USA). Real-time PCR was used to determine the transfection efficiency. The NC, miR-373 mimic and the miR-373 inhibitor were synthesized by BGI Technologies Co., Ltd. (China). The P2X7R overexpression was constructed with a P2X7R expression plasmid. After the vector was successfully constructed, it was harvested and transfected into chondrocytes cells.

Luciferase activity assay

Genbank (NM-001080927.2) was used to select the sequence of P2X7R. The P2X7R gene was cloned into the specific vector to establish the luciferase reporter plasmid (Luc-P2X7R-3′UTR-Mut and Luc-P2X7R-3′UTR-WT). Then the miR-373 mimic or the miR-373 inhibitor and the Luc-P2X7R-3′UTR-Mut or Luc-P2X7R-3′UTR-WT were co-transfected into cells to detect the relatively luciferase activity as well as the expression level of P2X7R.

Statistical analysis

Data were expressed as means±standard deviation (SD). SPSS 18.0 was used to analyse the data. One-way analysis of variance (ANOVA) with a *t*-test was used to determine the statistical difference. *P < 0.05 was considered a statistically significant difference.

RESULTS

Immunophenotypes of ADSCs and the effects of IL-1β and ADSCs on chondrocytes

ADSCs isolated from the abdominal surgery patients were used to detect the characteristics, as shown in Fig. 1A. The positive rates of CD29, CD90, CD49 were higher, while the positive rates of CD45, CD34 and CD14 were significantly lower, indicating the typical biological features of ADSCs. To detect the influence of IL-1β and ADSCs on OA chondrocytes' inflammation, the chondrocytes isolated from OA patients were incubated with IL-1β or pre-treated with IL-1β and then incubated with ADSCs. The results demonstrated that the level of NO (Fig 1B), the concentration of PGE2, IL-6 and MMP-3 (Figs. 1C, 1D, and 1F, respectively) and the expression of IL-6 and MMP-3were elevated (Figs. 1E and 1G, respectively) in IL-1β stimulated chondrocytes, compared with the control. In contrast, the levels of the inflammation-

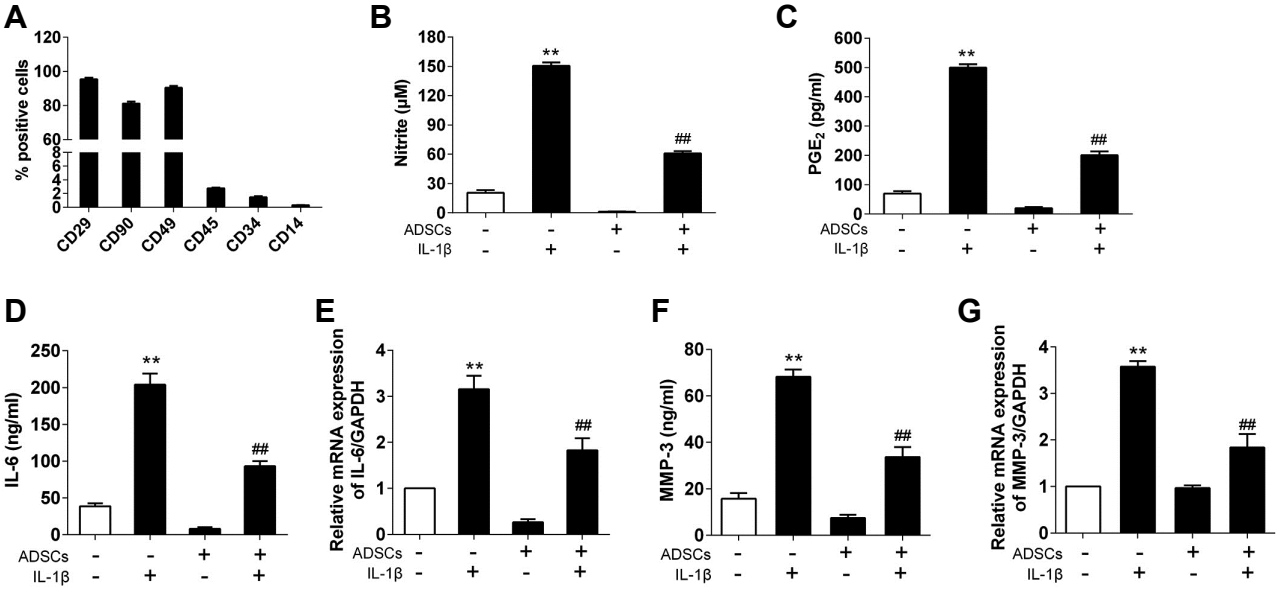

Fig. 1. Immunophenotypes of ADSCs and the co-culture of ADSCs with chondrocytes pre-treated by IL-1β. (A) Characteristics of ADSCs were detected by the method of flow cytometrc analysis. Chondrocytes treated with 10 ng/ml IL-1β for 2 h, and then with or without the co-culture of ADSCs for 24 h, (B) the level of NO was examined by Griess reagent; (C, D, and F) the concentrations of PGE2, IL-6 and MMP-3 were determined by ELISA; (E, G) real-time PCR was used to detect the expressions of IL-6and MMP-3. **P < 0.05 vs control, ##P < 0.05 vs IL-1β treated chondrocytes.

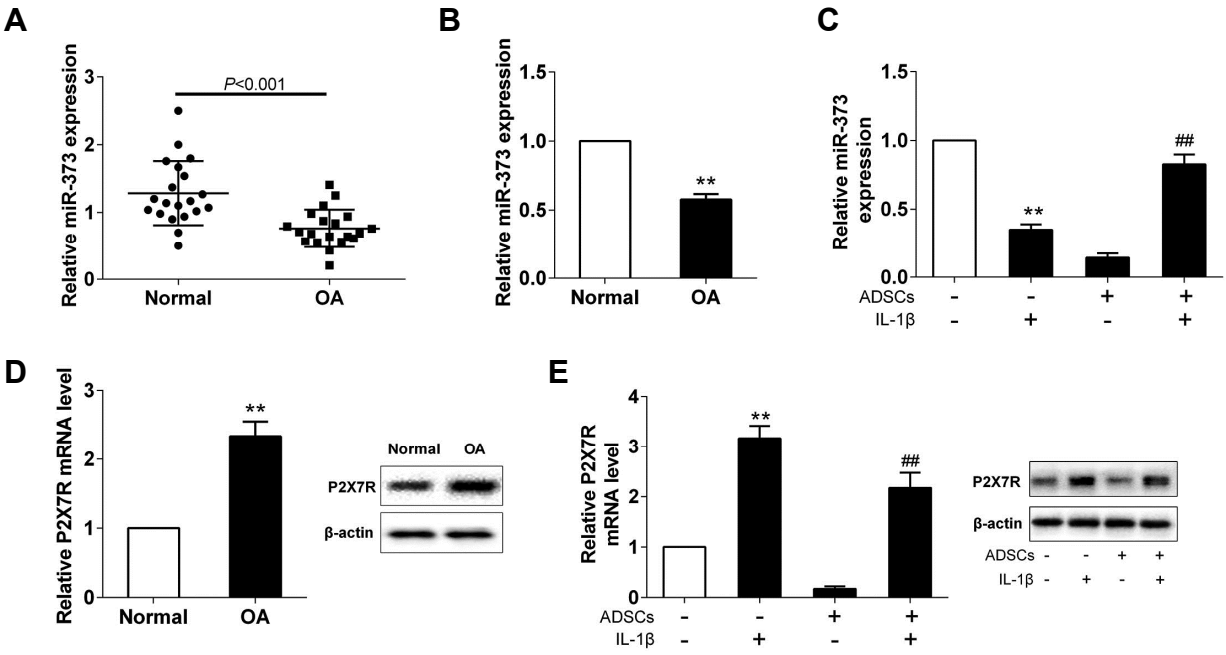

Fig. 2. The expression of miR-373 and P2X7R. The chondrocytes were isolated from 20 patients with OA and twenty normal participants. (A) the expression of miR-373 was significantly decreased in OA chondrocytes. (B) IL-1β supplementation significantly decreased the expression of miR-373 in OA chondrocytes, while ADSCs co-culture increased the expression. (C) the expression of P2X7R was increased in OA chondrocytes. (D) IL-1β stimulation increased the expression of P2X7R in OA chondrocytes; whereas, ADSCs co-culture attenuated the expression. **P < 0.05 vs Normal or control, ##P < 0.05 vs IL-1β treated chondrocytes.

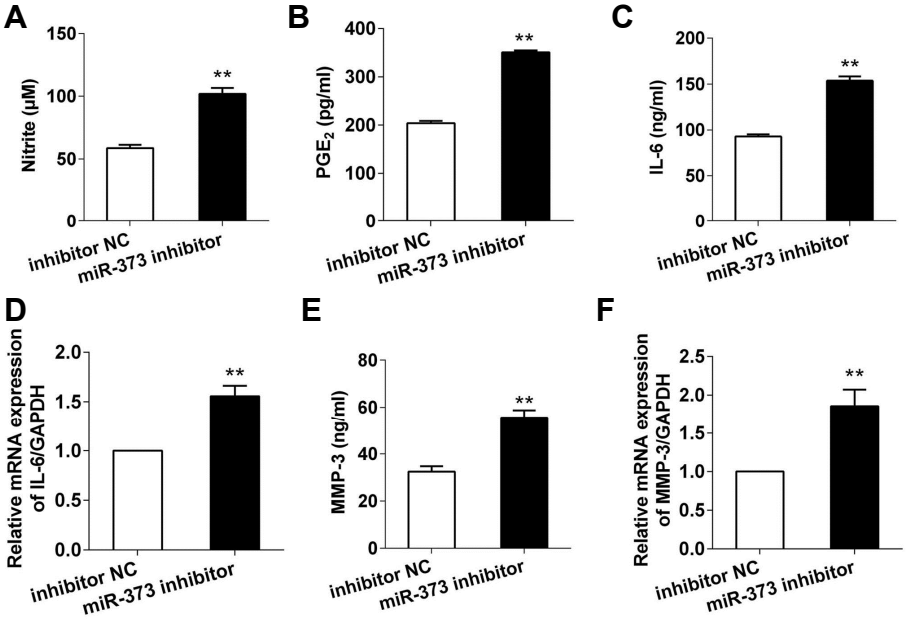

Fig. 3. MiR-373 inhibitor increased the level of inflammatory factors in OA Chondrocytes. OA Chondrocytes transfected with miR-373 inhibitor (inhibitor NC), pre-treatedwith IL-1β, and then co-cultured with ADSCs. (A) the level of NO was detected by Griessreagent; (B, C, and E) the densities of PGE2, IL-6 and MMP-3 were examined by ELISA; (D, F) the mRNA expressions of IL-6and MMP-3 were detected by real-time PCR.

related factors were decreased in chondrocytes pre-treated byIL-1β and then co-cultured with ADSCs, compared with that of chondrocytes treated with IL-1β alone.The results revealed that IL-1β supplementation promoted inflammatory reaction, while chondrocytes pre-treated with IL-1β and co-cultured with ADSCs eliminated the effect.

The expression pattern of miR-373 and P2X7R

Real-time PCR demonstrated that miR-373 expression levels in cartilage tissues and chondrocytes of patients with OA

were dramatically decreased, compared with the control participants (Figs. 2A and 2B). Chondrocytes incubated with IL-1β significantly suppressed the expression of miR-373 compared with the control. Co-cultured ADSCs promoted miR-373 expression, compared with chondrocytes incubated with only IL-1β (Fig. 2C). The expression of P2X7R in the chondrocytes of OA patients was obviously higher than in chondrocytes of the normal participants (Fig. 2D). However, IL-1β supplementation significantly increased the expression compared with the control, while co-cultured ADSCs attenuated the level that induced by IL-1β (Fig. 2E).The results demonstrated that IL-1β stimulation significantly suppressed the expression of miR-373, but increased the expression of P2X7R in OA chondrocytes, while co-cultured ADSCs reversed the effect induced by IL-1β.

Suppression of miR-373 promoted inflammation in chondrocytes

Chondrocytes isolated from OA patients were transfected with miR-373 inhibitor (inhibitor NC), then pre-treated with IL-1β and incubated with ADSCs. Results revealed that the levels of NO (Fig. 3A), PGE2 (Fig. 3B), IL-6 (Fig. 3C) and MMP-3 (Fig. 3E) were dramatically increased. The mRNA expressions of both IL-6 (Fig. 3D) and MMP-3 (Fig. 3F) were also up-regulated by the miR-373 inhibitor. All results indicated that inhibition of miR-373 dramatically promoted the inflammatory reaction.

MiR-373 targets P2X7R to regulate its expression

To explore the relationship between miR-373 and P2X7R, a target Scan database was used to predict the sequence of P2X7R. Results revealed that miR-373 has a highly-conserved target sequence with P2X7R 3′UTR (Fig. 4A). To verify the result, an miR-373 mimic or miR-373 inhibitor was constructed and co-transfected with P2X7R 3′UTR-WT or P2X7R 3′UTR-Mut into the chondrocytes of a normal participant. The luciferase reporter assay demonstrated that overexpression of miR-373 decreased the activity of P2X7R 3′UTR-WT, but not P2X7R 3′UTR-Mut. In addition, miR-373 suppression increased the activity of P2X7R 3′UTR-WT, but not P2X7R 3′UTR-Mut (Fig. 4B). On the other hand, overexpression of miR-373 suppressed the expression of P2X7R (Fig. 4C), while the down-regulated miR-373 promoted the expression of P2X7R (Fig. 4D). The results demonstrated that miR-373 targeted P2X7R to regulate its expression.

MiR-373 mediating P2X7R was involved in the inflammation in chondrocytes

We then determined thatP2X7R was involved in the inflammation of chondrocytes. Chondrocytes of OA patients were transfected with an miR-373 mimic and then exposed to IL-1β for 2 h and incubated with ADSCs for 24 h. Results showed that overexpression of miR-373 prominently decreased the level of NO (Fig. 5A), PGE2 (Fig. 5B), IL-6 (Fig. 5C) and MMP-3 (Fig. 5E),as well as the expression of IL-6

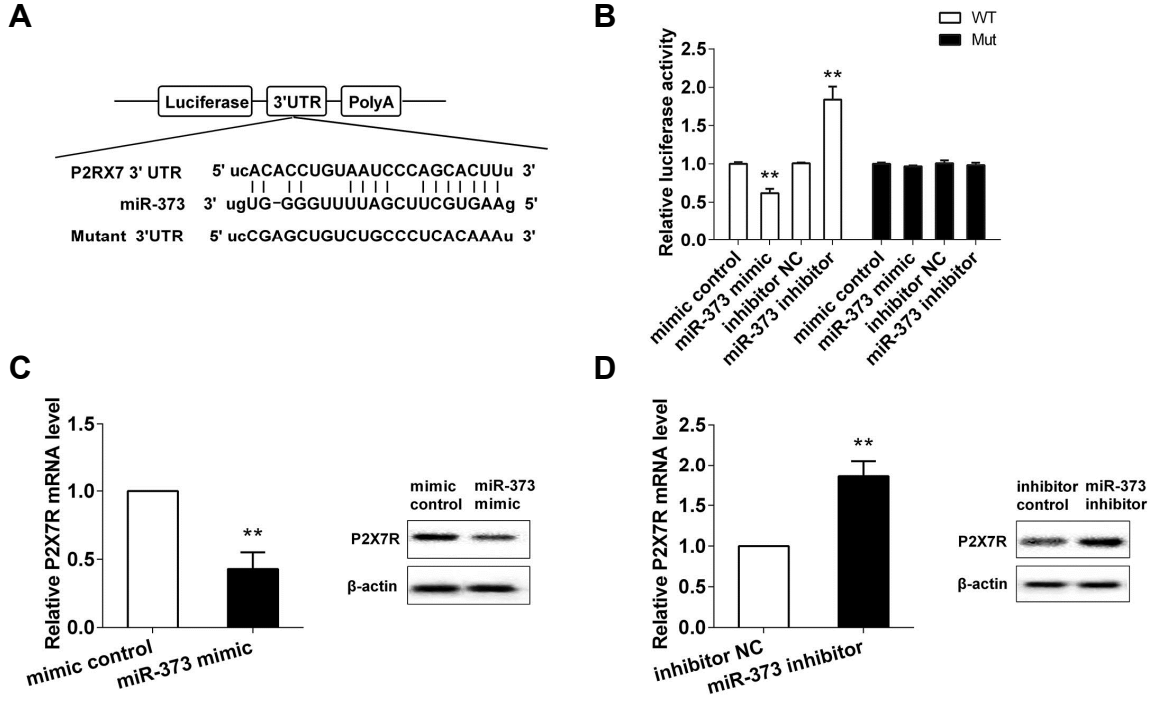

Fig. 4. The relationship between miR-373 and P2X7R. (A) miR-373 have a highly-conserved target sequence with P2X7R 3′UTR. (B) The relative luciferase activity of P2X7R 3′UTR was detected by luciferase reporter assay. (C) Chondrocytes transfected with miR-373 mimic, the mRNA expression and protein expression of P2X7R were detected by real-time PCR and Western blot, individually. (D) Chondrocytes transfected with miR-373 inhibitor, the expressions of P2X7R were detected by real-time PCR and Western blot.

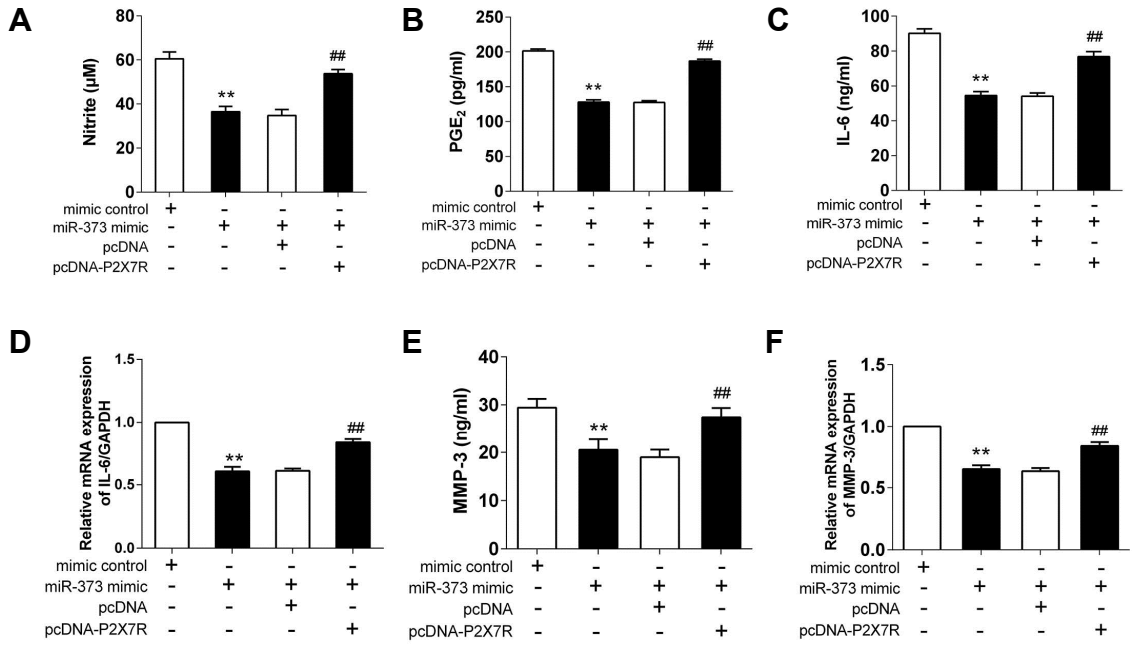

Fig. 5. The effects of over-expressed P2X7R on OA chondrocytes that over-expressed miR-373 mimic, then pre-treated by IL-1β and co-cultured with ADSCs. (A) the levels of NO were detected by Griessreagen; (B, C, and E) the PGE2, IL-6 and MMP-3 levels in densities were examined by ELISA; (D, F) real-time PCR detected the mRNA expressions of IL- MMP-3.

(Fig. 5D) and MMP-3 (Fig. 5F);whereas, the effects were reversed by the over-expressed P2X7R (Fig. 5).The results revealed that up-regulated miR-373 protected chondrocytes against inflammation, while over-expressed P2X7R abolished the effects.

DISCUSSION

IL-1β is an inflammatory factor, which was elevated in the inflamed synovium in OA. At the same time, the secretion and activation of IL-1β induces a pro-inflammation response (Dinarello, 2009; Takahashi et al., 1999). Our present study found that IL-1β stimulation significantly increases the level of NO, which is associated with oxidative stress, and the high level of oxidative stress is related to the degeneration of arthritic joints (Yudoh et al., 2005); thus, our results reveal that IL-1β stimulation might promote the degeneration of arthritic joints. Furthermore, the levels of inflammatory factors PGE2, IL-6 and MMP-3 are also increased in chondrocytes, indicating IL-1β supplementation significantly increases the inflammatory level of OA.

Increasingly evidence supports the hypothesis that the production of catabolic and pro-inflammatory mediators could cause destruction of the cartilage matrix in OA (Stone et al., 2014). More importantly, the ADSCs function has anti-inflammatory effects and regulates the inflammation by secreting the biological factors in co-cultured OA chondrocytes (Jiang et al., 2016). Moreover, the expression of several cytokines, such as MMPs, Runx2, is decreased in chondrocytes co-cultured with ASDCs. In the present study, we demonstrated that ADSCs are positive for CD29, CD90, CD49; whereas, the positive ratios of CD45, CD34, CD14 are low. Jiang et al. (2016) found that ADSCs isolated from SD mice show higher expression levels of CD44, CD90 and CD29, but low or absent expressions of CD34, CD45 and CD19. This is in agreement with our results, indicating the typical characters of ADSCs. As described above, IL-1β supplementation induces inflammatory conditions in OA chondrocytes, and our results found that ADSCs impair the level of inflammatory-related factors, indicating the protective effects of ADSCs on OA.

Previous microarray analysis has identified a large scale of miRNAs in OA, and discovered the up-regulated miRNAs, such as miR-16, miR-22, miR-30, miR-483, and the down-regulated miRNAs, such as miR-210, miR-26a, miR-337, as well as miR-373 (Tsezou, 2014). Circulating cell-free miR-373 is a member of miR-302 family. Previous studies have demonstrated that the miR-302 family is mediated in the onset and improvement of many diseases, such as tumour invasion and metastasis and the pathogenesis of degenerative diseases (Song et al., 2015). Furthermore, the miR-373 is involved in disease regulation, mainly via inflammatory regulation. In our study, miR-373 is down-regulated in both tissues and OA chondrocytes, which is consistent with the previous study. Then we found that the miR-373 inhibitor promotes oxidative stress and inflammatory factors. These results suggest that miR-373 participates in the inflammatory process and functions as an OA inhibitor. In addition, we found that IL-1β stimulation significantly decreases the expression of miR-373 in chondrocytes; however, the ADSCs supplementation significantly increases miR-373 expression, which confirms the pro-inflammatory effects of IL-1β and

the anti-inflammatory effects of ADSCs.

P2X purinoceptor 7 (P2X7R) is an ionotropic receptor that is controlled by adenosine triphosphate (ATP). Studies suggest that activated P2X7R promotes cell death, IL-1β release, as well as reactive oxygen species release (Cruz et al., 2007; Le Feuvre et al., 2002). Furthermore, the important role of P2X7R in inflammatory OA reactions has been verified,while the P2X7R mechanism at work in OA inflammation is obscure. The relationship between P2X7R and miR-373 also remains unclear. In this study, to comprehensively understand the role of miR-373 in OA chondrocytes, the potential target gene, P2X7R, was analysed. A Luciferase activity assay was performed, and the results found that P2X7R is the target gene of miR-373. The expression level of P2X7R is increased in OA, and IL-1β stimulation significantly promotes the expression, while ADSCs co-culture significantly attenuates the increase. Additionally, we observed that the miR-373 mimic prominently decreases the level of NO, PGE2, IL-6 and MMP-3 in OA chondrocytes co-cultured with ADSCs and exposed to IL-1β. However, the effects are reversed by the overexpression of P2X7R. These results suggest that miR-373 participates in the inflammation of chondrocytes through the regulation of the P2X7Rexpression. Therefore, the low-expression of P2X7R by up-regulated miR-373 is proposed to function as an anti-inflammatory in OA.

In summary, we found that IL-1β induces inflammation, while ADSCs function as anti-inflammatory factors in OA chondrocytes. MiR-373 is down-regulated in OA chondrocytes, and P2X7R is mediated by miR-373 and involved in the inflammatory process of OA. The role of miR-373 and P2X7R serves as potential biomarker for inflammatory regulation, and further study on miR-373 and P2X7R *in vivo* will be necessary for the clinical OA therapy.

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