



Upregulation of P2Y12 inhibits chondrocyte apoptosis in lumbar osteoarthritis through the PI3K/AKT signaling pathway

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Abstract

Lumbar facet osteoarthritis (FJOA) is a major cause of severe lower back pain and disability worldwide. However, the mechanism underlying cartilage degeneration in FJOA remains unclear. The purpose of this study was to investigate the regulation and mechanism of P2Y12 on chondrocyte apoptosis in FJOA. The experimental rats were randomly divided into non-operation (n=20) and operation groups (n=20). In the operation group, Sodium iodoacetate (MIA, Sigma, 200 mg/mL) was injected into the right L4/5 facet process using a blunt nanoneedle 26 (WPI, Sarasota, FL, USA) under the control of an injection pump. The final injection volume was 5 µL and the injection rate was 2 µL/min. The facet joint was removed four weeks after surgery. After the operation, samples were stored at -80 °C until further use, whereby the right facet joints in each group were tested. Hematoxylin and eosin (HE) and iron-red solid green staining were used to observe the degeneration of articular chondrocytes in rats. Immunohistochemistry and western blotting were used to observe the expressions of P2Y12, Matrix metalloproteinase 13 (MMP13), Collagen II (COL2), and other cartilage degeneration and apoptosis-related genes. Co-localization of P2Y12-cleaved caspase-3 in the apoptosis model was detected by dual-standard immunofluorescence staining. Apoptosis was also detected by flow cytometry and TUNEL assay. P2Y12 is highly expressed in OA cartilage tissue, and inhibits IL-1β-induced chondrocyte apoptosis through PI3K/AKT signaling pathway, thus playing a certain protective role on cartilage.

Keywords P2Y12 · FJOA · Osteoarthritis · Apoptosis · PI3K/Akt

Abbreviations

FJOA Lumbar facet osteoarthritis.
MMP13 Matrix metalloproteinase 13.
COL2 Collagen II.
MIA Sodium iodoacetate.
HE Hematoxylin and eosin.
TNF-α tumor necrosis factor α.
IL-1B interleukin 1B.
PVDF polyvinylidene difluoride.
HRP horseradish peroxidase.

TUNEL Terminal deoxynucleotide transferase dUTP notched-end labeling.

GIRKS G-protein-gated inward rectifier potassium channel.

Introduction

Facet joints exhibit the typical characteristics of synovial joints, wherein, the articular cartilage covers the opposite sides of each facet joint, located on the thickened layer of subchondral bone, and the synovial membrane connects to the edges of the articular cartilage [1], thus constituting three connected complexes of functional units [2]. Lumbar facet osteoarthritis (FJOA) is a common degenerative disease of the spine. Facet joint degeneration (FJD) is the pathological basis underlying lumbar degeneration. Chronic lower back pain can be caused by degenerative diseases and is common among middle-aged and elderly individuals [3]. The

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basic pathological features include articular chondrocyte damage, hyperplasia of the subchondral and periarticular bones, and osteophyte formation on lumbar facet joints [4]. The occurrence of osteoarthritis (OA) is affected by several inflammatory factors, including the tumor necrosis factor α (TNF- α) and interleukin 1B (IL-1B) [5]. In FJOA progression, these pro-inflammatory cytokines are considered to be the key factors promoting chondrocyte apoptosis. In addition, activation of Caspase-3 is involved in the occurrence of OA [6]. Caspase-3 is one of the most critical effector proteases in cellular apoptosis. However, a basic evaluation of FJOA and the underlying specific molecular mechanisms is lacking. P2Y12R gene is located on chromosome 3q21-q25. The P2Y receptor is a G-coupled transmembrane protein found in almost all cell types; its ligands are purine and pyrimidine nucleotides. From a phylogenetic and structural perspective, two P2Y receptor subgroups with high structural differences have been identified. The first subgroup includes the Gq-coupled subgroups (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11), while the second subgroup comprises the Gi coupling subtypes (P2Y12, P2Y13, and P2Y14) [7]. As a G protein coupled receptor, P2Y12 has typical characteristics of 7-transmembrane domain (TM) regions. Its inner and outer sides are connected with three intracellular loops (IL) and three extracellular loops (EL) respectively [8]. The human P2Y12 receptor is a 342 amino acid residue-long protein [9]. Like most G protein-coupled receptors, P2Y12 receptors are in the extracellular N-terminal (Cys17), the first extracellular ring (Cys97), the second extracellular ring (Cys175), and the third extracellular ring (Cys270) having four extracellular hormone residues [9]10. These are activated by nucleotides and extracellular signaling molecules released by damaged cells under inflammatory, ischemic, and hypoxia conditions [11]. P2y12-like receptors have been reported to be involved in platelets [12], microglia [10, 13–14] chemotactic, and phagocytosis in apoptosis and inflammation, and have played a role in bone metabolism, such as inhibiting osteoclast production and promoting osteogenesis [15]. Abnormal bone cell metabolism is a response to changes in the inflammatory microenvironment and may play a key role in chondrogenesis and OA progression [16]. Whether P2Y12 regulates the cartilage steady-state in the pathogenesis of OA remains unclear.

The activated P2Y12 receptor initially phosphorylates BAD through the PI3K Akt pathway, following which the liquid acidified BAD binds to the 14-3-3 protein, resulting in the separation of BAD from the BAD Bcl-XL complex and the release of free Bcl-XL. Free Bcl-XL can directly bind to BAK, thus downregulating the dimerization of BAK and inhibiting its activation. Although Bcl-XL and Bax do not bind directly in platelets, they may inhibit Bax activation by reducing its translocation from the cytoplasm to

the mitochondria in some indirect ways. In nucleated cells, BAD is an important downstream target gene of Akt [17] and belongs to the pro-apoptotic Bcl-2 family of proteins. The pro-apoptotic function of Bcl-XL is realized upon binding to the anti-apoptotic protein, Bcl-XL, thus inhibiting its anti-apoptotic activity. In nucleated cells, Akt activation inhibits the pro-apoptotic activity through flow acidification. As an important anti-apoptotic protein, dissociated Bcl-XL mainly binds to BAK or Bax and inhibits their pore-promoting activity [18] to achieve its anti-apoptotic function. These findings suggest that P2Y12 can potentially regulate cell death but its expression in FJOA and mechanistic role remain unclear. Considering the similarity of phenotypes between the SW1353 cells and human chondrocytes, which have been successfully used in previous scientific experiments, we decided to use the SW1353 cell line for a series of experiments in this study [19]. To the best of our knowledge, this is the first study aimed at evaluating and locating the differential expression and elucidating the role of P2Y12 in FJOA. The findings herein may provide useful clues and potential therapeutic targets for LFJ degeneration and arthritis-induced lower back pain.

Materials and methods

Experimental animals

All animal experimental protocols were in line with the National Health Center “Experimental Animal Nursing and Use Guide” and approved by the Jiangsu Branch of China’s National Medical Experimental Animal Committee. Male SD rats (n=40), aged 40–50 days, weighing 200–250 g, were housed in a room with constant temperature (37 ± 0.5 °C) with a light-dark cycle of 12 h. Based on the previously reported method for [20] intra-articular MIA injection, the surgical FJOA model was established for the facet joint. The rats were randomly divided into non-surgical (n=20) and surgical groups (n=20). In the surgical group, under the control of a syringe pump, using a 26-gauge blunt nano fil needle (WPI, Sarasota, FL, USA) MIA (Sigma, 200 mg/mL) was injected into the right L4/5 facet joint; the final injection volume was 5 μ L, and the injection rate was 2 μ L/min. The facet joints were collected four weeks after the operation and some samples were stored at -80 °C until further use. The remaining samples were fixed with 10% neutral buffered formalin, decalcified with 10% formic acid, and embedded in paraffin. The sagittal histological sections of the articular chondrocytes from the right facet joints were analyzed. The right facet joints of each group were used for further experiments.

Histopathological analysis

The lumbar facet joints of rats were obtained and fixed with 4% paraformaldehyde. The samples were decalcified in 10% EDTA solution for 21 days and subsequently embedded in paraffin. Tissue sections (5 μ m) were stained with Tibetan red O/ solid green solution following the standard regimen to determine cartilage degradation under light microscopy. The scoring system (OARSI) of the International Society for the Study of Osteoarthritis was used to evaluate articular cartilage degeneration [21]. Immunohistochemistry was further performed to analyze the protein expressions of P2Y₁₂, MMP13, and COL2 in the lumbar facet joint tissue sections of rats. Primary antibodies against P2Y₁₂ (Abcam), MMP13, and COL2 (Proteintech) were used in 1:100 to 1:200 dilution and incubated overnight at 4 °C. The sections were then incubated with the biotinylated secondary antibody (Vector Laboratories, USA). DAB kits were used for the reaction. The expressions of P2Y₁₂, MMP13 and COL2 were assessed by calculating the percentage of immune positive cells.

Cell culture and stimulation

Human SW1353 chondrosarcoma cells (Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in Lebovitz's L-15 medium, supplemented with 10% fetal bovine serum (GIBCO, USA) in a humidified atmosphere at 37 °C and 5% CO₂. The cells were passaged every three to four days. For stimulation, 20 ng/ml human IL-1 β (Proteintech) was added to the cells, 0, 6, 12, 24, or 48 h before harvesting.

siRNA transfection

P2Y₁₂-siRNA was synthesized by Guangzhou RiboBio Co. Ltd. The siRNA targeting the P2Y₁₂ sequence was: 5'-TGGGACTGATAACTATCGA-3'. Following the manufacturer's instructions, we transfected the SW1353 cells with P2Y₁₂-siRNA. After 48 h of transfection, these cells were collected for subsequent experiments.

Western blot analysis

After the necessary treatments, the SW1353 cells were harvested and the protein samples were extracted using the cell lysis buffer. 50 mg of total protein was loaded into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk at room temperature for 1 h, and subsequently incubated with primary

antibodies against P2Y₁₂ (anti-rabbit 1:500; proteintech), cleaved caspase-3 (anti-mouse, 1:1000, proteintech), MMP13 (anti-rabbit, 1:1000, proteintech), and GAPDH (anti-rabbit, 1:1000, abcam), overnight at 4 °C. After incubation with the secondary antibody conjugated with horseradish peroxidase (HRP), the proteins were visualized using an enhanced chemiluminescence kit (ECL, Pierce).

Double immunofluorescence staining

The cells were seeded in a 24-well plate (2 \times 10⁴ cells/cover glass) and treated with IL-1 β (20 ng/ml) for 36 h. The cells were incubated with primary antibodies for P2Y₁₂, cleaved caspase-3, and further with fluorescently labeled secondary antibodies to detect the corresponding protein localizations in SW1353 cells. Finally, the fluorescence was observed using a Zeiss fluorescence microscope (Germany) or Leica confocal microscope (Germany).

Measurement of apoptosis

According to the manufacturer's instructions (Becton Dickinson), the cells were stained in the dark with annexin V-FITC/ propidium iodide (PI) for 15 min. The stained cells were detected by flow cytometry and analyzed using the FlowJo software. Apoptosis of chondrocytes was detected using the Terminal deoxynucleotide transferase dUTP notched-end labeling (TUNEL) assay kit (Beyotime) following the manufacturer's instructions.

Statistical analysis

All data were analyzed using the GraphPad Prism 10 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance, Tukey's post hoc multiple comparison test and double comparison unpaired t-test were used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

P2Y₁₂ expression is upregulated in the articular cartilage of rat lumbar facet joints

In the FJOA model, P2Y₁₂ expression was elevated in the articular chondrocytes. However, whether P2Y₁₂ participates in the FJOA process herein, remains unclear. The experimental FJOA model of SD rats injected with MIA was constructed to elucidate the relationship between chondrocyte apoptosis and P2Y₁₂. The changes in articular facet chondrocytes between the operation and the non-operation

groups were investigated by the saffron solid green and H&E staining assays. As compared to the operation group, the facet joints of the rats in the non-operation group were smooth and the chondrocytes showed a more orderly arrangement (Fig. 1 A -B). OARSI grade scores confirmed cartilage damage caused by MIA injection (Fig. 1 C). Immunohistochemical analyses suggested that as compared to the non-surgical group, P2Y12 positive cells and MMP13 positive cells were increased but the proportion of COL2 positive cells showed a decline in the surgical group (Fig. 1D and G). Western blot analysis also confirmed that the expression of P2Y12 was consistent with those of the markers of facet joint degeneration (Fig. 1E and F).

IL-1 β induces an increase in P2Y12 expression and apoptosis in SW1353 cells

Given that the main pathogenic mechanism underlying FJOA is caspase-dependent apoptosis in chondrocytes, an IL-1 β induced SW1353 chondrocyte apoptosis model

was established, which showed the relationship between chondrocyte apoptosis and P2Y12. Western blot analysis showed the temporal expression patterns of P2Y12 in the chondrocyte apoptosis model. As shown in Fig. 2 A and B, the expression of P2Y12 was low at 0 h but after IL-1 β induction, P2Y12 levels were up-regulated within 12 h and reached the peak expression at 36 h ($P < 0.05$). Immuno-blot analysis was also performed to evaluate the expressions of the known apoptosis markers, caspase-3 and Bax. In the IL-1 β -induced SW1353 cell apoptosis model, the levels of expression of cleaved caspase-3 and Bax were up-regulated at 12 h and reached their peak at 36 h. In addition, cellular immunofluorescence assays exhibited the co-localization of P2Y12 and cleaved caspase-3 under IL-1 β stimulation, as shown in Fig. 2 C. These data indicated that during FJOA progression, the caspase-dependent apoptotic pathway may play an important role through P2Y12 and chondrocyte apoptosis.

Inhibition of P2Y12 promotes IL-1 β -mediation induction of SW1353 cell apoptosis

To further determine the correlation between P2Y12 and chondrocyte apoptosis, SW1353 cells were transfected with siRNAs to knock down the expression of the P2Y12 protein; 48 h post-transfection, the efficiencies in P2Y12-siRNA, non-specific siRNA, and negative control groups were detected. As shown in Fig. 3 A and B, in the IL-1 β -induced SW1353 cells, the siRNA could significantly

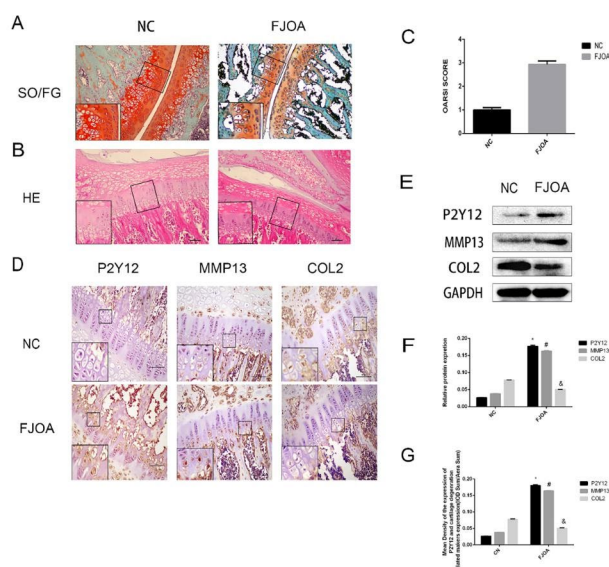


Fig. 1 P2Y12 expression is elevated in the FJOA rat model. A representative image of saffron solid green staining (scale=100 μ m). B Representative image of H&E staining (scale=100 μ m). C The chart shows the OARSI scores calculated using all sections of the facet joints. D and G Immunohistochemistry to detect the expressions of P2Y12, COL2, and MMP13 in rat FJOA and normal cartilage (scale=100 μ m) * # &P<0.05. E Western blotting shows the expressions of P2Y12, COL2, and MMP13 in rat FJOA and normal cartilage samples. F The bar chart shows the expressions of the ratio of P2Y12, MMP13, and COL2 normalized against GAPDH levels. Data are presented as mean \pm SEM (n=3, * # &P<0.05, statistically significant difference relative to the non-surgery group)

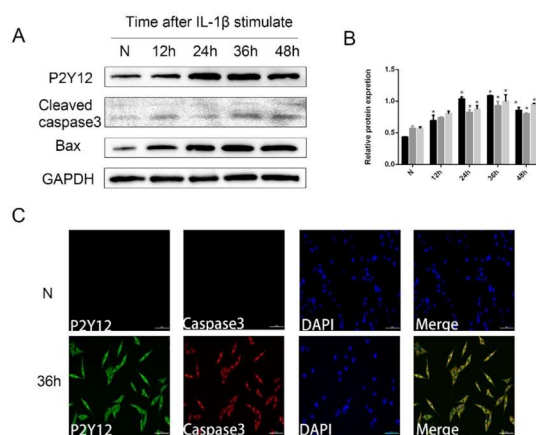


Fig. 2 Increased P2Y12 expression and apoptosis in SW1353 cells. A Western blot analysis shows that after IL-1 β (20ng/ml) stimulation, P2Y12 and apoptosis marker proteins (Bax and cleaved Caspase-3) are expressed in SW1353 cells. B The bar graph B shows the ratios of expressions of P2Y12, Bax, and cleaved caspase-3 relative to GAPDH. *P<0.001 vs. N. C immunofluorescence staining showing the co-localization P2Y12 (green) and caspase-3 lysis (red) in SW1353 cells(scale bar $\frac{1}{4}$ 20 mm) stimulated with IL-1 β

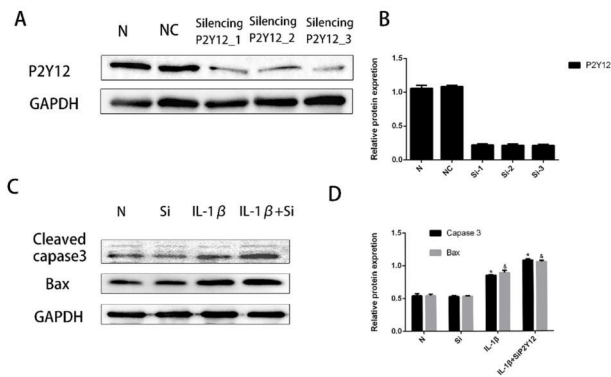


Fig. 3 Knocking down P2Y12 promotes IL-1 β -induced apoptosis in SW1353 cells. A and B demonstrate the effects of silencing P2Y12_1, silencing P2Y12_2 and silencing P2Y12_3 in SW1353 cells by western blotting. C Knocking down P2Y12 with siRNA and treatment with IL-1 β increases the levels of Caspase3/Bax in SW1353 cells. Bar graph D shows the quantification of the ratio of expression of cleaved Caspase-3 to GAPDH. * &P<0.05 Vs. untreated cells, * &P<0.05 Vs. IL-1 β -treated non-specific siRNA transfected cells

inhibit P2Y12 expression. Upon investigating the relationship between P2Y12 expression and cellular apoptosis, we observed increased expressions of caspase-3 and Bax proteins after P2Y12 silencing in the SW1353 cell apoptosis model (Fig. 3 C and D). These data indicated that P2Y12 may inhibit the expressions of caspase-3 and Bax in chondrocytes in FJOA.

P2Y12 negatively regulates the apoptosis of SW1353 cells induced by IL-1 β

As described above, our findings indicated that P2Y12 was closely related to chondrocyte apoptosis in FJOA, thus, it was necessary to further evaluate the effects of P2Y12 on IL-1 β -induced apoptosis in SW1353 cells through annexin V/PI staining based on flow cytometry. As compared to the control group, the number of apoptotic cells in the P2Y12-siRNA cells increased significantly (Fig. 4). Taken together, these data indicated that P2Y12 may inhibit inflammation-induced chondrocyte apoptosis during FJOA.

Effects of P2Y12 on PI3K/AKT signaling in IL-1 β -induced chondrocytes

To investigate the mechanism underlying the inhibition of P2Y12 function in chondrocytes in increasing IL-1 β -induced chondrocyte apoptosis, we performed a western blot analysis for the signaling molecules involved in the PI3K/AKT signaling pathway and found that reduced P2Y12 expression in chondrocytes could activate the PI3K/AKT

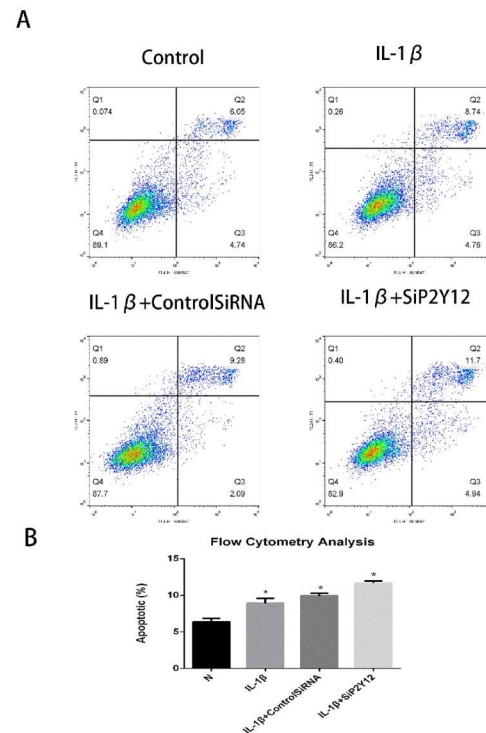


Fig. 4 The effects of P2Y12 inhibition on IL-1 β -induced apoptosis in SW1353 cells. Flow cytometry analysis shows that after IL-1 β stimulation, P2Y12 inhibits the increase in the levels of the phagocytic membrane protein v/pi cells. Bar graph B shows the analysis of apoptosis rates. * P<0.05 Vs. control group, *P<0.05 Vs. IL-1 β group

signaling pathway due to an increase in phosphorylation at Ser473, resulting in the active form of AKT. As shown in Fig. 5 A and 5B, after transfection with siP2Y12, the phosphorylation level of AKT in IL-1 β -treated chondrocytes increased significantly but the total AKT protein level in the treated cells remain unchanged. To investigate whether the effects of P2Y12 on chondrocytes were modulated by the PI3K/ AKT signaling pathway, we treated the chondrocytes with LY294002, a specific PI3K inhibitor, in the presence of IL-1 β . Western blot analysis showed that cleaved Caspase 3 expression decreased substantially, while that of BCL 2 increased after LY294002 treatment in siP2Y12-transfected chondrocytes (Fig. 5 C-5 F). Subsequently, the results of the TUNEL experiment verified the above results (Fig. 5G). Taken together, these findings suggested that the PI3K/AKT signaling pathway was involved in the regulation of P2Y12-mediated chondrocyte apoptosis.

Discussion

FJOA is a degenerative joint disease characterized by chondrocyte degeneration [22] and apoptosis [8]. Although

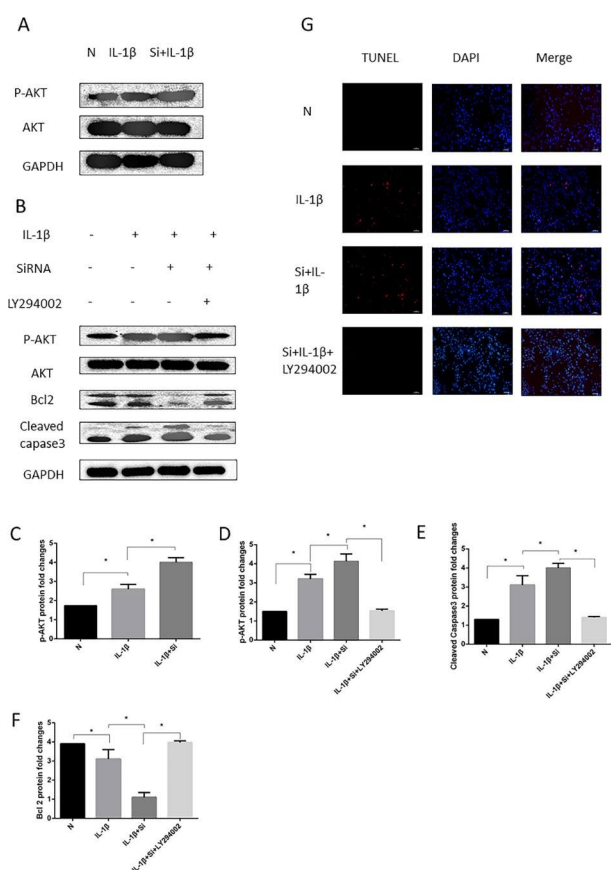


Fig. 5 Effects of P2Y12 on IL-1 β -induced PI3K/AKT signaling pathway in chondrocytes. A and C Representative western blotting and quantitative data for P-Akt and Akt levels in each group. * $P < 0.05$. B and D-F Representative western blotting and quantitative data for P-Akt, Akt, Cleaved Caspase3, Bcl 2. * $P < 0.05$. (G) TUNEL staining shows the apoptotic chondrocytes. The scale represents 100 μ m

factors such as age, genetics, mechanical stress, trauma, and metabolism are all associated with the progression of osteoarthritis, cellular signaling and metabolic changes in chondrocytes remain unclear. In this study, we focused on the role of P2Y12 in chondrocyte FJOA. We used MIA to induce OA to establish FJOA model of articular process in rats. MIA has been widely used in the study of OA pathogenesis [23–24]. Immunohistochemical staining and WB test results showed that P2Y12 expression was significantly enhanced in severely degenerated cartilage. In vitro western blot analysis showed that P2Y12 and Caspase-3 were significantly increased after 36 h of IL-1 β stimulation on SW1353 cells. Co-localization of P2Y12/ Cleaved caspase-3 in cell injury models was detected by immunofluorescence double-labeling. Silencing P2Y12 by siRNA increased IL-1 β -induced caspase-3 expression, suggesting that P2Y12 negatively regulated chondrocyte apoptosis

through caspase-dependent signaling. Flow cytometry showed that P2Y12 gene knockout significantly enhanced IL-1 β -induced apoptosis of SW1353 cells. western blot analysis and TUNEL showed that the expression of apoptosis-related genes was decreased after LY29004 was added into IL-1 β induced chondrocyte apoptosis. Based on our data, we conclude that: P2Y12 may play an important role in inhibiting the apoptosis of FJOA chondrocytes through PI3K/AKT signaling pathway.

P2Y12 receptor inhibits apoptosis by coupling with G α I2 subunit to activate the PI3K/Akt pathway [25]. After stimulation, decomposition of G α and G $\beta\gamma$ subunits leads to activation of signal transduction pathways [26]. G $\beta\gamma$ subunit stimulates the activity of phosphatidylinositol 3 kinase (PI3K), leading to late accumulation of phosphatidylinositol 3, 4-bisphosphonates [PtdIns (3,4) P2] and phosphatidylinositol 3, 4, 5-triphosphonates [PtdIns(3,4) rapid and transient accumulation, 5)P3] [27–29]. The PI3K pathway also activates Rap1b [14] and Akt [26]. G $\beta\gamma$ dimer activates g-protein-gated inward rectifier potassium channels (GIRKs) of Src tyrosine kinase [30]. Upregulation of P2Y12 induces Akt and undesirable phosphorylation. After phosphorylation by Akt, Bad is sequestered in the cytoplasm by the adapter protein 14-3-3, which prevents Bad from binding to Bcl-XL. Therefore, the free Bcl-XL heterodimer bound to Bak blocks the dimerization of Bak in mitochondria, thereby antagonizing its pro-apoptotic activity. Bcl-xl inactivates Bax indirectly by inhibiting translocation of Bax to mitochondria [15]. P2Y12 receptor is mainly expressed in platelets and neuronal tissues [28]. Only a few studies have reported the function of P2Y12 in articular chondrocytes of the lumbar facet. Based on these findings, we speculate that upregulation of P2Y12 may play an important role in inhibiting FJOA chondrocyte apoptosis. In our study, we did not study whether P2Y12 expression is involved in the development of FJOA and whether it is positively correlated with severity. Further research is needed to clarify.

Conclusions

In conclusion, P2Y12 expression was significantly up-regulated in FJOA. Inhibition of P2Y12 enhanced cellular apoptosis of SW1353 cells induced by IL-1 β . These observations indicated that the upregulation of P2Y12 may play an important role in inhibiting FJOA chondrocyte apoptosis. Further studies on the function of P2Y12 in chondrocytes may provide new insights into the pathophysiology and provide advanced strategies for the treatment of FJOA.

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Compliance with ethical standards

Conflict of interest The authors declare no financial or commercial conflict of interest.

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