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# Polydeoxyribonucleotide ameliorates IL-1 $\beta$ -induced impairment of chondrogenic differentiation in human bone marrow-derived mesenchymal stem cells

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Osteoarthritis (OA) is a degenerative disease of the joints, prevalent worldwide.

Polydeoxyribonucleotide (PDRN) is used for treating knee OA. However, the role of PDRN in IL-1 $\beta$ -induced inflammatory responses in human bone marrow-derived mesenchymal stem cells (hBMSCs) remains unknown. Here, we investigated the role of PDRN in IL-1 $\beta$ -induced impairment of chondrogenic differentiation in hBMSCs. hBMSCs treated with PDRN showed a large micromass, enhanced safranin O and alcian blue staining intensity, and increased expression of chondrogenic genes in IL-1 $\beta$ -induced inflammatory responses, in addition to regulation of catabolic and anabolic genes. In addition, PDRN treatment suppressed the expression of inflammatory cytokines and mitigated IL-1 $\beta$ -induced apoptosis in hBMSCs. Mechanistically, PDRN treatment increased the formation of cyclic adenosine monophosphate (cAMP) and upregulated the phosphorylation of cAMP-dependent protein kinase A (PKA)/cAMP response element binding protein (CREB) through the adenosine A2A receptor in hBMSCs and thus blocked the nuclear factor kappa-light-chain-enhancer of activated B cell (NF- $\kappa$ B) signaling pathway. Thus, IL-1 $\beta$ -induced expression of inflammatory cytokines in hBMSCs was directly reduced by adenosine A2A receptor activation. Based on our results, we suggest that PDRN may be a promising MSC-based therapeutic agent for OA.

**Keywords** Polydeoxyribonucleotide, Mesenchymal stem cell, Chondrogenic differentiation, Interleukin-1 $\beta$ , Osteoarthritis

## Abbreviations

OA	Osteoarthritis
PDRN	Polydeoxyribonucleotide
hBMSCs	Human bone marrow-derived mesenchymal stem cells
qRT-PCR	Real-time quantitative reverse transcription polymerase chain reaction
PKA/CREB	Protein kinase A/cAMP response element binding protein

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NF-κB      Nuclear factor kappa-light-chain-enhancer of activated B cell

Osteoarthritis (OA) is a common disease of the hand, hip, and knee joints that is characterized by pathological features including degradation of articular cartilage, inflammation in the synovial cavity, and subchondral sclerosis<sup>1</sup>. It leads to disability and causes serious socioeconomic problems worldwide<sup>2,3</sup>. The most common risk factors of OA are age, obesity, injury, gender, heredity, mechanical stress, joint damage, and genetic predisposition<sup>1</sup>. Excessive production of proinflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), plays an important role in OA pathogenesis<sup>4</sup>. Additionally, IL-1β and TNF-α activate the transcription factor nuclear factor-κB (NF-κB) and induce its nuclear translocation, which leads to enhanced expression of extracellular matrix (ECM)-degrading enzymes, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs)<sup>4</sup>. Several treatments for OA have been developed; these include nonsurgical therapies, such as nonsteroidal anti-inflammatory drugs, weight loss, exercise, intra-articular injection of viscosupplements, physical therapy, and nutritional supplements, and surgical therapies, such as arthroscopy, arthroplasty, osteotomy, and cartilage repair<sup>2</sup>. Stem cell-based therapy is also an emerging therapy for OA<sup>5</sup>.

Human bone marrow-derived mesenchymal stem cells (hBMSCs) are among the most promising options for OA treatment. hBMSCs are characterized by their ability to differentiate into multiple cell types including adipocytes, cardiomyocytes, chondrocytes, myocytes, and osteoblasts<sup>6,7</sup>. Moreover, hBMSCs have a positive effect on tissue regeneration, immune modulation, anti-inflammatory paracrine signaling, and stimulation of angiogenesis<sup>8</sup>. For these reasons, hBMSCs have been used in intra-articular injections in *in vivo* OA models<sup>9,10</sup> and in clinical trials for cartilage regeneration in patients with OA<sup>11,12</sup>. Proinflammatory cytokines, such as IL-1β, and TNF-α were reported to inhibit chondrogenic differentiation of hBMSCs by stimulating nuclear translocation of NF-KB and inducing inflammatory and apoptosis-regulating genes and ECM-degrading enzymes<sup>13–15</sup>. Moreover, proinflammatory cytokines suppressed the expression of sry-box transcription factor 9 (SOX9), the master transcription factor for chondrogenic differentiation of hBMSCs<sup>16</sup>. Therefore, inhibiting proinflammatory cytokines is a good option to enhance MSC chondrogenesis.

Polydeoxyribonucleotide (PDRN), mainly extracted from trout or salmon sperm, is known to enhance the migration and growth of cells, angiogenesis, and tissue repair activities. It has been shown to exhibit anti-inflammatory, antiapoptotic, and anti-ischemic effects in *in vitro*<sup>17,18</sup>, *in vivo*<sup>17,19,20</sup>, and clinical studies<sup>21,22</sup>. PDRN is also known as an adenosine A2A receptor (A2AR) agonist that exerts anti-inflammatory effects in immune cells, activates antitumor killer cells to reduce damage in cancer, and reduces the secretion of proinflammatory cytokines in neurological diseases<sup>23,24</sup>. PDRN showed anti-inflammatory and antiapoptotic effects in Achilles tendon-injury, carbon tetrachloride-induced acute liver injury, and cyclophosphamide-induced interstitial cystitis models<sup>25–27</sup>. Local injection of human umbilical cord blood-derived (UCB)-MSCs with PDRN showed potential therapeutic effects in full-thickness rotator cuff tendon tear rabbit models<sup>28,29</sup>. Moreover, PDRN has been used as a bioactive molecule in bone regeneration *in vivo*<sup>30–34</sup>. However, the role of PDRN in chondrogenic differentiation of hBMSCs remains unknown. Therefore, this study was aimed at investigating the effect of PDRN in chondrogenic differentiation of hBMSCs subjected to IL-1β-induced inflammatory stimuli. Our results indicate that PDRN may be a promising agent in improving the efficacy of MSC-based therapy of OA.

## Materials and methods

### Culture of hBMSCs and reagents

hBMSCs were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM-LG; Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin and streptomycin (P/S; Gibco). PDRN (Placentex, Mastelli Srl, Italy) was developed through selectively extraction from trout sperm. IL-1β was purchased from R&D Systems (Minneapolis, MN, USA) and dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. 3,7-Dimethyl-1-propargylxanthine (DMPX), a specific adenosine A<sub>2A</sub> receptor antagonist, was purchased from Sigma-Aldrich (St. Louis, MO, USA)<sup>35</sup>. CGS21680 and ZM241385 were purchased from Tocris Bioscience (Abingdon, UK)<sup>36</sup>.

### Chondrogenic differentiation of hBMSCs

Chondrogenic differentiation of hBMSCs was performed in chondrogenic differentiation medium—DMEM-high glucose (DMEM-HG; Gibco) containing 1% P/S, 1% insulin transferrin selenium-A (ITS; Invitrogen, Carlsbad, CA, USA), 50 mg/mL ascorbic acid (Sigma), and 10 ng/mL transforming growth factor (TGF)-β3 (R&D Systems; Minneapolis, MN, USA). For micromass culture,  $1 \times 10^5$  hBMSCs were dropped in the center of each well of a 24-well plate. After 2 h, chondrogenic medium was added to the wells. The medium was changed every 3 days, and the cells were induced with chondrogenic differentiation medium for 14 days<sup>37–39</sup>.

### Cell viability assay

hBMSCs were seeded in a 96-well plate and incubated for 24 h. The following day, CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well and the plate was kept for 1 h in a 37 °C incubator. Absorbance at 450 nm was measured using a microplate reader (VersaMax, Molecular Devices, San Jose, CA, USA).

### Alcian blue and safranin O staining

Alcian blue (Sigma, B8438) and safranin O staining solution (Sigma, TMS-009) were purchased from Sigma. The staining was performed according to the manufacturer's protocols<sup>40,41</sup>. To quantify alcian blue staining, 6 M guanidine hydrochloride was used, and absorbance was measured at 620 nm. To quantify the safranin O staining, isopropanol was used and similarly measured at 540 nm.

### Measurement of cyclic adenosine monophosphate (cAMP)

The cAMP concentration was determined using a cAMP ELISA Kit (Enzo Life Sciences; Plymouth Meeting, PA) according to the manufacturer's instructions.

### Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated using a RNeasy kit (74106, Qiagen, Valencia, CA, USA). RNA (2 µg) was reverse transcribed using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). qRT-PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and qPCR Bio SyGreen Mix (PB20.12-05; PCR Biosystems), according to the manufacturer's guidelines. The primer sequences are listed in Supplementary Table 1.

### Western blotting analysis

Total proteins were prepared from cells using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing 1X protease inhibitor cocktail (Cell Signaling Technology, cat. no. 5871) and 1X phosphatase inhibitor cocktail solution (GenDEPOT, cat. no. P3200). Proteins (30 µg) in each sample were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Laboratories, Richmond, CA, USA) and transferred onto 0.45 µm polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). After blocking with 5% bovine serum albumin, the blots were incubated overnight at 4 °C with the primary antibodies. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology) for 1 h. Finally, the western blots were imaged using Amersham ImageQuant 800 (Amersham Pharmacia Biotech). The western blot images were quantified using ImageJ. The primary antibodies used are listed in Supplementary Table 2.

### Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

hBMSC micromass was treated with vehicle, IL-1 $\beta$  (10 ng/mL), or IL-1 $\beta$  (10 ng/mL) and PDRN in chondrogenic differentiation medium for 14 days. The hBMSC micromass was fixed with 4% paraformaldehyde, embedded in paraffin blocks, and cut into 4 µm thick sections. The DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) was used to analyze the DNA fragmentation according to the manufacturer's protocol. The DNA fragmentation images were observed using a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss Micro Imaging GmbH, Jena, Germany).

### Immunofluorescence staining

hBMSCs were treated with vehicle, IL-1 $\beta$  (10 ng/mL), or IL-1 $\beta$  (10 ng/mL) and PDRN in the chondrogenic differentiation medium for 14 days. Thereafter, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. The cells were then blocked with 5% bovine serum albumin in PBS for 1 h. The cells were subsequently washed with PBS and incubated overnight with NF-κB p65 (1:100, sc-8008; Santa Cruz Biotechnology, Santa Cruz, CA) in a cold room. The following day, the cells were incubated with Alexa Fluor 488 (Abcam, Cambridge, MA, USA) for 1 h in the dark. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vectashield, Vector, Burlingame, CA, USA). A Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH) was used for nuclear localization of NF-κB p65.

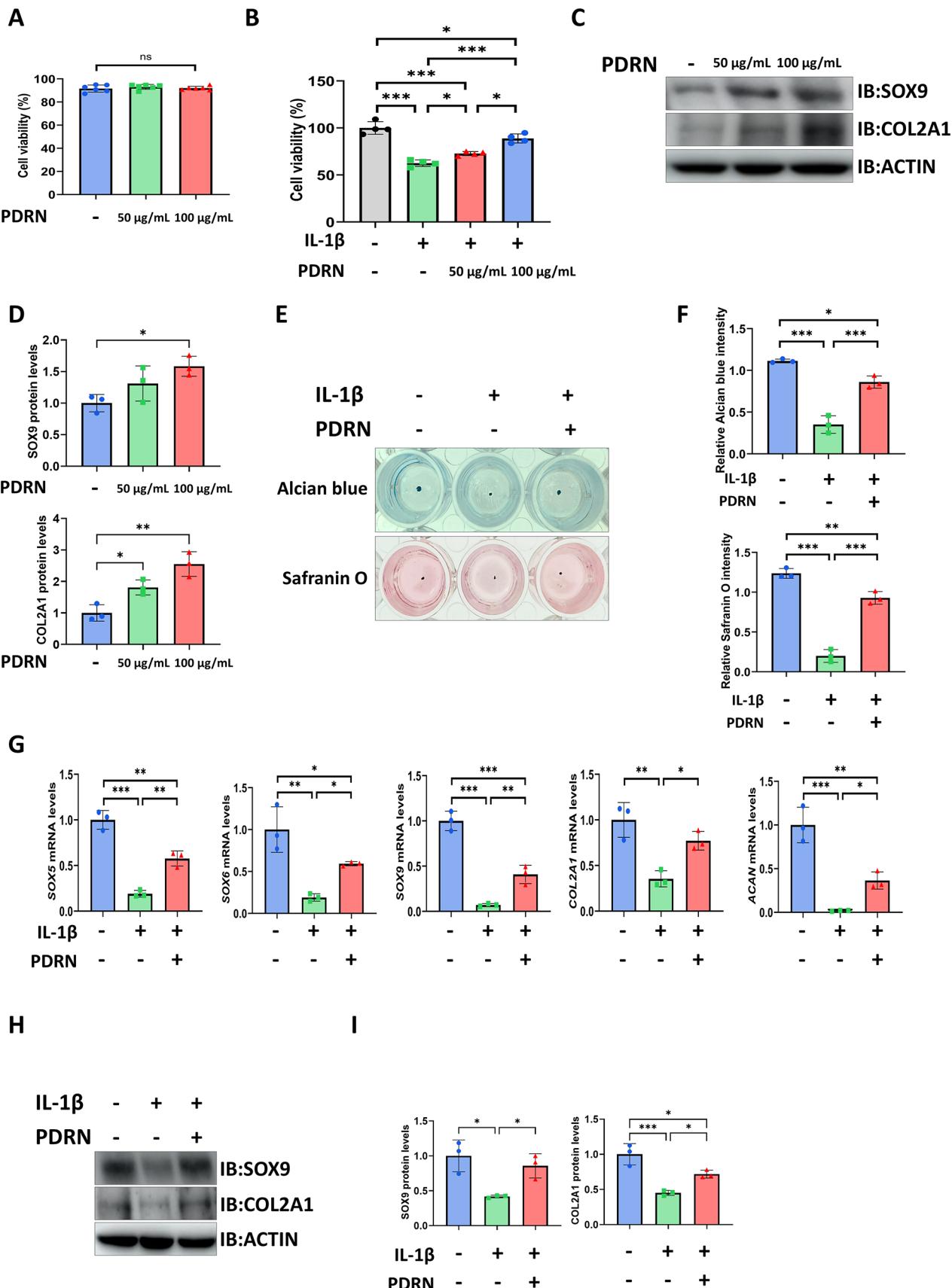
### Statistical analysis

All data are presented as means  $\pm$  standard deviations (SDs). One-way analysis of variance with Bonferroni post hoc tests were used for multi-group comparisons. The GraphPad PRISM software (version 10.0) and the Statistical Package for Social Sciences (version 25.0) were used for statistical analysis.

## Results

### PDRN rescues inflammation-mediated inhibition of chondrogenesis in IL-1 $\beta$ -treated hBMSCs

To investigate the effects of PDRN on cell viability, hBMSCs were treated with 50 and 100 µg/mL PDRN. PDRN was not cytotoxic to hBMSCs at either of the tested concentrations (Fig. 1A). Moreover, while IL-1 $\beta$ -treated hBMSCs had decreased cell viability, treatment of hBMSCs with 100 µg/mL PDRN attenuated IL-1 $\beta$ -induced inhibitory effects on cell viability as compared to 50 µg/mL PDRN (Fig. 1B). Additionally, protein levels of SRY-type high mobility group box 9 (SOX9), the main chondrogenic transcription factor, were increased in a dose-dependent manner, suggesting that PDRN may positively regulate the chondrogenic differentiation of hBMSCs. (Fig. 1C, D). We previously showed that PDRN (100 µg/mL) promoted angiogenesis and wound healing in an *in vitro* model of OA<sup>17</sup>. We therefore used the same concentration (100 µg/mL) in this study. To examine the effect of PDRN on chondrogenic differentiation of hBMSCs in an IL-1 $\beta$ -induced inflammatory environment, we performed alcian blue and safranin O staining for cartilage matrix synthesis and proteoglycan, respectively. IL-1 $\beta$ -treated hBMSCs showed smaller micromass and lower alcian blue and safranin O staining intensity than hBMSCs treated with the vehicle; however, hBMSCs treated with IL-1 $\beta$  and PDRN showed bigger micromass and higher glycosaminoglycan and proteoglycan deposition than any of the groups (Fig. 1E, F). We also investigated the expression of chondrogenic differentiation markers using qRT-PCR and western blot analyses. The expression levels of chondrogenesis-related genes, such as SOX-trio, COL2A1 and ACAN were reduced in IL-1 $\beta$ -induced hBMSCs whereas this effect was significantly attenuated in hBMSCs treated with IL-1 $\beta$  and PDRN (Fig. 1G). The results of western blot analysis showed a reduction in the expression levels of SOX9 and COL2A1 in IL-1 $\beta$ -treated hBMSCs compared to that in vehicle-treated hBMSCs; however, the reduction in expression was attenuated upon treatment of hBMSCs with IL-1 $\beta$  and



PDRN (Fig. 1H-I). These results suggested that IL-1 $\beta$  suppressed the chondrogenic differentiation of hBMSCs, and PDRN treatment of hBMSCs prevented this effect.

**Fig. 1.** Polydeoxyribonucleotide (PDRN) rescued IL-1 $\beta$ -induced inhibition of chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs). (A) hBMSCs were treated with different doses (50 and 100  $\mu$ g/mL) of PDRN or left untreated, and cell viability was determined using the CCK-8 reagent. (B) hBMSCs were treated with vehicle, IL-1 $\beta$  (10 ng/mL) and different doses (50 and 100  $\mu$ g/mL) of PDRN, and cell viability was determined using the CCK-8 reagent. (C) hBMSCs were treated with different doses (50 and 100  $\mu$ g/mL) of PDRN or left untreated, and then cultured with chondrogenic differentiation medium for 14 days. Thereafter, protein levels of SOX9 and COL2A1 were investigated by western blot analysis. (D) The western blot images were quantified using ImageJ. (E) hBMSCs were treated with vehicle, IL-1 $\beta$  (10 ng/mL), or IL-1 $\beta$  (10 ng/mL) with PDRN (100  $\mu$ g/mL) and then subjected to chondrogenic differentiation for 14 days. Thereafter, alcian blue and safranin O staining of hBMSCs was performed. (F) Absorbance quantification of alcian blue and safranin O staining. (G) mRNA levels of chondrogenesis-related genes were analyzed using qRT-PCR; GAPDH used for normalization. (H) Expression levels of SOX9 and COL2A1 were determined using western blot analysis. (I) Densitometry of the protein bands shown in (H), using the ImageJ software. Results are means  $\pm$  SDs.  $n = 3$ ; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . (A, B, D, F, G, and I)  $P$ -values were determined using a one-way analysis of variance with Bonferroni post hoc tests.

### Expression of key genes and inflammatory cytokines associated with IL-1 $\beta$ -induced catabolism is regulated by PDRN treatment

To further explore the effects of PDRN in IL-1 $\beta$ -induced degradation of ECM in hBMSCs, we determined the expression levels of anabolic and catabolic genes at mRNA and protein levels. The expression of catabolic genes, namely *MMP9*, *ADAMTS4*, and *MMP13*, was upregulated, both at mRNA and protein levels, in IL-1 $\beta$ -induced hBMSCs compared with that in vehicle-treated hBMSCs; however, PDRN treatment of IL-1 $\beta$ -induced hBMSCs downregulated their expression (Fig. 2A-C). In contrast, the expression of *T1MP1*, an anabolic gene, was reduced in IL-1 $\beta$ -induced hBMSCs, whereas it was restored in IL-1 $\beta$ -induced hBMSCs treated with PDRN (Fig. 2A-C). These results indicate that PDRN may regulate the imbalance of catabolic and anabolic gene expression in IL-1 $\beta$ -induced hBMSCs.

To examine the role of PDRN on IL-1 $\beta$ -induced expression of inflammatory cytokines in hBMSCs, we quantitated the expression of *COX2*, *iNOS*, *IL-1 $\beta$* , and *TNF $\alpha$*  at mRNA and protein levels. The expression of these cytokines was significantly enhanced in IL-1 $\beta$ -treated hBMSCs compared with that in vehicle-treated hBMSCs; however, this enhanced expression was significantly reversed in hBMSCs treated with IL-1 $\beta$  and PDRN (Fig. 3A-C). These results indicated that IL-1 $\beta$  increased the proinflammatory response in hBMSCs, which was mitigated by PDRN.

### IL-1 $\beta$ -induced apoptosis of hBMSCs is mitigated by PDRN treatment

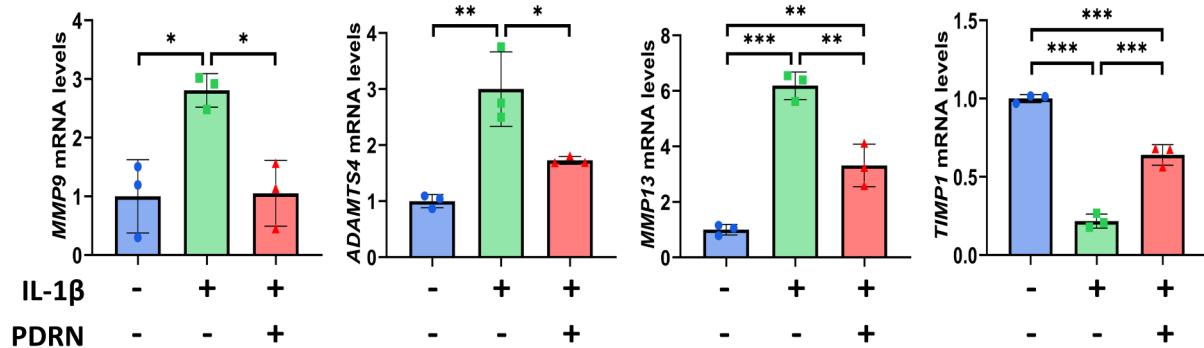
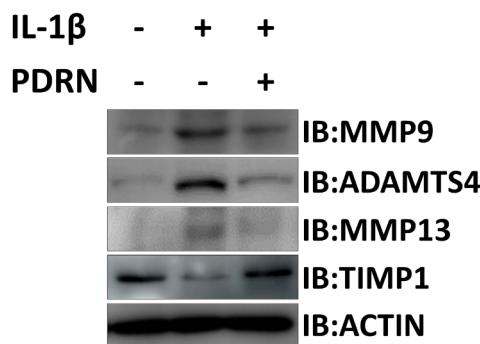
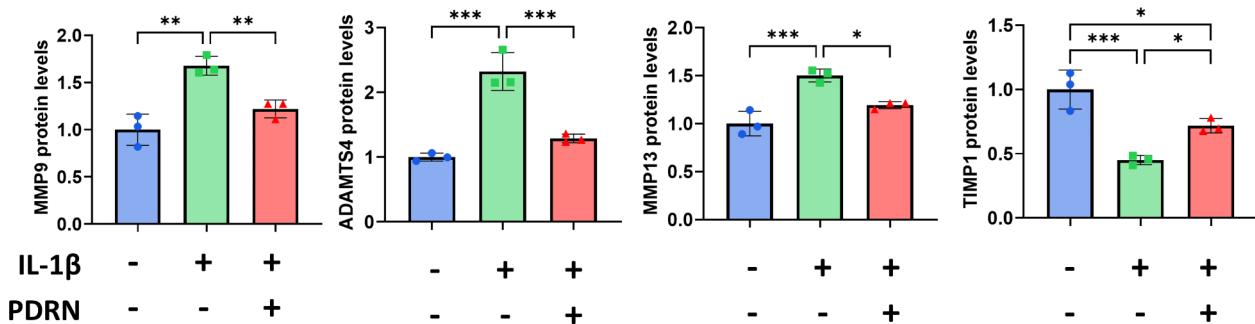
To investigate the effect of PDRN in regulating IL-1 $\beta$ -induced apoptosis in hBMSCs, we performed TUNEL staining to determine the number of apoptotic cells. The percentage of TUNEL-positive cells was higher among IL-1 $\beta$ -treated hBMSCs than among vehicle-treated hBMSCs, whereas treatment of hBMSCs with IL-1 $\beta$  and PDRN reduced the percentage of TUNEL-positive cells compared to those among IL-1 $\beta$ -treated hBMSCs (Fig. 4A, B). We also determined the expression levels of apoptosis-related genes, namely Bcl-2-associated X protein (BAX2) and B-cell lymphoma 2 (BCL-2), using qRT-PCR and western blot analysis. IL-1 $\beta$  treatment enhanced the expression of BAX and reduced that of BCL2 in hBMSCs, whereas these effects were mitigated upon treatment of hBMSCs with IL-1 $\beta$  and PDRN (Fig. 4C-E). These results indicated that IL-1 $\beta$ -induced apoptosis of hBMSCs was partially alleviated by PDRN.

### Anti-inflammatory effects of PDRN are mediated via activation of the cAMP/PKA/CREB signaling pathway through the adenosine A2A receptor

PDRN is an adenosine A2A receptor agonist that increases the levels of 3',5'-cyclic adenosine monophosphate (cAMP) and thereby regulates the activation of cAMP protein kinase A (PKA) and cAMP responsive element binding protein (CREB) signaling pathways, which have important anti-inflammatory effects. We therefore hypothesized that PDRN may regulate the cAMP/PKA/CREB signaling pathway and inhibit the expression of proinflammatory cytokines in hBMSCs. To test this, we first investigated the intracellular cAMP level in IL-1 $\beta$ -treated hBMSCs by ELISA assay. As predicted, intracellular cAMP levels were decreased. Conversely, after exposing IL-1 $\beta$ -treated hBMSCs to PDRN, they exhibited increased levels of intracellular cAMP. Thus, we confirmed that the levels of intracellular cAMP were increased compared to IL-1 $\beta$ -treated hBMSCs alone. (Fig. 5A). Next, we determined the expression levels of PKA/CREB components in IL-1 $\beta$ -treated hBMSCs with or without PDRN treatment using western blot analysis. IL-1 $\beta$ -treated hBMSCs showed decreased p-CREB/CREB and p-PKA/PKA ratios compared to those treated with the vehicle (Fig. 5B, C). PDRN treatment partially restored the IL-1 $\beta$ -induced decrease in the ratio of p-CREB/CREB and p-PKA/PKA in hBMSCs, but this effect was reduced in the presence of DMPX, which is an inhibitor of the adenosine A2A receptor (Fig. 5B, C). This suggested that PDRN's action via the A2A receptor exhibits anti-inflammatory activity by activating the cAMP/PKA/CREB signaling pathway.

### Activation of the adenosine A2A receptor is sufficient to decrease IL-1 $\beta$ -induced expression of inflammatory cytokines in hBMSCs

To determine whether treatment with an adenosine A2A receptor (A2AR) agonist could prevent inflammation in IL-1 $\beta$ -induced hBMSCs, CGS21680, an A2A receptor agonist and ZM241385, an A2A receptor antagonist were supplemented into the media during chondrogenic differentiation of hBMSCs. As shown in Fig. 6A and

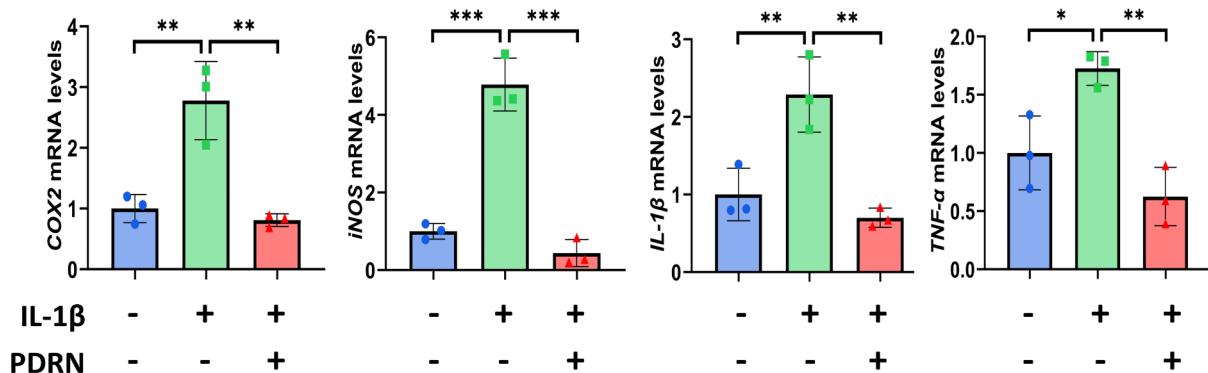
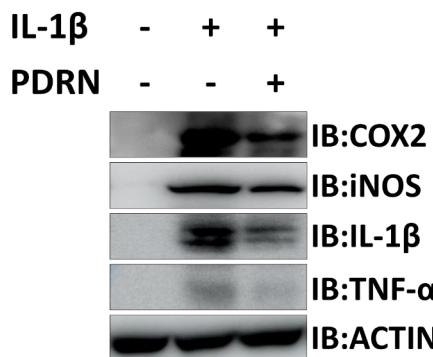
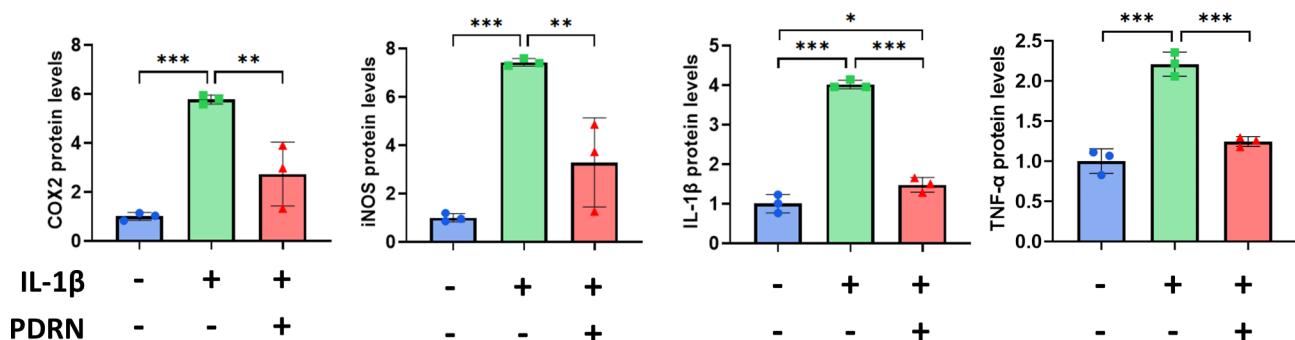
**A****B****C**

**Fig. 2.** Polydeoxyribonucleotide (PDRN) downregulates the expression of catabolic markers and upregulates that of an anabolic marker in an IL-1 $\beta$ -induced inflammatory microenvironment. **(A)** mRNA expression of catabolic and anabolic genes was determined using qRT-PCR; GAPDH used for normalization. **(B)** Expression of catabolic and anabolic proteins was measured using western blot analysis. **(C)** Densitometry analysis of the protein bands shown in **(B)** using the ImageJ software. Results are means  $\pm$  SDs.  $n=3$ ; \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.001$ . **(A,C)**  $P$ -values were determined using a one-way analysis of variance with Bonferroni post hoc tests.

B, the CGS21680 treatment group reduced proinflammatory cytokine expression levels, but this effect was reversed by PDRN and A2A receptor antagonist (ZM241385) treatment. These results demonstrated that PDRN downregulates proinflammatory cytokines in IL-1 $\beta$ -treated hBMSCs through adenosine A2A receptor modulation.

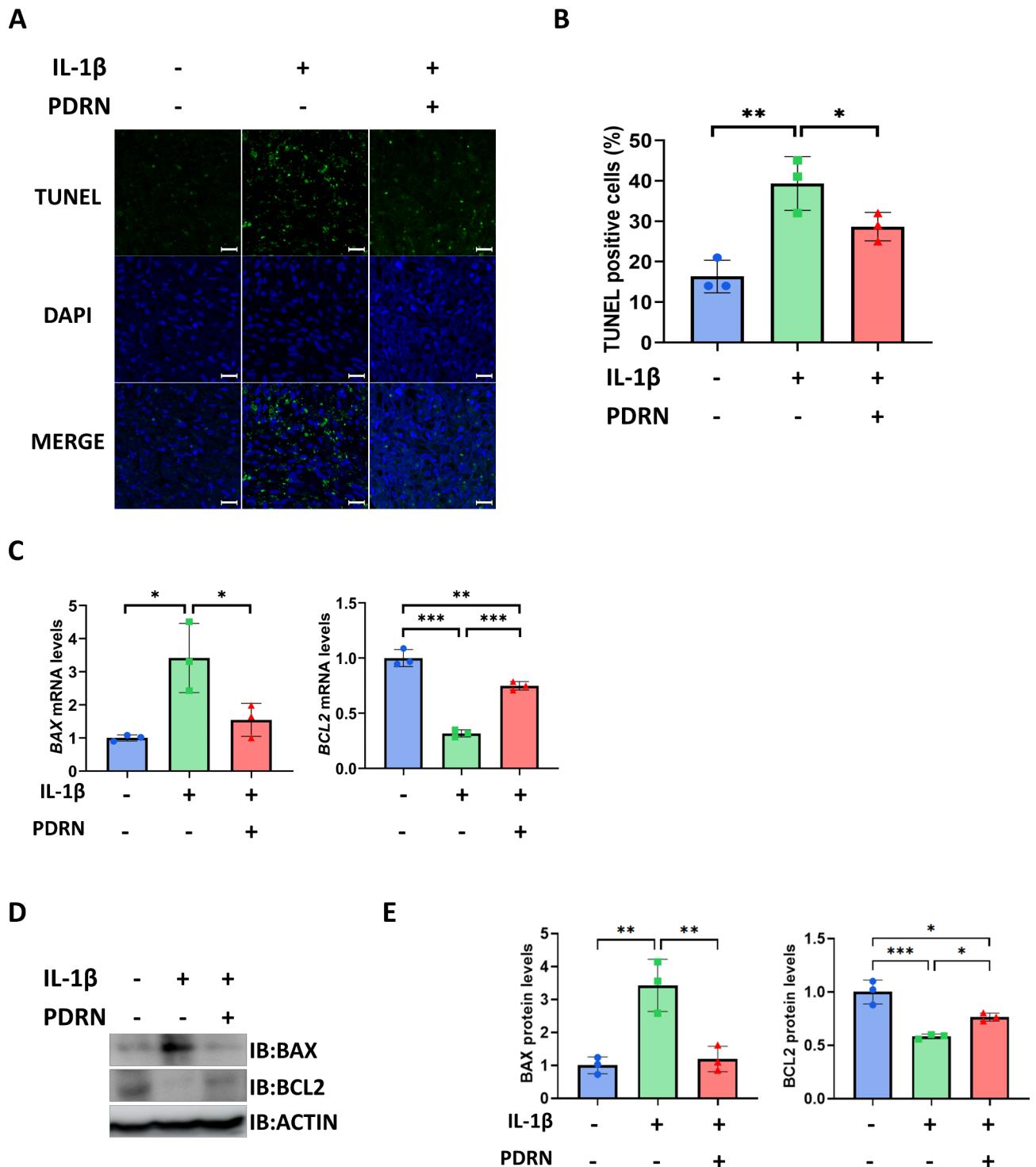
#### PDRN exerts anti-inflammatory effects via NF- $\kappa$ B signaling pathway modulation

The NF- $\kappa$ B signaling pathway is activated by IL-1 $\beta$  and plays an important role in regulating inflammation and apoptosis in hBSMCs. To examine whether PDRN exerts anti-inflammatory effects in hBMSCs via

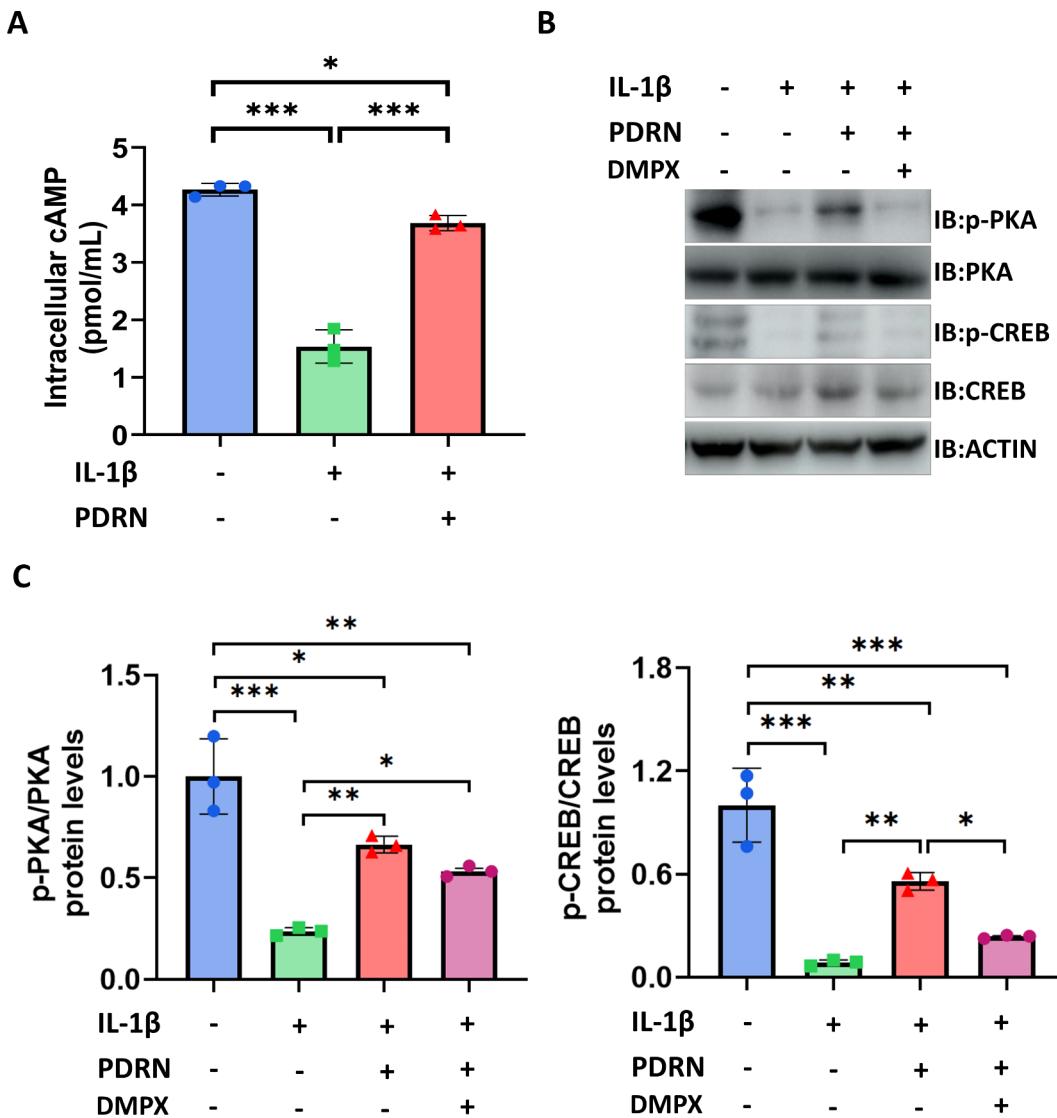
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**Fig. 3.** Polydeoxyribonucleotide (PDRN) decreased the expression of inflammatory cytokines in chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. (A) mRNA expression of proinflammatory cytokine genes was evaluated using qRT-PCR; GAPDH used for normalization. (B) Expression of proinflammatory cytokines was determined using western blot analysis. (C) Densitometry analysis of protein bands shown in (B) using the ImageJ software. Results are means  $\pm$  SDs.  $n=3$ ; \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.001$ . (A,C)  $P$ -values were determined using a one-way analysis of variance with Bonferroni post hoc tests.

modulation of the NF- $\kappa$ B signaling pathway, the expression of NF- $\kappa$ B mediators was evaluated using western blot analysis. IL-1 $\beta$ -treated hBMSCs showed increased phosphorylation of I $\kappa$ B $\alpha$  and p65 compared with vehicle-treated hBMSCs, indicating activation of the NF- $\kappa$ B signaling pathway. In contrast, PDRN treatment of IL-1 $\beta$ -induced hBMSCs suppressed the phosphorylation of I $\kappa$ B $\alpha$  and p65, suggesting that part of PDRN's mechanism of action works by inhibition of the NF- $\kappa$ B signaling pathway (Fig. 7A, B). Next, we confirmed the effect of PDRN on the nuclear translocation of p65, which is required for NF- $\kappa$ B signal



**Fig. 4.** Polydeoxyribonucleotide (PDRN) rescued IL-1 $\beta$ -induced apoptosis in chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. (A) Cell apoptosis was determined using the TUNEL assay. Scale bar = 20  $\mu$ m. (B) Percentage of TUNEL-positive cells. (C) mRNA expression of apoptosis-related genes (BAX and BCL2) was evaluated using qRT-PCR; GAPDH used for normalization. (D) Expression of apoptosis-related proteins was evaluated using western blot analysis. (E) Densitometry analysis of protein bands shown in (D) using the ImageJ software. Results means  $\pm$  SDs.  $n=3$ ; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . (B-D)  $P$ -values were determined using a one-way analysis of variance with Bonferroni post hoc tests.



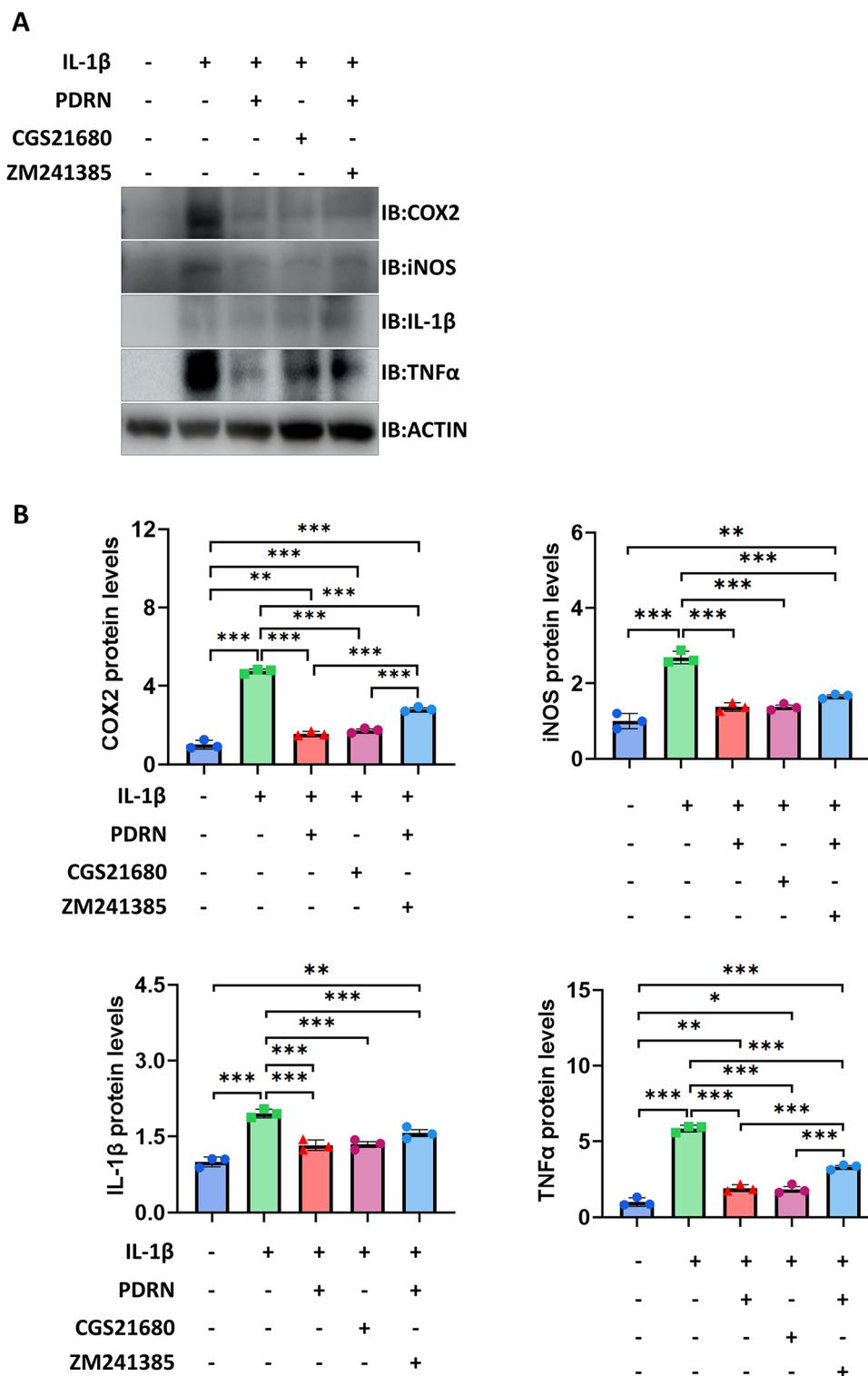
**Fig. 5.** Polydeoxyribonucleotide (PDRN) upregulates the phosphorylation of the cAMP/PKA/CREB pathway proteins under IL-1 $\beta$ -induced inflammation. **(A)** Intracellular cAMP levels of hBMSCs treated with vehicle, IL-1 $\beta$  (10 ng/mL), or IL-1 $\beta$  (10 ng/mL) with PDRN (100  $\mu$ g/mL). **(B)** Expression of PKA/CREB signaling pathway mediators in hBMSCs treated with vehicle, IL-1 $\beta$  (10 ng/mL), PDRN (100  $\mu$ g/mL), or DMPX (100  $\mu$ g/mL) were evaluated using western blot analysis. **(C)** Densitometry analysis of protein bands shown in **(B)** using the ImageJ software. Results are means  $\pm$  SDs.  $n = 3$ ; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . **(A,C)**  $P$ -values were determined using a one-way analysis of variance with Bonferroni post hoc tests.

pathway activation, using immunofluorescence staining. IL-1 $\beta$ -treated hBMSCs showed translocation of NF- $\kappa$ B from the cytoplasm to the nucleus, whereas this transfer was inhibited upon PDRN treatment (Fig. 7C). Taken together, these findings suggest that PDRN inhibits the nuclear translocation of p65.

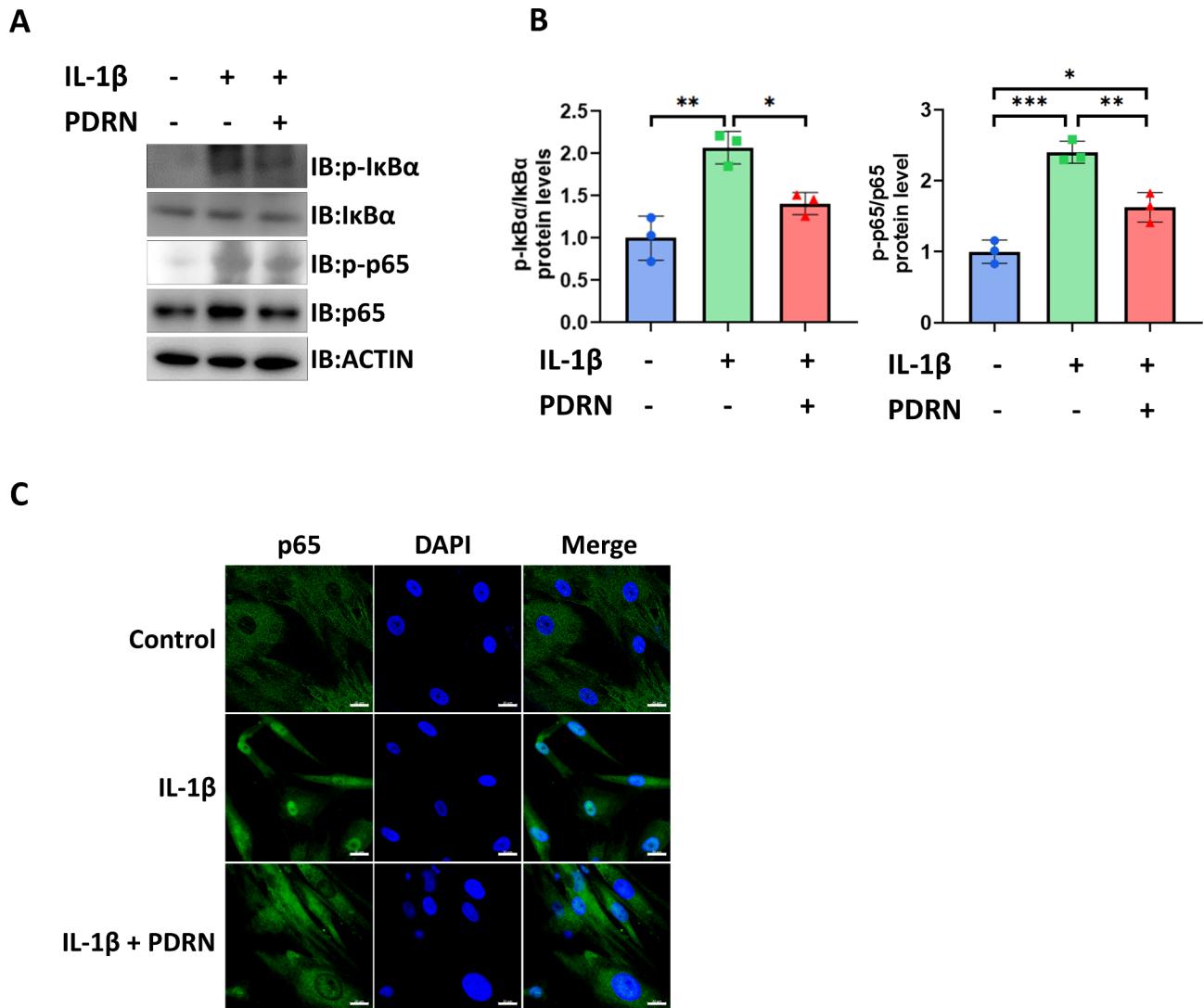
## Discussion

PDRN is widely known to have angiogenic, anti-inflammatory, antiapoptotic, bone-regenerative, wound-healing, collagen-synthesizing, and scar-preventive effects through activation of the A2a receptor and the salvage pathway<sup>42</sup>. *De novo*DNA synthesis is impaired in damaged tissues, and the DNA salvage pathway acts through recovery of bases and nucleosides<sup>42</sup>. PDRN-derived nucleotides can act through a functional salvage pathway, which is an efficient energy-saving metabolic pathway for cell proliferation and growth<sup>43,44</sup>. PDRN may also promote fast wound healing by facilitating efficient DNA synthesis through this energy-saving pathway<sup>43</sup>. However, whether PDRN affects chondrogenic differentiation of hBMSCs remains unknown.

Here, we found that PDRN inhibited the deleterious effects of IL-1 $\beta$  on chondrogenesis and exerted antiapoptotic effects via modulation of the cAMP/PKA/CREB signaling pathway, thereby blocking NF- $\kappa$ B



**Fig. 6.** Adenosine A2A receptor agonist suppressed the expression of inflammatory cytokines in chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. (A) hBMSCs were treated with PDRN (100  $\mu$ g/mL), IL-1 $\beta$  (10 ng/mL) with PDRN (100  $\mu$ g/mL), IL-1 $\beta$  (10 ng/mL) with CGS21680 (1  $\mu$ M) and IL-1 $\beta$  (10 ng/mL) with ZM241385 (1  $\mu$ M) or left untreated in chondrogenic differentiation medium for 14 days. The expression of proinflammatory cytokines was determined using western blot analysis. (B) Densitometry analysis of protein bands shown in (A) using the ImageJ software. Results are means  $\pm$  SDs.  $n=3$ ; \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.001$ .  $P$ -values were determined using a one-way analysis of variance with Bonferroni post hoc tests.



**Fig. 7.** Polydeoxyribonucleotide (PDRN) downregulates the NF- $\kappa$ B signaling pathway under IL-1 $\beta$ -induced inflammation. (A) Expression of NF- $\kappa$ B signaling pathway mediators in human bone marrow-derived mesenchymal stem cells (hBMSCs) treated with IL-1 $\beta$  (10 ng/mL) in the presence or absence of PDRN (100  $\mu$ g/mL) was evaluated using western blot analysis. (B) Densitometry analysis of protein bands shown in (A) using the ImageJ software.  $P$ -values were determined using a one-way analysis of variance with Bonferroni post hoc tests. (C) Immunofluorescence images showing nuclear translocation of p65 in hBMSCs treated with IL-1 $\beta$  (10 ng/mL) in the presence or absence of PDRN (100  $\mu$ g/mL). Nuclei were counterstained with DAPI. Scale bar = 20  $\mu$ m. Results are means  $\pm$  SDs.  $n = 3$ ; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

activation in hBMSCs. To our knowledge, this is the first study to provide an understanding of the molecular mechanism underlying the effect of PDRN on chondrogenic differentiation of hBMSCs.

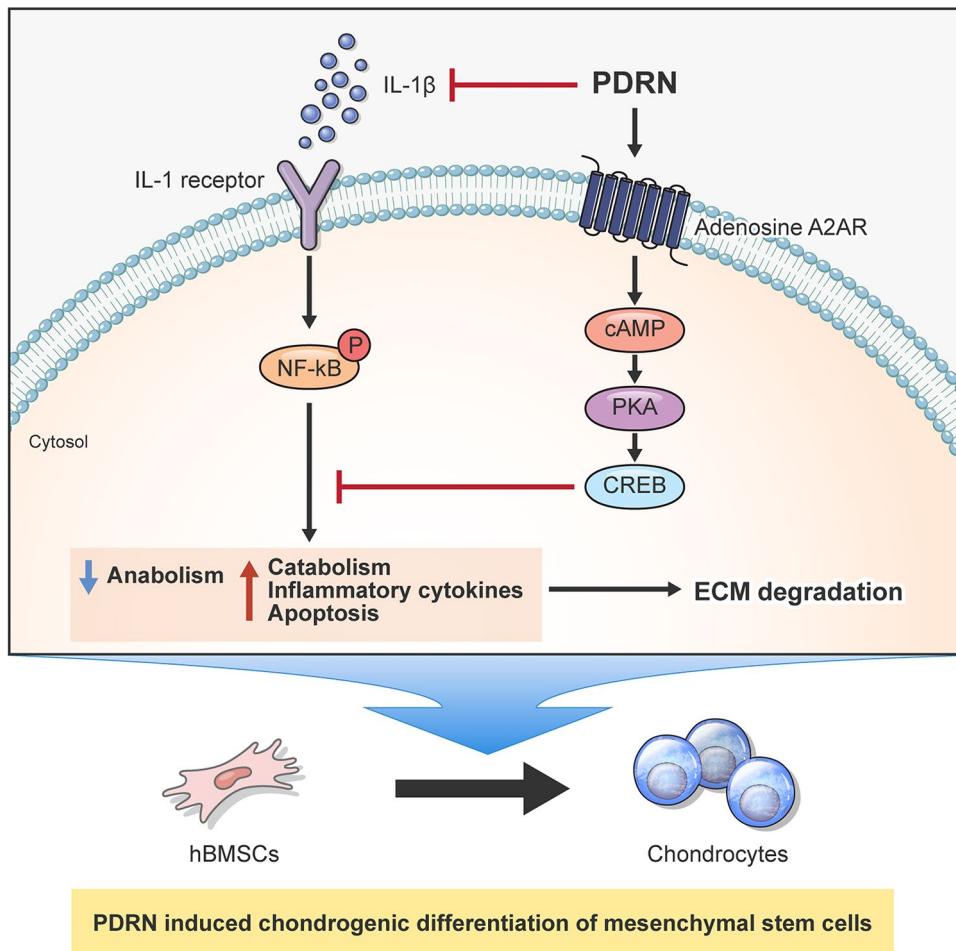
PDRN is a known agonist of A2AR, which triggers elevation of intracellular cAMP levels and enhances the phosphorylation of PKA/CREB leading to inactivation of NF- $\kappa$ B, manifested as an anti-inflammatory effect<sup>23,45</sup>. NF- $\kappa$ B is a key regulator of proinflammatory mediators and can aggravate OA pathogenesis<sup>46</sup>. The NF- $\kappa$ B signaling pathway is stimulated by mechanical stress or inflammatory cytokines, such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, and induces phosphorylation-induced degradation of I $\kappa$ B. Subsequently, active NF- $\kappa$ B p50/p65 heterodimers move into the nucleus and stimulate the transcription of target genes<sup>47</sup>. Thus, inactivation of NF- $\kappa$ B is regarded as a potential therapeutic target in OA.

Previously, various studies have demonstrated the negative effects of inflammatory mediators (IL-1 $\beta$ , TNF- $\alpha$ , and IL-6) on the chondrogenic differentiation of hBMSCs<sup>16,48</sup>. Concurrently, attempts to identify candidates that can reduce the negative effects of these inflammatory factors on hBMSC differentiation have become a significant focus in therapeutic research. For example, melatonin was found to promote chondrogenesis of hBMSCs following exposure to IL-1 $\beta$ -induced inflammation<sup>14</sup>. Additionally, Diallyl disulfide, a constituent of garlic, attenuated IL-1 $\beta$ -induced oxidative stress response in human adipose-derived mesenchymal stem cells and promoted the expression of chondrogenic marker genes<sup>49</sup>. Honokiol,

a natural biphenolic compound, enhanced chondrogenic differentiation of human umbilical cord-derived mesenchymal stem cells under IL-1 $\beta$  stimulation and inhibited inflammatory response by inactivation of the NF- $\kappa$ B signaling pathway<sup>50</sup>. Finally, resveratrol, a natural phytoalexin, cocultured on chitosan-gelatin scaffolds (CGS) blocked the catabolic effect of IL-1 $\beta$  by suppressing the nuclear translocation of NF- $\kappa$ B elements and enhanced the expression of chondrogenic markers in rat MSCs under IL-1 $\beta$  stimulation<sup>51</sup>. These findings, together with our findings in this study, suggest that blocking the proinflammatory cytokine-induced impairment of chondrogenesis could be a potential strategy for the treatment of damaged cartilage.

Our study has some limitations, the important one being the lack of evidence from *in vivo* experiments. Further investigation is needed to demonstrate the role of PDRN in an animal model of OA. Moreover, further experiments are needed to determine the effector pathway of PDRN using A2a receptor knockout (A2aR KO) mice. Previous studies have extensively reported that the adenosine A2A receptor (A2AR) is preferentially stimulated by PDRN. However, adenosine is known to be an agonist of not only the A2AR receptor (of which DMPX is a direct and specific antagonist) but also of the A3R adenosine receptor<sup>52</sup>. PDRN, as a mixture of deoxyribonucleotides, has sufficiently overlapping structural similarities to adenosine, and despite preferentially activating the A2A receptor, may be able to additionally activate A3R or other purigenic receptors<sup>53</sup>. While there is only limited research on the subject, and it is beyond the scope of this study, there is a possibility that direct action of PDRN via another purigenic/adenosine receptor is responsible for supplementary mediation of the inflammatory response. Additionally, use of PDRN in other settings, such as models of neurodegeneration, liver injury, or colitis, have suggested direct actions of PDRN on various other inflammatory cytokines and pathways<sup>26,54</sup>. Similarly to our study, use in these various models confirmed anti-apoptotic regulation of Bax and Bcl-2 by PDRN. However, additional effects of PDRN on TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, VEGF-mediated growth and angiogenesis pathways, and the MAPK pathways<sup>26,54</sup> were also observed. The extent to which these same pathways are implicated in facilitating hBMSC chondrogenesis, tissue regeneration and cartilage regrowth remain to be investigated. Verification of the detailed molecular mechanisms through methods such as transcriptome analysis using RNA-sequencing will also allow a more complete understanding of PDRN's mechanism of action in hBMSCs and provide new insights into the various uses for this promising therapeutic agent.

Taken together, we have demonstrated that PDRN inhibits the negative effects of IL-1 $\beta$  on chondrogenic differentiation of hBMSCs and has a protective effect against IL-1 $\beta$ -induced inflammatory conditions by promoting the expression of anabolic genes through the cAMP/PKA/CREB pathway and blocking NF- $\kappa$ B activation (Fig. 8). Based on these results, hBMSCs in combination with PDRN could be part of an effective therapeutic strategy in cartilage regenerative medicine.



**Fig. 8.** Schematic diagram of polydeoxyribonucleotide (PDRN) treatment in IL-1 $\beta$ -induced impairment of chondrogenic differentiation. PDRN protects against IL-1 $\beta$ -induced inflammation and promotes chondrogenic differentiation of hBMSCs by activating the cAMP/PKA/CREB pathway and inhibiting NF- $\kappa$ B activation. Binding of PDRN to the adenosine A2A purigenic receptor activates the downstream kinase cascade which ultimately results in CREB-mediated suppression of NF- $\kappa$ B. Inhibition of this pathway prevents suppression of growth factors necessary for chondrocyte development such as growth hormone (GH) and insulin-like growth factor 1 (IGF-1) as well as bone morphogenic protein 2 (BMP2) expression. Suppression of these factors normally leads to apoptosis and catabolism. Thus, PDRN action via the A2A receptor effectively prevents apoptosis and supports development and differentiation of hBMSCs.

## Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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## Author contributions

AB, DB: conducted the study design, data interpretation, and wrote the manuscript draft. SHK: completed the data acquisition, analysis, and intellectual discussion. JK: helped the sample preparation and data analysis. GRN, D-WL: performed experiments and analyzed data. HJK, S-RC: contributed to the funding acquisition, study design, data interpretation, manuscript editing.

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## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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