Novel molecule BBC0901 inhibits BRD4 and acts as a catabolic regulator in the pathogenesis of osteoarthritis

Hyemi Lee [a], Jiho Nam [a], Hahyeong Jang [a], Young-Sik Park [b], Min-Hee Son [b], In-Hyun Lee [b], Seong-il Eyun [c], Jimin Jeon [a], Siyoung Yang [a,*]

[a] Department of Biological Sciences, Sungkyunkwan University, Suwon 16419, Republic of Korea
[b] Benobio Co., Ltd., Gyeonggi-do 13494, Republic of Korea
[c] Department of Life Science, Chung-Ang University, Seoul 06974, Republic of Korea

ARTICLE INFO

ABSTRACT

Osteoarthritis (OA) is induced by matrix degradation and inflammation mediated by bromo-domain-containing protein 4 (BRD4)-dependent catabolic factors. BRD4 acts as both a transcriptional regulator and an epigenetic reader. BBC0901 was identified as an inhibitor of BRD4 using a DNA-encoded library screening system. We aimed to demonstrate the effects of BBC0901 on OA pathogenesis by in vitro, *ex vivo*, and in vivo analyses. BBC0901 inhibited the expression of catabolic factors that degrade cartilage without significantly affecting the viability of mouse articular chondrocytes. Additionally, *ex vivo* experiments under conditions mimicking OA showed that BBC0901 suppressed extracellular matrix degradation. RNA sequencing analysis of gene expression patterns showed that BBC0901 inhibited the expression of catabolic factors, such as matrix metalloproteinases (MMPs) and cyclooxygenase (COX)2, along with reactive oxygen species (ROS) production. Furthermore, intra-articular (IA) injection of BBC0901 into the knee joint blocked osteoarthritic cartilage destruction by inhibition of MMP3, MMP13, COX2, interleukin (IL)6, and ROS production, thereby obstructing the nuclear factor kappa-light-chain-enhancer of activated B cell and mitogen activated protein kinase signaling. In conclusion, BBC0901-mediated BRD4 inhibition prevented OA development by attenuating catabolic signaling and hence, can be considered a promising IA therapeutic for OA.

1. Introduction

Osteoarthritis (OA), a degenerative joint disease, is a primary cause of disability in the elderly population worldwide. More than 500 million older adults are diagnosed with this degenerative condition across the globe, which manifests as excruciating joint pain, swelling, and loss of mobility [1]. The majority of individuals are likely to experience pain caused by OA during various phases of their lives, with advanced age emerging as a pivotal risk factor for its onset [2]. OA pathogenesis involves tissue-level failure that affects more than just the articular cartilage surface [3]. The involvement of epigenetic regulation is required during gene regulation to induce OA pathogenesis with various phenotypes [4–6]. Epigenetics could potentially exert a significant influence on determination of the activation or deactivation timings of pathological catabolic factors involved in OA pathogenesis [7]. The inhibition of epigenetic regulation could offer an ideal approach for OA

therapy. JQ1 is a known BRD4 inhibitor.

Bromodomain-containing protein 4 (BRD4) belongs to the family of bromo and extra-terminal domain-containing proteins, binds to hyper-acetylated histone regions, and accumulates in transcriptionally regulated areas during transcriptional initiation and elongation [8,9]. It is divided into four distinct domains, namely BD1, BD2, ET, and CTM [10]. Each BD1 and BD2 tandem bromodomain is assembled via four alpha helices connected by two loops, and both BD1 and BD2 can interact with acetylated lysine residues of target proteins [11]. Deficiencies in BD1 and BD2 domains are associated with the induction of nephrocalcinosis and colorectal cancer. [12,13]. The ET domain exhibits a structure with three helices and a single loop. The CTM domain is associated with an increase in gene transcription and corresponding activities via interaction with RNA polymerase II and the transcription elongation factor, P-TEFb [11,14]. The defects in ET and CTM domains induce Cornelia de Lange Syndrome via disruption of the interaction with NIPBL,

interruption of binding to cellular components, and an uncontrolled activation of RNA polymerase II [14–16]. As BRD4 is an epigenetic and transcriptional regulator that modifies chromatin activity, corresponding inhibitors are effective treatments for a wide variety of malignancies, including acute lymphoblastic leukemia and multiple myeloma [17–19]. Although JQ1 is a well-known BRD4 inhibitor for OA treatment [20–22], clinical trials of JQ1 have failed owing to its short half-life and associated side effects [23,24].

At present, patients with OA commonly use nonsteroidal anti-inflammatory drugs (NSAIDs) to treat pain associated with OA; however, the dose has to be carefully adjusted because of the serious adverse effects associated with higher doses [25]. The possible adverse effects of NSAIDs include gastrointestinal distress, which manifests as symptoms such as nausea, vomiting, and diarrhea, and more serious complications including stomach ulcers, blood loss in the intestines, and anemia [26].

In addition to oral administration methods, such as NSAIDs, OA drugs can also be administered via intra-articular (IA) injection [27]. IA injections have many advantages, such as increased bioavailability of therapeutic drugs in affected areas, decreased systemic exposure, and low total costs [28]. However, few treatments can be administered via IA. IA injections of corticosteroids are commonly used to treat OA-associated pain and relieve joint effusion [29]. While they might provide temporary relief from symptoms, these treatments do not halt the progression of the condition. Hyaluronic acid (HA) is a visco-supplement approved by the Food and Drug Administration (FDA). HA is commonly used to treat OA; however, there is no evidence that HA can delay or prevent the need for joint replacement [30]. To exploit the potency of IA injections as an administration route, an IA-compatible drug must be developed. An ideal IA drug should guarantee a disease-modifying effect and/or cartilage regeneration, pose no or few safety issues, and provide regulated release of the therapeutic agent over a long period. However, recent studies have not identified any IA injection-compatible drug that meets all criteria.

Therefore, we developed BBC0901, a novel BRD4 inhibitor demonstrating no cytotoxic effects when compared to JQ1. Furthermore, BBC0901 was compatible with IA injection, and it acted as an anti-osteoarthritic agent by inhibiting matrix degradation, inflammation, and production of reactive oxygen species (ROS).

2. Materials and methods

2.1. Screening of DNA-encoded chemical library (DEL) for bromodomains of BRD4

Novel bromodomain and extra-terminal motif (BET) inhibitors (BETis) were identified by screening a DEL (WuXi AppTec, Shanghai, China) of BD1s and BD2s of the BRD4 protein. These proteins were generated by amplifying the relevant sections of the BRD4-encoding genes, placing the sections into pET28a vectors (Novagen, Northumberland, UK), transforming *Escherichia coli* BL21(DE3) (Novagen, Northumberland, UK) with each plasmid, and inducing protein expression by treatment with 0.2 mM isopropyl β-D-thiogalactopyranoside (Sigma, St. Louis, MO) for 16 h at 18 ºC. The bacteria were collected, resuspended in a lysis buffer (50 mM Tris [pH 8.2], 300 mM NaCl, and 20 mM imidazole), and sonicated. The lysates were centrifuged at 1550 g for 1 h at 4 °C, after which the supernatant was loaded onto a nickel affinity HisTrap HP column (GE Healthcare, Chicago, USA). After washing the column, BRD4 was eluted with 50 mM Tris buffer (pH 8.2) containing 300 mM NaCl and 500 mM imidazole.

2.2. Time-resolved fluorescence resonance energy transfer (TR-FRET) assay

TR-FRET assay kits obtained from BPS Bioscience (CA, USA) that were specific for BRD4 (BD1 + BD2) were used. The BD ligands were diluted in water. A master mix comprising 1x BRD Homogeneous Assay

Buffer and diluted BD ligands was prepared. BRD proteins were thawed on ice and diluted with 1x BRD Homogeneous Assay Buffer. Subsequently, 1.5 µl of the master mix was added to each well of a microplate (Labcyte, San Jose, USA), and reactions were initiated by adding 5 µl of diluted BRD protein to each well. The plates were then incubated at room temperature for 30–60 min. GSH acceptor beads (Perkin Elmer, Waltham, USA) and streptavidin-conjugated donor beads (Perkin Elmer, Waltham, USA) were diluted with 1x BRD Homogeneous Detection Buffer 1. Subsequently, 10 µl of the acceptor bead mixture was added to each well. After incubation at room temperature for 30 min, 10 µl of the donor bead mixture was added to each well, followed by incubation at room temperature for 15–30 min. Alpha counts were measured using the EnVision 2105 multimode plate reader (Perkin Elmer, Waltham, MA, USA).

2.3. Binding kinetics assay

Analysis of binding kinetics was performed using a Sartorius Octet® R2 system (Sartorius, Gottingen, German). The super streptavidin (SSA) biosensors were loaded with biotinylated BRD4 BD1, BRD4 BD2, or biotin (used as a control) in PBST buffer (PBS, 0.02% Tween20, 5% DMSO) and dipped into the compound solution for 10 min. The binding (represented by kon values) of the compound to proteins was examined by immersing an individual sensor into a series of dilutions of proteins for 180 s. Subsequently, the sensor was immersed into the blank buffer PBST to measure the off-rate (koff). Simultaneously, another compound sensor was immersed into the blank buffer PBST, which served as the blank control. Competitive binding was assessed by immersing the compound sensor into a mixture of proteins and test chemicals.

2.4. Cells and reagents for treatment

Primary mouse chondrocytes isolated from five-day-old ICR (DBL, Chungbuk, South Korea) mice were used for in vitro assays, as previously reported [31]. Briefly, the femoral condyles and tibial plateaus were isolated from the knees of each mouse after removing the skin. Subsequently, they were incubated at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) containing collagenase and trypsin-EDTA in a thermal incubator. After cartilage pieces were transferred to a new petri dish, they were incubated in DMEM containing collagenase at 37 °C in a thermal incubator. After few hours, the digestion solution containing the cartilage fraction and cells in DMEM containing 10% fetal bovine serum were filtered with cell strainer. The solution was centrifuged, and chondrocytes were isolated as single cells and cultured. The animal experiments conducted at Sungkyunkwan University were approved by the University's Institutional Animal Care and Use Committee (IACUC). BBC0901 was dissolved in dimethyl sulfoxide. The recombinant protein interleukin (IL)− 1β (1 ng/mL) was purchased from GenScript (Piscataway, NJ, USA) and was used to treat chondrocytes at concentrations of 5, 10, and 20 µM for 12 h.

2.5. Lactate dehydrogenase (LDH) assay

Chondrocytes were isolated from the cartilage explants obtained from the knee joints of five-day-old mice. The cells were cultured in a 96-well plate at a density of 1.5×10^4 cells/well. Cell survival was evaluated by the LDH assay after 12 h. The LDH colorimetric assay kit was used to conduct the experiments as described previously (BioVision, Milpitas, USA) [32]. Samples viable at 100% and Triton X-100-killed samples at 0% were used for standardization. Chondrocyte supernatants were examined following 12 h of incubation with various concentrations of BBC0901 and JQ1. The cell viability was calculated as follows:100 - (sample LDH - negative control)/(maximum LDH - negative control)− 100. A SYNERGY H1 microplate reader was used to analyze all signals at 495 nm (Biotek, Vermont, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative (q)RT-PCR

Reverse transcription was performed to generate cDNA from total RNA isolated from mouse articular chondrocytes as described previously [33]. Primer and PCR conditions (annealing temperature and cycles) used for each gene are described as follows: mouse matrix metalloproteinase (MMP)3 (5′-TCCTGATGTTGGTGGCTTCAG-3′ and 5′-TGTCTTGGCAAATCCGGTGTA-3′, 58 °C, 21 cycles), mouse MMP13 (5′-TGATGGACCTTCTGGTCTTCTGG-3′ and 5′-CATCCACATGGTTGGGAAGTTCT-3′, 58 °C, 21 cycles), mouse cyclooxygenase (COX)2 (5′-GGTCTGGTGCCTGGTCTGATGAT-3′ and 5′-GTCCTTTCAAGGAG-3′, 64 °C, 26 cycles), and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-TCACTGCCACCCAGAAGAC-3′ and 5′-TGTAGGCCATGAGGTCCAC-3′, 58 °C, 21 cycles). Real-time PCR (qRT-PCR) was performed using SYBR premix ExTaq (Takara Bio, Shiga, Japan) to quantify gene transcript levels, which were normalized to those of GAPDH.

2.7. Protein isolation and western blotting

Protease and phosphatase inhibitors were added to the lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, 0.2% sodium dodecyl sulfate, and 5 mM NaF) and used to extract proteins from chondrocytes (Roche, Basel, Switzerland). MMP3 and MMP13 in the conditioned medium were precipitated using trichloroacetic acid (TCA). The protein pellet was treated with acetone and centrifuged to remove TCA. Western blotting was performed using antibodies against MMP3 (Abcam, Cambridge, UK), MMP13 (Abcam), COX2 (Abcam), extracellular signal-regulated kinase (ERK)1/2, pERK, c-Jun N-terminal kinases (JNK), pJNK, p38, pp38, and IκB (Cell Signaling Technology). The protein levels were evaluated using the anti-ERK1/2 antibody. Band intensities were normalized to those corresponding to ERK activity using densitometric analysis (AlphaEase FC 4.0; Alpha Innotech, San Leandro, USA).

2.8. Destabilization of the medial meniscus (DMM) surgery and IA injection

The IACUC of Sungkyunkwan University provided permission to conduct all experiments included in this project. DMM surgery destabilizes the medial meniscus by transecting the tibial ligament of the medial meniscus (MM). A DMM operation was performed on male C57BL/6 mice aged ten weeks, following a previously published protocol [33]. Experimental OA in DMM mice was treated with BBC0901 administered via IA injection once a week for seven weeks (three weeks after the surgery). Ten weeks after the DMM surgery, the mice were euthanized for histopathological examination. [34].

2.9. Safranin O staining and immunohistochemistry

Safranin O staining was used for histopathological analysis as described previously [35]. The knee joints of dead mice were removed and preserved in paraformaldehyde before decalcification with EDTA for two weeks. Paraffin blocks were used to cast the knee joints, which were dehydrated using an ethanol/xylene gradient. Microtomal serial sections were obtained from blocks of paraffin with a thickness if 5 μm. Before safranin O staining, the slide sections were hydrated using a process involving deparaffinization, xylene substitution, and ethanol gradients. Cartilage damage was assessed by examining stained knee joint slices based on Osteoarthritis Research Society International (OARSI) grading and the thickness of the subchondral bone plate. OARSI grading is a representative method used to assess severity of OA, which is evaluated from grade 0 to grade 6 according to severity as previously described [36]. Briefly, grade 0 implies intact cartilage morphology, and grades 1–6 indicate abnormal matrix and presence of cells in superficial zone, surface discontinuity, the presence of vertical fissures, erosion, denudation, and deformation, respectively. Protein expression in the cartilage was analyzed using immunohistochemistry. The hydration process was similar to that used for safranin O staining. Specifically, we used an antigen-retrieval reagent, a background blocking reagent, a primary antibody against each protein (MMP3 [Abcam, Cambridge, UK], MMP13 [Abcam, Cambridge, UK], and COX2 [ProteinTech Group, Rosemont, USA]), and a secondary antibody against each primary antibody. Finally, Detection using aminoethyl carbazole was employed to obtain visuals of the samples before quantification using the Image J software [37].

2.10. Gene Set Enrichment Analysis (GSEA) and heatmap analysis using RNA-seq and Ingenuity Pathway Analysis (IPA)

To analyze the effect of BBC0901 on the OA, NF-κB, and MAPK signaling pathways, GSEA was conducted with RNA-seq data. RNA-seq was performed using RNA isolated from chondrocytes of three groups: control, IL-1β only, and co-treating with 20 M BBC0901 and IL-1β. Genes related to the OA, NF-κB, and MAPK signaling pathways were sorted from IPA. The software for GSEA was the Broad Institute Java Desktop software (ver. 4.3). GSEA utilizes nonparametric Kolmogorov-Smirnov statistics to determine whether there is a significance level between the members of a specific gene set and the control group [33]. Heatmap analysis was conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) and RNA-seq data. Gene expression changes by BBC0901 in gene sets whose expression was increased by IL-1β were presented in a heatmap.

2.11. Statistical analysis

At least three independent repetitions of each experiment ($n = 3$) were conducted. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc testing to analyze three or more groups, and the results are presented as the mean $\pm$ SD [33]. In this study, significance was determined at 5% probability ($P < 0.05$), and GraphPad Prism 9 was used for all analyses.

3. Results

3.1. Small molecule BBC0901 selected using the DEL screening system binds to BRD4

BBC0901 was synthesized in the salt form (Fig. 1A) and exhibited good solubility in water. The substance was selected from the WUXI DNA-encoded chemical library (DEL) (Fig. 1B). TR-FRET and Kd values revealed that BBC0901 exhibited high binding specificity for BRD4 (Fig. 1C, D, and Table S1). All in vitro experiments were performed using primary chondrocytes isolated from mice. Cytotoxicity of BBC0901 was evaluated in primary chondrocytes. The LDH assay indicated that treatment with BBC0901 at concentrations ranging from 0.5 μM to 20 μM did not result in cell death compared to control treatment at a concentration of 0 μM (Fig. S1).

3.2. BBC0901 prevents IL-1β-induced gene expression associated with OA

The anti-osteoarthritic effects of BBC0901, a novel BRD4 inhibitor, were evaluated at concentrations ranging from 5 to 20 μM. The chondrocyte viability observed with this concentration range did not differ from that observed with 0 μM. Co-treatment with IL-1β was performed to stimulate an OA-like condition by elevating the expression of catabolic factors MMP3, MMP13, COX2, and IL-6, all of which degrade the cartilage or are associated with inflammation. BBC0901 suppressed the expression of catabolic factors in mouse primary chondrocytes incubated with IL-1β. RT-PCR and qRT-PCR results showed the anti-osteoarthritic effects of BBC0901 on mRNA levels, and western blotting results confirmed these effects (Fig. 2A-2D). The production of prostaglandin E_2 (PGE_2) is activated in osteoarthritic cartilages. PGE_2 is
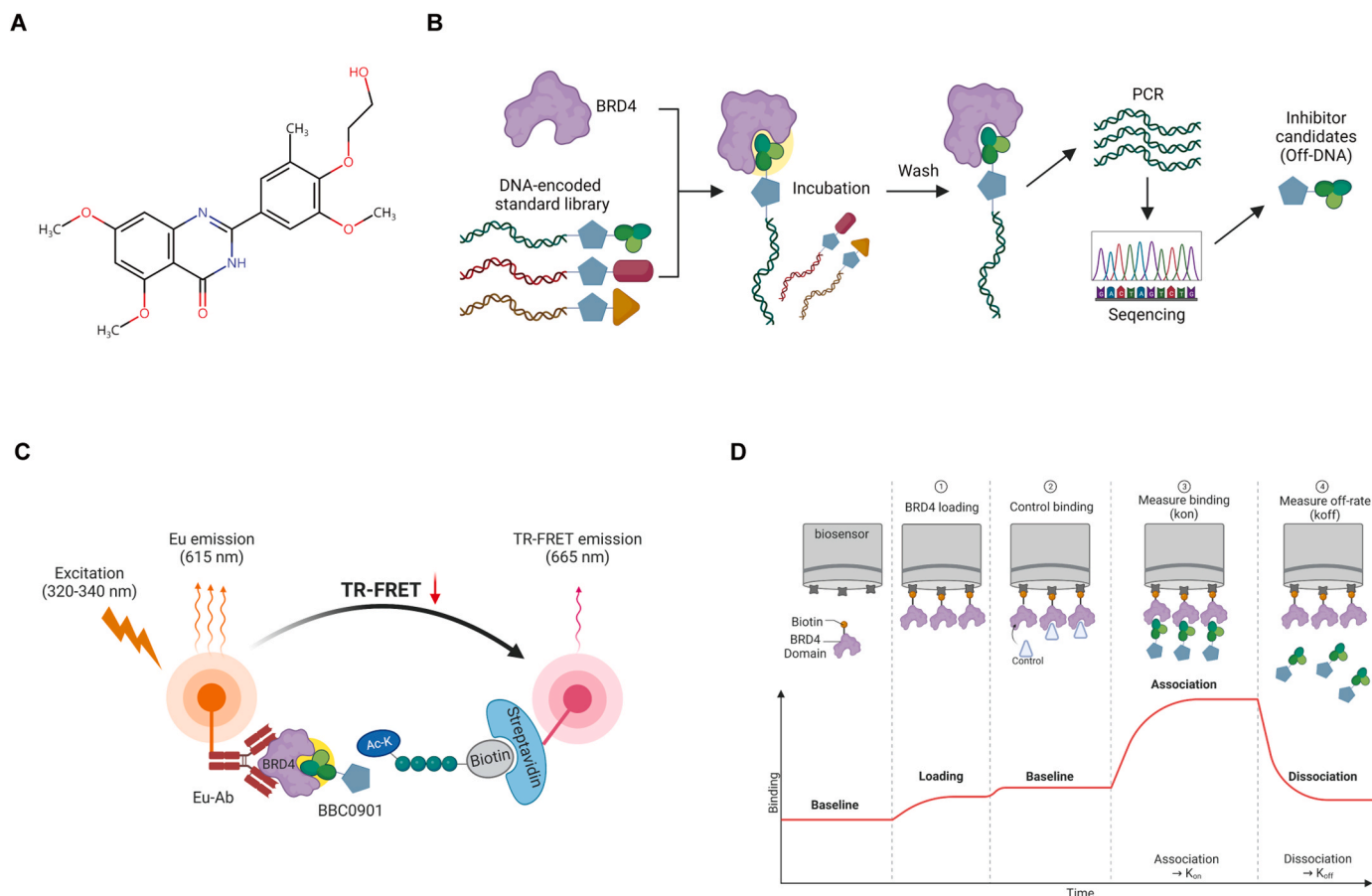
Fig. 1. Chemical properties of BBC0901. (A) The chemical structure of BBC0901 was selected using the DEL screening system. (B) Schematic representing the DEL screening method. DEL screening is used to identify chemicals binding to protein targets using a DNA-encoded library. (C) The binding affinity of BBC0901 toward BRD4 was evaluated by TR-FRET assay. The schematic of the TR-FRET assay is shown in (C). (D) The binding kinetics between BBC0901 and the BD domains of BRD4 were analyzed using a biosensor system. The schematic of the experiment is outlined in (D).

not produced in COX2 tissue-specific knockout mice. BBC0901 inhibited IL-1β-induced PGE$_2$ production in the chondrocyte culture medium (Fig. 2E). To characterize the relationship between BBC0901 and OA signatures, RNA-seq and gene set enrichment analysis (GSEA) were conducted. OA gene signatures comprise sets of genes associated with for matrix degradation, inflammation, and ROS production. As shown in Fig. 2F, from GSEA, we found that BBC0901 downregulated the expression of MMP3, MMP13, COX2, IL-6, and ROS, which was associated with osteoarthritic cartilage destruction. Next, we examined the recovery of extracellular matrix (ECM) synthesis using an *ex vivo* explant culture system. As expected, reduced ECM synthesis under conditions mimicking OA was restored following treatment with BBC0901 (Fig. 2G). Collectively, these data suggest that BBC0901, a novel BRD4 inhibitor, protects catabolic factor expression and promotes ECM synthesis.

3.3. IA injection of BBC0901 administered to mice subjected to DMM surgery halts degeneration of cartilage mediated by OA

With the destabilization of the MM, the mouse model of DMM-induced OA is a highly representative in vivo model for OA. [37]. Next, we examined the effect of BBC0901 on DMM-induced OA in mice. The onset of cartilage destruction was initiated four weeks after DMM surgery in mice [33]. To test whether BBC0901 is suitable as an IA injectable therapeutic agent, we injected BBC0901 intra-articularly on a weekly basis from four to nine weeks after DMM surgery. Finally, after ten weeks after DMM surgery, the animals were euthanized for analysis (Fig. 3A). Injection of 10 μpk of BBC0901 into the knee following the

DMM surgery reduced the OARSI grade and subchondral bone plate thickness compared to those observed in the PBS control group (Fig. 3B–3D). Next, we examined the expression of catabolic factors in mice with DMM-induced OA treated with or without IA injection of BBC0901. As shown in Figs. 3E and 3F, the expression of MMP3, MMP13, and COX2 was reduced upon IA injection of BBC0901. To evaluate ROS production in mouse cartilage, we measured the expression of 8-Hydroxyguanosine (8-OHdG), a representative biomarker for ROS production, which is formed when DNA is oxidized by ROS. As shown by GSEA, BBC0901 exerted protective effects against ROS-mediated damage in the cartilage of IA-injected mice with DMM-induced OA. These results suggest that BBC0901 is a safe and suitable IA-injectable drug for OA treatment.

3.4. BBC0901 inhibits NF-κB and MAPK signaling, thereby reducing the risk of OA

To characterize the signaling pathways regulated by BBC0901, we performed RNA-Seq and ingenuity pathway analysis (IPA) analyses. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways were primarily associated with the IL-1β signaling pathway in articular chondrocytes [38,39]. GSEA suggested that BBC0901 downregulated NF-κB and MAPK signaling enhanced by IL-1β (Fig. 4A). We analyzed alterations in the expression pattern of genes related to each pathway mediated by BBC0901. Lists of genes involved in each signaling pathway were derived using IPA. Alterations in the expression pattern have been presented as a heatmap (Fig. 4B). We have presented only genes related
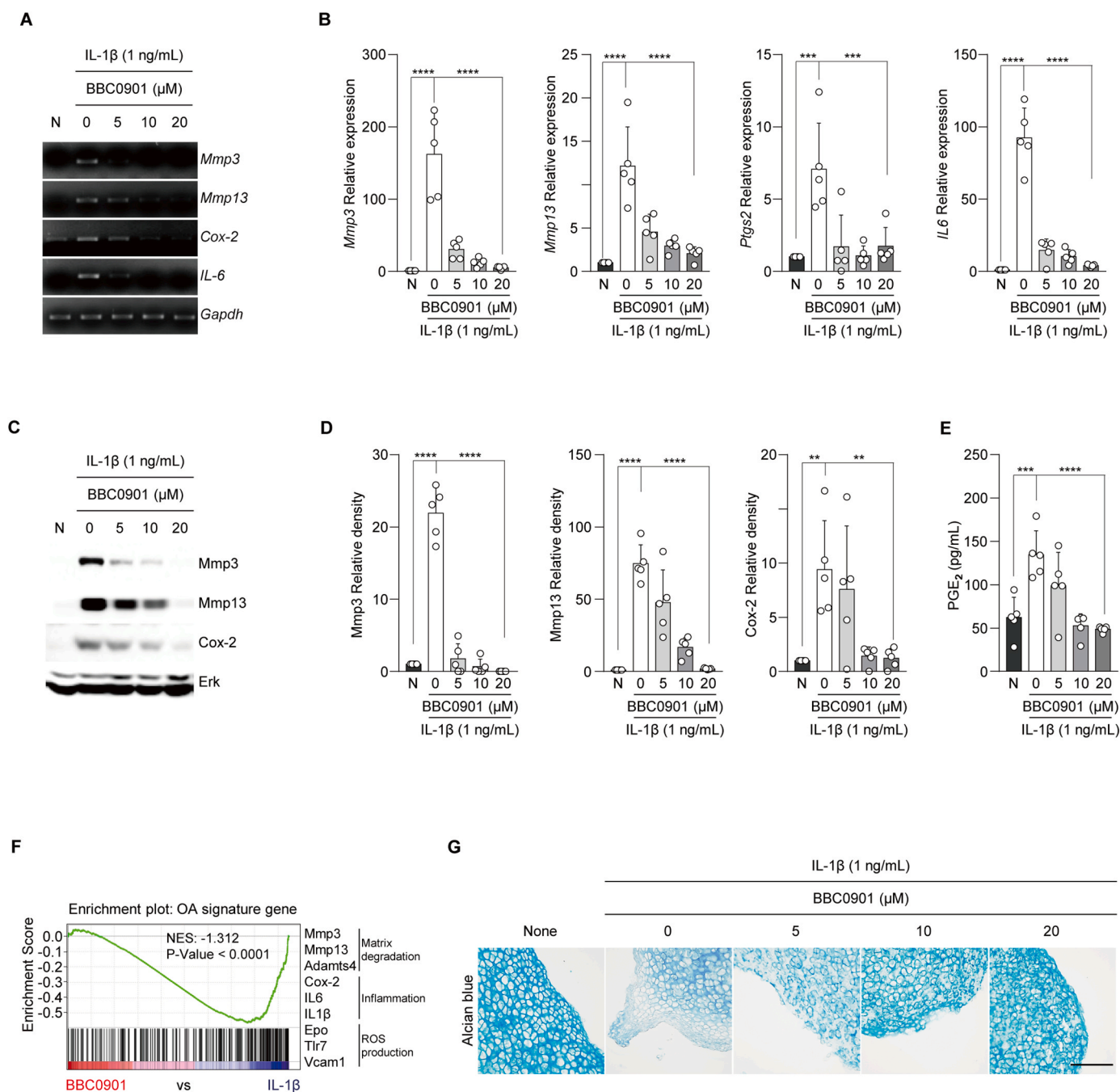
Fig. 2. Under OA-like conditions, BBC0901 reduced the levels of catabolic factors and prevented ECM degradation in chondrocytes. (A-E) Mouse articular chondrocytes were treated with a range of concentrations of BBC0901 and IL-1β (1 ng/mL) for 12 h to observe the anti-osteoarthritic effect of BBC0901 under in vitro conditions. PCR (A), qRT-PCR (B), western blotting (C), and densitometry (D) analyses were performed to assess the expression of MMP3, MMP13, and COX2 ($n = 5$). The level of PGE$_2$ was evaluated from the culture media using ELISA ($n = 5$) (E). The expression levels of each gene and protein were relatively standardized using those of GAPDH (gene) and ERK (protein). (F) The chondrocyte RNA-seq data was used for GSEA. (G) Cartilage explants were stained with an alcian blue solution. After obtaining explants from five-day-old mice, we administered BBC0901 and IL-1β (1 ng/mL) for three days. Values are presented as means ± SD and were assessed using one-way ANOVA with Bonferroni's posthoc test. * *P < 0.01, * **P < 0.001, * ** *P < 0.0001. Scale bar: 100 μm.

to each pathway whose expression was upregulated by IL-1β. We compared the expression patterns with those of the BBC0901 treatment sample.

Among the genes whose expression was increased by IL-1β, the number of genes whose expression showed a higher increase upon co-treatment with BBC0901 was evaluated (Fig. 4C). A total of 25 of 97 genes related to the NF-κB signaling pathway, 64 of 22 genes related to Jnk signaling, 18 of 59 genes associated with p38, and 42 of 105 genes associated with Erk signaling showed a higher increase in the expression

upon co-treatment with BBC0901 when compared to that observed with IL-1β-only treatment. Many activated genes of each signaling pathway were downregulated. The results showed that BBC0901 blocked four signaling pathways. Next, we investigated whether the protein expression levels correlated with the GSEA outcomes. IL-1β-treated chondrocytes were treated with BBC0901 in a dose-dependent manner. As shown by GSEA, the protein level of pIκB was altered by BBC0901 treatment (Figs. 4D, 4E). This indicates that BBC0901 inhibited the NF-κB and MAPK signaling pathways related to catabolic factor expression
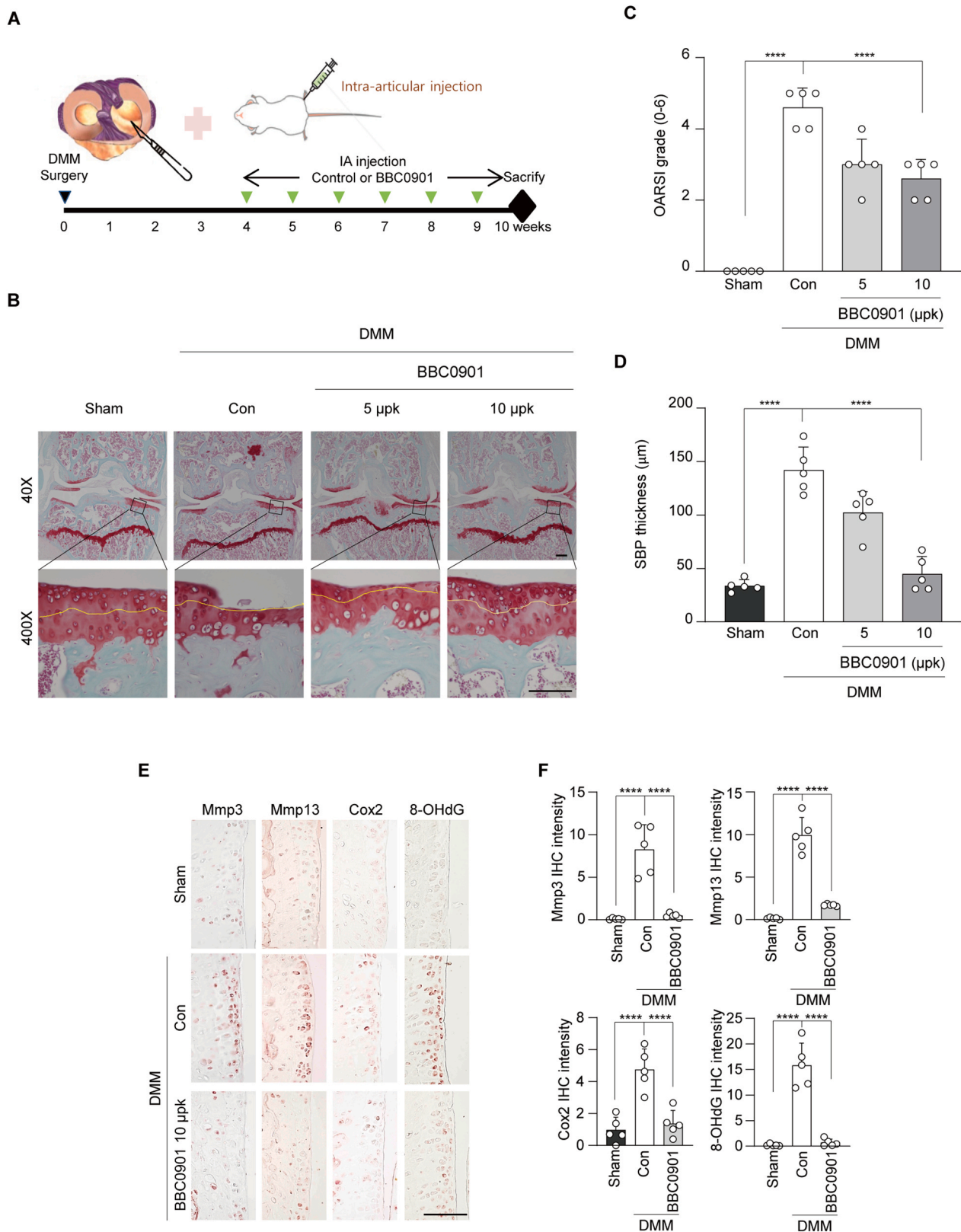
Fig. 3. BBC0901 protected cartilage from breakdown by mediating downregulation of the expression of catabolic factors following DMM surgery. (A) An in vivo experimental plan to assess the impact of BBC0901 on OA is outlined. Ten-week-old mice were subjected to DMM surgery to develop an animal model of OA. BBC0901 was injected intravenously (IV) every alternate day for seven weeks, beginning three weeks after surgery. (B) Safranin O staining was used to examine the histopathology of the knees after injections of BBC0901 at 5 and 10 μpk. (C, D) Anti-arthritic effects of BBC0901 were evaluated by analysis of histopathology based on OARSI scoring (C) and measurement of subchondral bone plate (SBP) thickness (D) ($n = 5$). (E) Immunohistochemistry was analyzed to observe changes in catabolic factor expression after the injection of BBC0901. (F) The level of each protein was quantified using the Image J software ($n = 5$). Values are presented as means ± SD and were assessed using one-way ANOVA with Bonferroni's posthoc test. * ** *P < 0.0001. Scale bar: 100 μm.
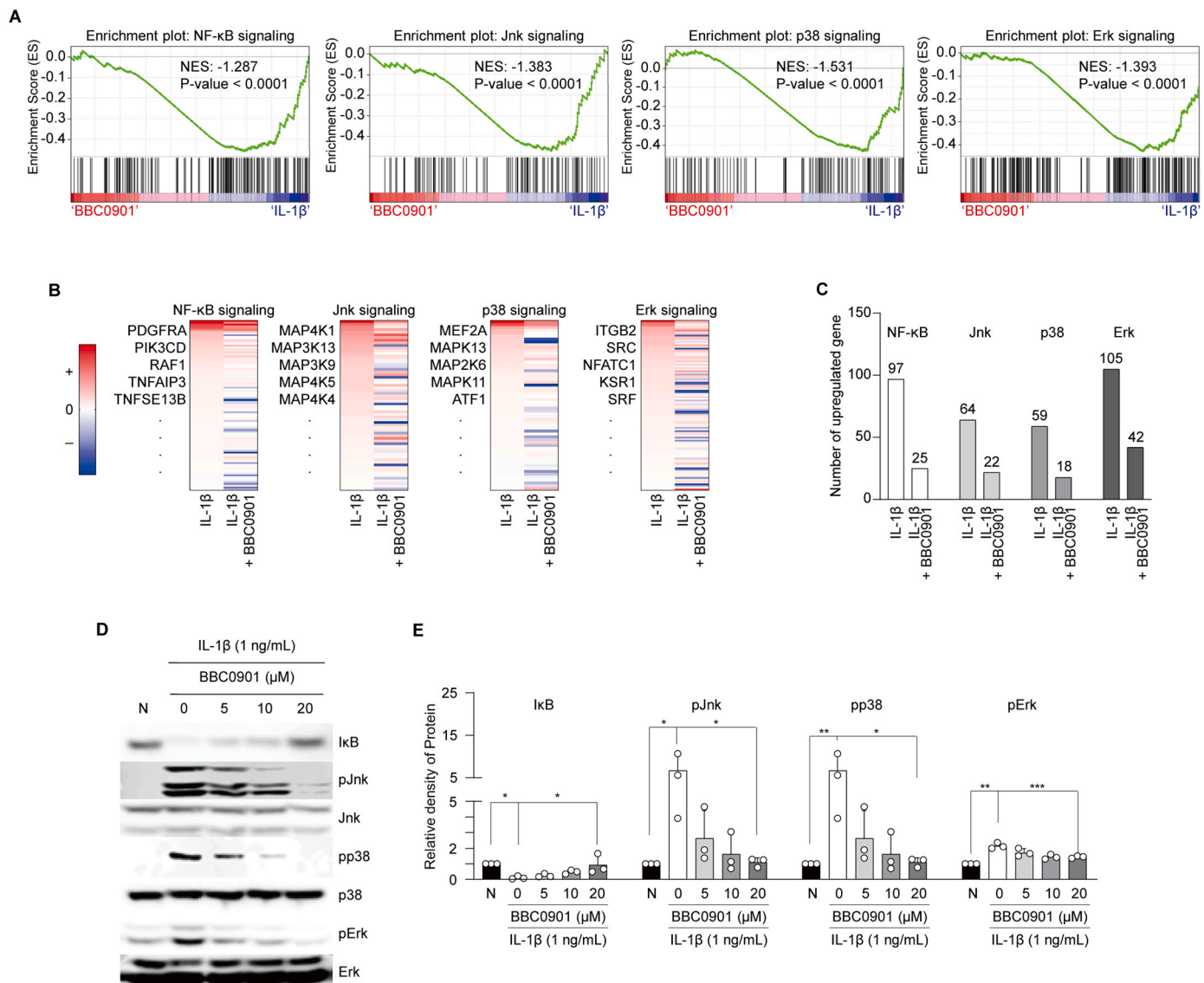
Fig. 4. Ability of BBC0901 to inhibit OA is linked to its capacity to restrain IL-1β-activated NF-κB and MAPK signaling. (A-C) Analyses were performed using RNA-seq data obtained from chondrocytes cotreated with BBC0901 and IL-1β and those treated only with IL-1β as a control to examine associations with each signaling pathway and changes in expression patterns. First, GSEA was performed to examine associations with each signaling pathway (A). Next, heatmaps showed changes in expression pattern associated with only IL-1β treatment and those observed with co-treatment with BBC0901. Heatmaps were analyzed with genes whose expression was upregulated by IL-1β in each signaling pathway (B). Furthermore, some genes whose expression was downregulated by co-treatment with BBC0901 have been presented on the left side of each heatmap. Among the genes whose expression was increased by IL-1β, the number of genes whose expression showed a higher increase upon co-treatment with BBC0901 was evaluated in association with each signaling pathway (C). In the graph, the first bar of each pathway represents the number of IL-1β-upregulated genes, and the second bar represents the number of BBC0901-upregulated genes among these genes. (D-E) The ability of BBC0901 to prevent OA was verified by treating mouse articular chondrocytes with the BBC0901 for 12 h at varying concentrations and then with IL-1β (1 ng/mL) for 10 min before harvesting them ($n = 3$). Western blotting was performed to assess the extent of each signaling pathway (D), and the results were validated by densitometry analysis (E). Values are presented as means ± SD and were assessed using one-way ANOVA with Bonferroni's posthoc test. *$P < 0.05$, * *$P < 0.01$, * **$P < 0.001$. NES, normalized enrichment score.

(Fig. 5).

4. Discussion

BETis can modify epigenetics and have significant potential for therapeutic use as small molecule inhibitors. These inhibitors bind to BD1 and BD2 in BET proteins and prevent their interactions with acetylated lysine residues on histone tails and transcription factors [40]. The pan-BETi JQ1 inhibits atherogenesis in hypercholesterolemic mice, prevents inflammation-induced redistribution of BRD4 on chromatin, reduces monocyte (THP-1 cells) adhesion to human umbilical vein endothelial cells, and downregulates the expression of inflammatory and

adhesion molecules [41]. However, pan-BETis cannot be used chronically because of their toxic effects [42]. JQ1 has a fatal disadvantage of short half-life and toxicity and has not been subjected to evaluation using clinical trials [23,24]. Therefore, selective BETis are important. While pan-BETi binds to BET proteins, selective BETis show higher affinity for one BD than that for the other domain. Hence, it is essential to determine whether selective BETis can maintain their therapeutic properties without toxicity.

With advancing age, there is a growing interest in OA. It is a representative degenerative disease with high treatment costs [43]. However, the drugs currently in use cannot completely treat OA and are primarily administered for pain relief. These medications are primarily
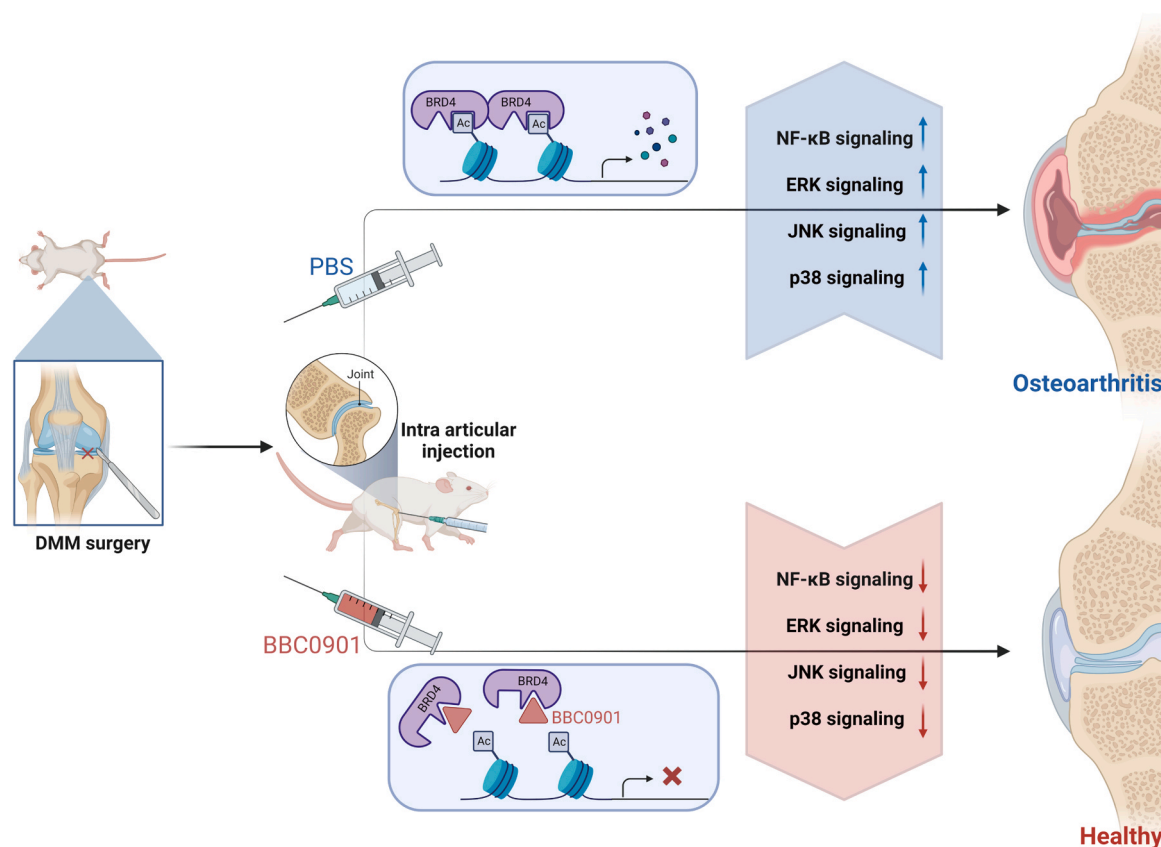
Fig. 5. BBC0901 is the chemical that targets BRD4 and was selected by DEL screening. When BBC0901 was injected in the knee joints of mice after DMM surgery, cartilage destruction was inhibited, and expression of catabolic factors was suppressed. These effects were attributed to the inhibitory impact of BBC0901 on the NF-κB and MAPK signaling pathways.

administered orally, with NSAIDs serving as a representative example [25]. NSAIDs control pain, inflammation, and fever; however, they are associated with side effects, such as gastrointestinal irritation (indigestion, nausea, ulcers, and stomach), cardiovascular risk (stroke and heart attack), allergic reactions, bleeding risk, and tinnitus [26]. Instead of relying on oral administration, recent research has shown active exploration of intra-articular (IA) injections as an alternative route. Pharmacologically, IA injections are highly advantageous compared to systematic oral administration. IA injections are effective with fewer doses than those required for oral administration because active drugs can be delivered directly into the joint space. Therefore, IA injection are associated with fewer side effects and higher bioavailability of active drugs.

As mentioned earlier, JQ1 is an already known BRD4 inhibitor that suppresses the NF-κB signaling pathway [44]. IA injection of JQ1 attenuates OA induced cartilage destruction through inhibition of inflammation and catabolic factor expression [20]. Indeed, intraperitoneal injections of both JQ1 and cyclin-dependent-kinase-9 inhibitor Flavopiridol synergistically reduces DMM induced OA severity [22]. However, JQ1 has a short half-life of an hour and has failed in clinical trials because of its cytotoxicity [23,24]. Hence, screening efforts have shifted towards identifying BRD4 inhibitors suitable for IA injection. DEL screening systems have been used to identify new drugs that target BRD4. The DEL screening system is used to identify drugs that bind to the target proteins by using chemicals combined with barcode DNA. This method has the advantage of screening a wide range of compounds for target proteins. To confirm the binding of BRD4, TR-FRET and kinetic assays were performed using a biosensor.

We screened BBC0901 as a new BRD4 inhibitor through DEL, and TR-FRET assay. BBC0901((2-(4-(2-hydroxyethoxy)− 3-methoxy-5-methylphenyl)− 5,7-dimethoxyquinazolin-4(3 H)-one)) is based on a quinazolin-4(3 H)-one core which is a fused ring structure. Attached to it are several groups, including a benzene ring with methoxy, hydroxyethoxy, and methyl branches. Herein, we firstly reported the function of BBC0901 in this research.

BBC0901 did not show cytotoxicity in chondrocytes and inhibited the expression of catabolic factors that degrade cartilage without toxicity. Catabolic factors have many functions; however, their representative functions include matrix degradation, inflammation, and ROS production. Typical examples of factors that promote matrix degradation include MMP3 and MMP13 [45]. These two molecules are metalloproteinases that break down aggrecan and collagen, which are representative cartilage ECM molecules. This process mediates ECM destruction following the increase in MMP expression [45,46]. Although inflammation is not the main factor inducing OA, it can contribute to disease progression and symptom severity. Representative molecules involved in mediating inflammation include COX2 and IL6. The expression of COX2 increases the production of prostaglandins, especially PGE$_2$, which induces inflammation and pain. It also stimulates the expression of MMP. IL6 is a cytokine that activates an inflammatory reaction and induces the expression of cartilage degradation factors, such as MMPs. ROS production is the representative phenomenon involved in the onset and progression of OA. ROS are byproducts of cell metabolism produced due to oxidative stress and can directly damage cartilage components [47,48]. 8-OHdG serves as a biomarker of ROS production because its presence indicates oxidative stress and DNA damage. [49]. Hence, the concentration of 8-OHdG serves as an indicator for conditions linked to diseases like aging, cancer, and chronic kidney disease. [50–52]. Elevated levels of oxidative stress may disrupt metabolic processes in chondrocytes [48]. Additionally, 8-OHdG levels

increase during OA pathogenesis [33,53].

The expression of these catabolic factors is attenuated by the inhibition of BRD4. For instance, BRD4 inhibition suppresses the expression of MMP13 by regulating autophagy in diabetic intervertebral disc degeneration. Furthermore, BRD4 inhibitor suppresses LPS-induced inflammatory acute colon injury [54,55]. Additionally, blocking BRD4 induces the relaxation of cardiac hypertrophy by reducing ROS generation [56]. In this study, RNA-seq analysis confirmed that among the catabolic factors whose expression is increased by IL-1β, BBC0901 suppressed the expression of genes related to matrix degradation, inflammation, and ROS production. Additionally, the levels of these catabolic factors were reduced in the knee joints of mice after IA injection of BBC0901 following DMM surgery.

Cartilage destruction results from ECM degradation mediated by the upregulation of catabolic factors [38]. Proteoglycan, a cartilage component, binds to water and helps protect the cartilage. A higher fraction of proteoglycan fragments is observed in the synovial fluid of an injured knee than in that of a healthy knee [46]. To conduct an *ex vivo* experiment, cartilage explants were incubated with or without IL-1β and BBC0901 for 72 h. The accumulation or loss of extracellular-sulfated proteoglycans was evaluated by Alcian blue staining, and BBC0901 prevented the loss of the ECM. To confirm the anti-osteoarthritic effect of BBC0901, DMM surgery was performed with an IA injection of BBC0901. These experiments demonstrated that IA injection of BBC0901 after DMM surgery prevented cartilage destruction and the expression of catabolic factors in mouse cartilage via reduction of the expression of catabolic factors, such as MMP3, MMP13, COX2, and ROS production. Upregulated expression of the aforementioned catabolic factors and the destruction of cartilage are primarily induced by NF-κB and MAPK signaling pathways, mainly implicated in OA [39,57]. NF-κB is located in the cytoplasm as a dimer in an inactive state bound to IkB [58]. Upon stimulation by IL-1β, IκB is phosphorylated by IκB kinase and decomposed by the ubiquitin-proteasome system [59]. As a result, NF-κB becomes active and translocates to the nucleus. This induces the expression of genes encoding MMPs, COX2, and those related to ROS production [60–62].

Much like the impact of NF-κB signaling on chondrocyte changes, MAPK signaling is a paradigm example of a signaling pathway that influences chondrocyte alterations. Phosphorylation of p38, a member of the MAPK family, activates p38 signaling. Many age-related diseases have been linked to p38 [63–65]. As a key component of cartilage, type II collagen is destroyed when the p38 signaling pathway is activated, which occurs specifically in OA and is associated with the disease [39]. Phosphorylation of JNK and ERK activates protein-1 (AP-1) [66]. Multiple studies have linked OA development to JNK and ERK signaling activity [67–69]. They are also associated with the developmental, pathogenic, and proliferative processes. Synovial fluid from patients with OA shows elevated levels of chemokines such as CXCL8 and CXCL11, and these chemokines promote apoptosis and limit chondrocyte growth by activating the JNK signaling pathway [70].

In this study, western blot results showed inhibition of the expression of representative molecules of NF-κB and MAPK signaling pathways. Furthermore, RNA-seq analysis using GSEA was conducted to identify alterations in the expression patterns of the overall molecular signatures related to each signaling pathway. Due to the effect of BBC0901, the expression of most of the molecules associated with the NF-κB, JNK, p38, and ERK signaling pathways was reduced compared to that in the control group, indicating that BBC0901 completely inactivated both NF-κB and MAPK signaling pathways.

Overall, our study demonstrates the evaluation of the anti-osteoarthritic effects of BBC0901, a novel BRD4 inhibitor. BBC0901 exhibited high binding specificity for BRD4 and did not affect the viability of primary chondrocytes. BBC0901 reduced the expression of MMP3, MMP13, and COX2 along with ROS production via modulation of the NF-κB and MAPK signaling pathways. This study suggested that BBC0901 can be developed as a potential IA-injectable therapeutic agent for OA.

CRediT authorship contribution statement

Hyemi Lee: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Jiho Nam:** Validation, Formal analysis. **Hahyeong Jang:** Validation. **Young-Sik Park:** Validation, Formal analysis. **Min-Hee Son:** Validation, Formal analysis. **In-Hyun Lee:** Validation, Formal analysis, Investigation, Resources, Writing – original draft. **Seong-il Eyun:** Validation, Formal analysis. **Jimin Jeon:** Validation, Investigation, Writing – original draft, Funding acquisition. **Siyoung Yang:** Conceptualization, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115426.

References

[1] Q. Yao, et al., Osteoarthritis: pathogenic signaling pathways and therapeutic targets, Signal Transduct. Target Ther. 8 (1) (2023) 56.

[2] T. Matsuzaki, et al., FoxO transcription factors modulate autophagy and proteoglycan 4 in cartilage homeostasis and osteoarthritis, Sci. Transl. Med 10 (2018) 428.

[3] J. Martel-Pelletier, et al., Osteoarthritis, Nat. Rev. Dis. Prim. 2 (1) (2016) 16072.

[4] R. Assi, et al., Inhibition of KDM7A/B histone demethylases restores H3K79 methylation and protects against osteoarthritis, Ann. Rheum. Dis. 82 (7) (2023) 963–973.

[5] H. Iijima, et al., Age-related matrix stiffening epigenetically regulates α-Klotho expression and compromises chondrocyte integrity, Nat. Commun. 14 (1) (2023) 18.

[6] Y. Zhang, et al., Triclocarban triggers osteoarthritis via DNMT1-mediated epigenetic modification and suppression of COL2A in cartilage tissues, J. Hazard Mater. 447 (2023), 130747.

[7] P. Smeriglio, et al., Inhibition of TET1 prevents the development of osteoarthritis and reveals the 5hmC landscape that orchestrates pathogenesis, Sci. Transl. Med 12 (2020) 539.

[8] B. Donati, E, et al., BRD4 and Cancer: going beyond transcriptional regulation, Mol. Cancer 17 (1) (2018) 164.

[9] T. Kanno, et al., BRD4 assists elongation of both coding and enhancer RNAs by interacting with acetylated histones, Nat. Struct. Mol. Biol. 21 (12) (2014) 1047–1057.

[10] R.K. Modukuri, et al., Discovery of potent BET bromodomain 1 stereoselective inhibitors using DNA-encoded chemical library selections, Proc. Natl. Acad. Sci. USA 119 (22) (2022) e2122506119.

[11] F. Vollmuth, et al., Structures of the dual bromodomains of the P-TEFb-activating protein Brd4 at atomic resolution, J. Biol. Chem. 284 (52) (2009) 36547–36556.

[12] C.M. Gorvin, et al., Mice with a Brd4 mutation represent a new model of nephrocalcinosis, J. Bone Min. Res 34 (7) (2019) 1324–1335.

[13] W. Wang, et al., Stromal induction of BRD4 phosphorylation results in chromatin remodeling and BET inhibitor resistance in colorectal cancer, Nat. Commun. 12 (1) (2021) 4441.

[14] B.L. Crowe, et al., Structure of the Brd4 ET domain bound to a C-terminal motif from γ-retroviral integrases reveals a conserved mechanism of interaction, Proc. Natl. Acad. Sci. USA 113 (8) (2016) 2086–2091.

[15] N. Luna-Peláez, et al., The Cornelia de Lange Syndrome-associated factor NIPBL interacts with BRD4 ET domain for transcription control of a common set of genes, Cell death Dis. 10 (8) (2019) 548.

[16] B. Zheng, et al., Distinct layers of BRD4-PTEFb reveal bromodomain-independent function in transcriptional regulation, 00508-7, in: Molecular cell, S1097–2765, Advance online publication, 2023.

[17] B.N. Devaiah, et al., BRD4 is a histone acetyltransferase that evicts nucleosomes from chromatin, Nat. Struct. Mol. Biol. 23 (6) (2016) 540–548.

[18] C.J. Ott, et al., BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia, Blood 120 (14) (2012) 2843–2852.

[19] J. Lovén, et al., Selective inhibition of tumor oncogenes by disruption of super-enhancers, Cell 153 (2) (2013) 320–334.

[20] Y. Jiang, et al., BRD4 has dual effects on the HMGB1 and NF-κB signalling pathways and is a potential therapeutic target for osteoarthritis, Biochim Biophys. Acta Mol. Basis Dis. 1863 (12) (2017) 3001–3015.

[21] Q.D. An, et al., Inhibition of bromodomain-containing protein 4 ameliorates oxidative stress-mediated apoptosis and cartilage matrix degeneration through activation of NF-E2-related factor 2-heme oxygenase-1 signaling in rat chondrocytes, J. Cell Biochem 119 (9) (2018) 7719–7728.

[22] T. Fukui, et al., Bromodomain-containing-protein-4 and cyclin-dependent-kinase-9 inhibitors interact synergistically in vitro and combined treatment reduces post-traumatic osteoarthritis severity in mice, Osteoarthr. Cartil. 29 (1) (2021) 68–77.

[23] H. Yang, et al., BRD4: an emerging prospective therapeutic target in glioma, Mol. Ther. Oncolytics 21 (2021) 1–14.

[24] Y. Wang, et al., Synergistic therapy for cervical cancer by codelivery of cisplatin and JQ1 inhibiting Plk1-mutant Trp53 Axis, Nano Lett. 21 (6) (2021) 2412–2421.

[25] B.R. da Costa, et al., Effectiveness of non-steroidal anti-inflammatory drugs for the treatment of pain in knee and hip osteoarthritis: a network meta-analysis, Lancet 390 (10090) (2017) e21–e33.

[26] P. McGettigan, et al., Use of non-steroidal anti-inflammatory drugs that elevate cardiovascular risk: an examination of sales and essential medicines lists in low-, middle-, and high-income countries, PLoS Med. 10 (2) (2013), e1001388.

[27] R.R. Bannuru, et al., OARSI guidelines for the non-surgical management of knee, hip, and polyarticular osteoarthritis, Osteoarthr. Cartil. 27 (11) (2019) 1578–1589.

[28] X. Li, et al., Nanoparticle-cartilage interaction: pathology-based intra-articular drug delivery for osteoarthritis therapy, Nanomicro Lett. 13 (1) (2021) 149.

[29] N. Bellamy, et al., Intraarticular corticosteroid for treatment of osteoarthritis of the knee, Cochrane Database Syst. Rev. 2 (2006), Cd005328.

[30] A. Jørgensen, et al., Intra-articular hyaluronan is without clinical effect in knee osteoarthritis: a multicentre, randomised, placebo-controlled, double-blind study of 337 patients followed for 1 year, Ann. Rheum. Dis. 69 (6) (2010) 1097–1102.

[31] M. Gosset, et al., Primary culture and phenotyping of murine chondrocytes, Nat. Protoc. 3 (8) (2008) 1253–1260.

[32] H. Lee, et al., Seomae mugwort and jaceosidin attenuate osteoarthritic cartilage damage by blocking IκB degradation in mice, J. Cell Mol. Med 24 (14) (2020) 8126–8137.

[33] J. Jeon, et al., Blockade of activin receptor IIB protects arthritis pathogenesis by non-amplification of activin A-ACVR2B-NOX4 axis pathway, Adv. Sci. 10 (14) (2023), 2205161.

[34] J.H. Kim, et al., Regulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1 axis, Cell 156 (4) (2014) 730–743.

[35] C. Cho, et al., Prussian blue nanozymes coated with Pluronic attenuate inflammatory osteoarthritis by blocking c-Jun N-terminal kinase phosphorylation, Biomaterials 297 (2023), 122131.

[36] K.P.H. Pritzker, et al., Osteoarthritis cartilage histopathology: grading and staging, Osteoarthr. Cartil. 14 (1) (2006) 13–29.

[37] J. Jeon, et al., TRIM24-RIP3 axis perturbation accelerates osteoarthritis pathogenesis, Ann. Rheum. Dis. 79 (12) (2020) 1635–1643.

[38] Y. Deng, et al., Reciprocal inhibition of YAP/TAZ and NF-κB regulates osteoarthritic cartilage degradation, Nat. Commun. 9 (1) (2018) 4564.

[39] C.R. Liao, et al., Advanced oxidation protein products increase TNF-α and IL-1β expression in chondrocytes via NADPH oxidase 4 and accelerate cartilage degeneration in osteoarthritis progression, Redox Biol. 28 (2020), 101306.

[40] E. Kulikowski, et al., Inhibitors of bromodomain and extra-terminal proteins for treating multiple human diseases, Med Res Rev. 41 (1) (2021) 223–245.

[41] J.D. Brown, et al., NF-κB directs dynamic super enhancer formation in inflammation and atherogenesis, Mol. Cell 56 (2) (2014) 219–231.

[42] D.B. Doroshow, et al., BET inhibitors: a novel epigenetic approach, Ann. Oncol. 28 (8) (2017) 1776–1787.

[43] Y. Li, et al., Progress in osteoarthritis research by the National Natural Science Foundation of China, Bone Res 10 (1) (2022) 41.

[44] J. Dong, et al., Transcriptional super-enhancers control cancer stemness and metastasis genes in squamous cell carcinoma, Nat. Commun. 12 (1) (2021) 3974.

[45] Y. Zhang, et al., Dual functions of microRNA-17 in maintaining cartilage homeostasis and protection against osteoarthritis, Nat. Commun. 13 (1) (2022) 2447.

[46] Y. Luo, et al., The minor collagens in articular cartilage, Protein Cell 8 (8) (2017) 560–572.

[47] C. Deng, et al., Bioceramic scaffolds with antioxidative functions for ROS scavenging and osteochondral regeneration, Adv. Sci. (Weinh.) 9 (12) (2022), e2105727.

[48] D. Kang, et al., Selenophosphate synthetase 1 deficiency exacerbates osteoarthritis by dysregulating redox homeostasis, Nat. Commun. 13 (1) (2022) 779.

[49] Z. Guo, et al., Constructing a novel 8-hydroxy-2′-deoxyguanosine electrochemical sensor and application in evaluating the oxidative damages of DNA and guanine, Biosens. Bioelectron. 86 (2016) 671–676.

[50] G. Cavallini, et al., Evidence for selective mitochondrial autophagy and failure in aging, Autophagy 3 (1) (2007) 26–27.

[51] S. Kondo, et al., Overexpression of the hOGG1 gene and high 8-hydroxy-2′-deoxyguanosine (8-OHdG) lyase activity in human colorectal carcinoma: regulation mechanism of the 8-OHdG level in DNA, Clin. Cancer Res 6 (4) (2000) 1394–1400.

[52] M. Sanchez, et al., Plasma concentrations of 8-hydroxy-2′-deoxyguanosine and risk of kidney disease and death in individuals with type 1 diabetes, Diabetologia 61 (4) (2018) 977–984.

[53] F. Renaudin, et al., NADPH oxidase 4 deficiency attenuates experimental osteoarthritis in mice, RMD Open 9 (2023) 1.

[54] L. Chen, et al., JQ1 as a BRD4 Inhibitor Blocks Inflammatory Pyroptosis-Related Acute Colon Injury Induced by LPS, Front Immunol. 12 (2021), 609319.

[55] J. Wang, et al., BRD4 inhibition regulates MAPK, NF-κB signals, and autophagy to suppress MMP-13 expression in diabetic intervertebral disc degeneration, Faseb J. 33 (10) (2019) 11555–11566.

[56] W. Zhu, et al., BRD4 blockage alleviates pathological cardiac hypertrophy through the suppression of fibrosis and inflammation via reducing ROS generation, Biomed. Pharm. 121 (2020), 109368.

[57] F. Zhou, et al., Kinsenoside attenuates osteoarthritis by repolarizing macrophages through inactivating NF-κB/MAPK signaling and protecting chondrocytes, Acta Pharm. Sin. B 9 (5) (2019) 973–985.

[58] Uchida, T., et al., Promotion of Knee Cartilage Degradation by IκB Kinase ε in the Pathogenesis of Osteoarthritis in Human and Murine Models. Arthritis Rheumatol, 2022.

[59] P.B. Shambharkar, et al., Phosphorylation and ubiquitination of the IkappaB kinase complex by two distinct signaling pathways, Embo J. 26 (7) (2007) 1794–1805.

[60] S. Frank, et al., Regulation of matrixmetalloproteinase-3 and matrixmetalloproteinase-13 by SUMO-2/3 through the transcription factor NF-κB, Ann. Rheum. Dis. 72 (11) (2013) 1874–1881.

[61] C.H. Ma, et al., Homocysteine causes dysfunction of chondrocytes and oxidative stress through repression of SIRT1/AMPK pathway: A possible link between hyperhomocysteinemia and osteoarthritis, Redox Biol. 15 (2018) 504–512.

[62] S.H. Chang, et al., Excessive mechanical loading promotes osteoarthritis through the gremlin-1-NF-κB pathway, Nat. Commun. 10 (1) (2019) 1442.

[63] D. He, et al., Gut stem cell aging is driven by mTORC1 via a p38 MAPK-p53 pathway, Nat. Commun. 11 (1) (2020) 37.

[64] P.J. Barnes, J. Baker, L.E. Donnelly, Cellular senescence as a mechanism and target in chronic lung diseases, Am. J. Respir. Crit. Care Med 200 (5) (2019) 556–564.

[65] M.O.J. Grootaert, et al., SIRT6 protects smooth muscle cells from senescence and reduces atherosclerosis, Circ. Res 128 (4) (2021) 474–491.

[66] J.E. Park, et al., Oncogenic roles of PRL-3 in FLT3-ITD induced acute myeloid leukaemia, EMBO Mol. Med 5 (9) (2013) 1351–1366.

[67] W. Tong, et al., Wnt16 attenuates osteoarthritis progression through a PCP/JNK-mTORC1-PTHrP cascade, Ann. Rheum. Dis. 78 (4) (2019) 551–561.

[68] C. Cho, et al., Prussian blue nanozymes coated with pluronic attenuate inflammatory osteoarthritis by blocking c-Jun N-terminal kinase phosphorylation, Biomaterials 291 (2022), 121851.

[69] Z. Lin, et al., JUNB-FBXO21-ERK axis promotes cartilage degeneration in osteoarthritis by inhibiting autophagy, Aging Cell 20 (2) (2021), e13306.

[70] P. Yang, et al., Expression profile of cytokines and chemokines in osteoarthritis patients: Proinflammatory roles for CXCL8 and CXCL11 to chondrocytes, Int Immunopharmacol. 40 (2016) 16–23.