Inhibition of p38 pathway leads to osteoarthritis like changes in a rat animal model

Indira Prasadam[1], Xinzhan Mao[1, 2, 3], Yanping Wang[1, 4], Wei Shi[1], Ross Crawford[1, 2], Yin Xiao[1]

[1]*Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia*

[2] *Prince Charles Hospital, Brisbane, Queensland, Australia*

[3] *Department of Orthopaedic Surgery, The Second Xiangya Hospital, Central South University, China*

[4] *Department of Rheumatism, The Xiangya Hospital, Central-South University, China*

Running title: p38 inhibitors aggravate *osteoarthritis development*

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Corresponding authors

1. Associate Professor Yin Xiao, Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove Campus, Brisbane, Qld 4059 Australia, Tel: +61 7 3138 6240,

Fax: +61 7 3138 6030, Email: yin.xiao@qut.edu.au

2. Dr Indira Prasadam, Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove Campus, Brisbane, Qld 4059 Australia, Tel: +61 7 3138 6137,

Fax: +61 7 3138 6030, Email: i.prasadam@qut.edu.au

ABSTRACT

Objectives: The p38 mitogen-activated protein kinase (MAPK) signal transduction pathway is involved in a variety of inflammatory responses, including cytokine generation, cell differentiation proliferation and apoptosis**.** Here, we examined the effects of systemic p38 MAPK inhibition on cartilage cells and osteoarthritis (OA) disease progression by both *in vitro* and in *vivo* approaches.

Methods: p38 kinase activity was evaluated in normal and OA cartilage cells by measuring the amount of phosphorylated protein. To examine the function of p38 signaling pathway in *vitro*, normal chondrocytes were isolated and differentiated in the presence or absence of p38 inhibitor; SB203580 and analysed for chondrogenic phenotype. Effect of systemic p38 MAPK inhibition in normal and OA (induced by menisectomy) rats were analysed by treating animals with vehicle alone (DMS0) or p38 inhibitor (SB203580). Damage to the femur and tibial plateau was evaluated by modified Mankin score, histology and immunohistochemistry.

Results: Our *in vitro* studies have revealed that a down-regulation of chondrogenic and increase of hypertrophic gene expression occurs in the normal chondrocytes, when p38 is neutralized by a pharmacological inhibitor. We further observed that the basal levels of p38 phosphorylation were decreased in OA chondrocytes compared with normal chondrocytes. These findings together indicate the importance of this pathway in the regulation of cartilage physiology and its relevance to OA pathogenesis. At *in vivo* level, systematic administration of a specific p38 MAPK inhibitor, SB203580, continuously for over a month led to a significant loss of proteoglycan; aggrecan and cartilage thickness. On the other hand, SB203580 treated normal rats showed a significant increase in TUNEL positive cells, cartilage hypertrophy markers such as Type 10 collagen, Runt-related transcription factor and Matrix metalloproteinase-13 and substantially induced OA like phenotypic changes in the normal rats. In addition, menisectomy induced OA rat models that were treated with p38 inhibitor showed aggravation of cartilage damage.

Conclusions: In summary, this study has provided evidence that the component of the p38 MAPK pathway is important to maintain the cartilage health and its inhibition can lead to severe cartilage degenerative changes. The observations in this study highlight the possibility of using activators of the p38 pathway as an alternative approach in the treatment of OA.

INTRODUCTION

Osteoarthritis (OA), the most common multifactorial degenerative joint disease in the elderly, is characterized by the degeneration of articular cartilage, changes in subchondral bone, osteophyte formation and synovial inflammation. The cause of OA is still unknown and many mechanical, biochemical and molecular factors are known to be involved. In the progression of OA, the balance of synthesis and degradation of articular cartilage shifts towards catabolism [1]. These synthetic changes involve alterations in specific signaling molecules and their respective downstream pathways [2]. Among the most conserved signal transduction systems in the cartilage is the mitogen-activated protein kinase (MAPK) cascade, which consists of sequentially acting protein kinases resulting in the activation of three terminal MAP kinases: p38 kinase, extracellular signal-regulated protein kinase (ERK), and c-jun N-terminal protein kinase (JNK)[3].

It has been reported that p38 signaling controls terminal differentiation [4], proliferation and differentiation of chondrocytes with a delicate balance, interacting with the TGF-β1/Smads signalling pathway [5, 6]. Our observations indicate that the p38 pathway plays a major positive role in regulating the articular chondrogenic phenotype and its expression negatively regulates cartilage hypertrophic phenotype [7]. Although p38 kinases were originally cloned as mediators of growth and development, recent results demonstrate important roles of these proteins in stress and inflammatory signals [8]. Studies have documented a key role for proinflammatory cytokine overproduction as a potential driving force for cartilage catabolism in OA [9]. One of the key signal transduction pathway involved in the production of proinflammatory cytokines is the p38 mitogen-activated protein kinase (MAPK) pathway [10]. p38 MAPK has been reported as a primary signal transduction pathway activated by the degradative cytokines such as IL-1β and TNFα [2, 11]. This suggests that strategies to target signaling pathways that lead to cytokine overproduction should be explored in attempts to develop new OA therapeutics with the potential for disease modification. Given the dual anabolic and catabolic role of p38 pathway in cartilage, it remains unknown whether the inhibition of p38 pathway has some impact on cartilage from undergoing degeneration.

SB203580, a pyridinyl imidazole that selectively inhibits p38 MAPK phosphorylation, has been widely used *in vivo* and *in vitro* studies to explore the function of p38 pathway. SB203580 has been demonstrated to attenuate the synthesis of inflammatory cytokines and MMPs in cartilage cells in several previous studies [12, 13]. One study has shown the decrease of chondrogenic phenotype in cartilage cells upon the addition of SB203580 [14]. Preclinical *in vivo* studies have shown that SB203580 inhibitors reduce inflammatory lung injury [15], rheumatoid arthritis [16] and cardiac dysfunction [17, 18]. Analysis of these data, collectively, suggests that the level of p38 phosphorylation plays a direct role in cartilage homeostasis. Therefore, the aim of this study was to

investigate p38 pathway in OA tissue samples and assess the effects of SB203580 in normal rats and the rats challenged with OA.

MATERIALS AND METHODS

The study was conducted in compliance with the ethical principles derived from the Declaration of Helsinki and with the local institutional review board. All patients gave their informed consent to participate in this in vitro study (Ethics Number: 0700000157). Animal ethics approval for this project has been granted from the Queensland University of Technology and the Prince Charles Hospital Ethics Committees (Ethics number: 0900001134). Male Wistar Kyoto rats (11-12 weeks old) were purchased from Medical Engineering Research Facility (MERF) (Brisbane, Australia). Each animal weighing about 300-350 grams were used for this experiment.

Isolation of cartilage cells: Normal articular cartilage chondrocytes (ACCs) were obtained from knee medial compartment tibial joint cartilage from tissue donors (n = 4) who were undergoing above the knee amputations due to traumatic injury. Normal patients were healthy adults with no clinical signs or symptoms of joint, metabolic or hormonal diseases (osteoporosis). None of the patients were taking medications which might affect cartilage or bone metabolism. All patients selected for this study had ceased taking anti-inflammatory medication at least two weeks prior to surgery. Early stage OA patients were excluded if the samples showed any evidence of cartilage changes such as: softening of the hyaline articular cartilage; thinning and fibrous dislocation; ulcerations of the cartilage; and light sclerosis of the subchondral bone. OA ACCs (n=4) were sourced from the main defective area of medial compartment knee tibial joint cartilage from patients undergoing total knee replacement surgery. All radiographs were reviewed, and the patient samples were classified accordingly into three categories, on the basis of modified Mankin score [19, 20]. This score assesses structure (0-6 points), cellularity (0-3 points), matrix staining (0-4 points), and tidemark integrity (0-1points), and has a maximum of 14 points. The final score for each cartilage was based on the most severe histologic changes observed in multiple sections from each specimen. The Mankin score was again divided into three stages depending on the score: grade 0-1 (normal cartilage), grade II (mild to moderate degenerative change, 2-9 points), and grade III (severe degenerative change, 10 or more points. In this study normal cartilage showed grade 0 and OA cartilage showed grade III cartilage. Chondrocytes were isolated from both normal and OA patients using enzymatic digestion as described previously [7, 21]. Passage 2-3 ACCs were used for the studies.

Detection of MAPK-p38 activation in articular cartilage chondrocytes (ACC) pellet cultures: $2x10^5$ cells of normal ACCs and OA ACCs were resuspended in a serum free chondrogenic media (high

glucose DMEM supplemented with 10 ng/mL TGF-β3, 10nM dexamethasone, 50 mg/mL L-ascorbic acid, 10 mg/mL sodium pyruvate, 10 mg/mL proline, and ITS+ (final concentration: 6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 mg/mL selenious acid, 5.33 mg/mL linoleic acid, and 1.25 mg/mL bovine serum albumin)) and centrifuged at 600 × *g* for 20 min to form a pellet. After two weeks of differentiation protein from pellets were isolated and western blot technique was applied to see the expression difference of P-p38 in normal and OA ACCs.

***In vitro* inhibition of P-p38 pathway using SB203580 in normal ACC pellets:** P-p38 specific inhibitor, SB203580 was used to study the pathway mediated cellular phenotypic changes in the normal ACC pellets. Briefly, normal ACCs were incubated with or without the SB280580 after dissolving the concentrated stock solutions of each inhibitor in DMSO. The final concentration of DMSO never exceeded 0.1% (v/v) and the same amount of DMSO vehicle was added to the control medium. The medium was replenished every 3 days. Our previous experiments demonstrated an optimum concentration of 5uM of SB203580 for p38 inhibition in ACC pellets [7]. At these concentrations there was no observable change in the proliferation rates between control cells and inhibitor treated cells, nor was there any evidence of cytotoxicity, as assessed by LDH (lactose dehydrogenase) assays (data not shown). All experiments were performed in triplicate. After 2 weeks of differentiation in the presence or absence of inhibitor some pellets were stained with Alcian blue or immunostained with Type 10 Collagen (COL10) and Aggrecan (AGG) antibodies as described previously [7]. Some pellets were used to extract the total RNA to see the gene expression of hypertrophy and chondrogenic markers using protocols as described previously [7, 21].

Rat OA models: Rats were anesthetized *via* intra-peritoneal injection with Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg) and Xylazil (xylazine 10 mg/kg). OA was induced by transecting the medial collateral ligament just below its attachment to the meniscus, so that when the joint space opens, the meniscus is reflected toward the femur. The meniscus was then cut at its narrowest point without damaging the tibial surface, resulting complete medial meniscus transection. The surgical wound was then closed by suturing in two layers. A sham group on the left knee was subjected to the same surgical procedure, without the excision of the ligament or any meniscus manipulation. After the surgery, all rats received pain killer (Buprenorphine 0.05 mg/kg) and antibiotics (Cephalothin sodium 20 mg/kg, and Gentamicin 5 mg/kg).

Study design and Drug administration: The rats were divided randomly into following four groups (n=12 (6 animals were used for histology + 6 animals for western blotting)). Group 1: Normal rats treated with DMSO + saline (vehicle alone), Group 2: normal rats treated with SB203580, Group 3: OA rats treated with DMSO + saline, and Group 4: OA rats treated with SB203580. Rats received

either intraperitoneal (i.p.) SB203580 (50 mg/kg in 0.25 mL) or diluents only (DMSO + saline). This concentration was chosen based on the published reports known to sufficiently raise plasma drug levels [22-24]. On the 30th day of the experimental period, the rats were euthanized with lethabarb (200 mg kg, i.p.); and the knee joints were collected for downstream observations.

Morphological characterization: Whole knee joints were removed by dissection, fixed in 4% paraformaldehyde, and decalcified in 10% EDTA. After dehydration and paraffin embedding, serial 5 µm sagittal sections from the lateral and medial compartment of the joint were cut. Two sections obtained at 100 µm intervals from the non weight bearing region and weight-bearing region of each knee joint were stained with safranin O–fast green. OA severity in the tibial plateau was evaluated according to modified Mankin histologic grading system (Mankin score: 0 to 14) [19, 20], and a cartilage destruction score was assigned for each knee sample by three independent assessors. For Safranin-O/Fast Green staining, 5µm paraffin-embedded sections of tibia from mice were counter-stained with Haematoxylin before being stained with 0.02% aqueous Fast Green for 4 min (followed by 3 dips in 1% acetic acid) and then 0.1% Safranin-O for 6 min. The slides were then dehydrated and mounted with crystal mount medium.

Cartilage thickness: The depths of articular cartilage in medial compartment tibial knee was measured using semi automatic Image J software (NIH) using sections stained with saffranin-o which provided excellent color discrimination between bone and cartilage. The regions of interest on the femoral condyles were drawn using software and divided on the basis of the load bearing areas of the knee during locomotion. The total thickness of the cartilage was determined by measuring the distance from the medial compartment of superficial border of non calcified cartilage to boundary with the zone of calcified cartilage according to the length proportion. For each condylar section, the average of three measurements was used for statistical analysis.

Western blotting : Total protein lysates were harvested by lysing the cartilage tissue samples with a lysis buffer containing 1 M Tris HCl (pH 8), 5 M NaCl, 20% Triton X140, 0.5 M EDTA and a protease inhibitor cocktail (Roche, Castle Hill, Australia). The cell lysate was clarified by centrifugation and the protein concentration determined by a bicinchoninic acid protein assay (Sigma, Castle Hill, Australia). 5 microgram of protein was separated by electrophoresis on a 12 % sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked with a Tris-Tween buffer containing 5% non-fat milk. The membranes were incubated with phosphorylated p38 or tubulin primary antibodies (1:000) overnight at 4^{0}C. After washing the membranes three times in TBS-Tween buffer they were incubated with anti-rabbit secondary antibody at 1:2000 dilutions for 1 hr. The

protein bands were visualized using the ECL Plus™ Western Blotting Detection Reagents (Amersham Biosciences, Castle Hill, Australia) and exposed on X-ray film (Fujifilm, Stafford, Australia).

Immunohistochemistry: Immunohistochemistry was carried out using an indirect immunoperoxidase method. Tissue slices were dewaxed in xylene and dehydrated in ethanol. Endogenous peroxidases were blocked by incubation in 0.3% peroxide in methanol for 30 min following repeated washing in PBS. The sections were then incubated with proteinases K (DAKO Multilink, CA, USA) for 20 min for antigen retrieval. Next, all sections were treated with 0.1% bovine serum albumin (BSA) with 10% swine serum in PBS. Sections were then incubated with optimal dilution of primary antibody overnight at $4^{o}C$ (P-p38 (Gene search PTY. Ltd, Queensland, Australia)=1:100, COL10 (Santa Cruz biotechnology, USA) =1:50, AGG (Millipore, New South Wales, Australia) =1:200, MMP-13 (Labvison, Fremont, CA)= 1:200). Optimum concentration of antibodies was determined by using a series of dilutions. Next day, sections were incubated with a biotinylated swine-anti-mouse, rabbit, goat antibody (DAKO Multilink, CA, USA) for 15 min, and then incubated with horseradish perioxidase-conjugated avidin-biotin complex for 15 min. Antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 min and the reaction was stopped by immersion and rinsing of the sections in PBS. Sections were lightly counterstained with Mayer's haematoxylin and Scott's blue for 40 sec each, in between 3 min rinses with running tap water. Following this, they were dehydrated with ascending concentrations of ethanol solutions, cleared with xylene and mounted with a coverslip using DePeX mounting medium. Controls for the immunostaining procedures included conditions where the primary antibody or the secondary (anti-mouse IgG) antibodies were omitted. In addition, an irrelevant antibody (anti CD-15), which was not present in the test sections, was used as a control. For semi-quantitative data, at 400x magnifications, the positive cells were counted from each field of observation from medial compartment femur knee. At least three observation fields were selected from each section and three sections were selected from each sample. To eliminate the difference in total cell number in each observation field, the positive cell numbers were normalized to the cell number per 100 total cells in each specific group.

TUNEL analysis: TUNEL is a specific immunohistochemical technique that enables sensitive and specific staining of the high concentrations of DNA 3-OH ends that are localized in apoptotic bodies. TUNEL analysis on tissue sections was performed by first permeabilizing the tissue with proteinase K solution for 30 min at $37^{o}C$. Following permeabilization, the slides were washed with PBS and the TUNEL reaction was performed using the fluorescein in situ cell death detection kit (Roche, Germany) following manufacturer's protocol. As a positive control, sections were treated with DNase1 for 10

min at room temperature prior to labelling procedure to induce DNA strand breaks. The TUNEL reaction mixture / terminal transferace was omitted for the negative control. Slides were viewed and analysed using the microscope and % positive cells were calculated as described above.

Behavioral studies: Behavior of the animals was monitored throughout the entire study period to assess any SB203580 side effects in drug treated *vs.* untreated animals. Some of the most common signs indicating the health status of a rat include observation of behavior (such as observation of unprovoked behaviour and responses to external stimuli), assessment of physical appearance (exophthalmia or enophthalmia (bulging or sunken eyes, respectively), nasal or ocular discharge, rough coat, and hunched back), and measurement of body weight. All these parameters were monitored and recorded carefully during the entire period of this study.

Statistical analysis: Statistical analysis was performed by using the statistical Package from Graphpad prism (version 4.0).The unpaired student's t-test was used for comparisons of P-p38 expression in grade 0 and grade 4 cartilage, the differential expression of COL2, AGG, COL10, CBFA1, MMP-13 in the ACC culture, and the difference of positive cell numbers for AGG, COL10, MMP-13 in normal, sham and SB203580 application rats. The Mankin score and cartilage thickness in different treatment groups (normal+vehicle, normal+SB203580, OA+vehicle, and OA+SB203580) were subjected to one-way ANOVA and SNK tests. Data were expressed as the mean+/-SD. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Patient Demographics: The mean age and body mass index (BMI) of OA study participants were 63.7±2.8 years and 26.8±0.6 kg/m2 respectively. The mean age and BMI of normal study participants were 59.3±2.4 years and BMI 25.1±0.7 kg/m2 respectively. Both normal and OA cartilage samples were obtained from only males. No significant statistical differences were observed with respect to all the above parameters when compared in between the groups.

Increased p38 expression in cartilage tissue and ACCs collected from OA patients: Characterisation of normal and OA ACCs were performed to ensure the phenotypic stability by analysing the gene expression of COL2 and AGG. It was found that both normal and OA ACCs expressed significantly higher levels of COL2 and AGG. However, reduced levels of COL2 and AGG were observed in OA compared to normal ACCs ***(Fig 1A)***. Levels of phosphorylated p38 were determined in the protein lysate collected from normal articular cartilage chondrocytes (ACCs) and OA ACCs pellets after differentiating for two weeks under chondrogenic conditions. It was found that the levels of P-p38

were reduced significantly in OA ACCs compared to normal ACCs ***(Fig1B)***. Knee tibia joint sections from OA patients were collected and graded according to the disease severity based on Mankin scale. Immunohistochemistry further confirmed the *in vitro* findings showing the decrease of p38 phosphorylated forms in OA cartilage tissue, suggesting that the p38 MAPK pathway may be more active in normal compared to OA cartilage ***(Fig 1C,D&E)***.

In vitro **inhibition of P-p38 pathway using SB203580 led to decrease of chondrogenic and increase of hypertrophic phenotype in normal ACCS:** Normal ACC pellets were differentiated for two weeks in the presence or absence of SB203580. After two weeks, ACC pellets were stained with alcian blue to analyse the GAG deposition and chondrogenic phenotype. When normal ACC pellets were differentiated in the presence of SB203580, a decrease in the GAG deposition and AGG expression and the increase of hypertrophic marker, COL10, were found in ACC pellet cultures ***(Fig 2A)***. These observations were further confirmed by the gene expression showing the decrease in the expression of COL2 and AGG in the presence of SB203580 ***(Fig 2B)***. On the other hand, COL10, Runt-related transcription factor 2 (RUNX2) and MMP-13 were increased in the presence of SB203580, emphasizing the importance of this p38 signaling pathway in maintaining the cartilage function ***(Fig 2C).***

Tissue p38 MAPK measures in rats: High basal activity of p38 was observed in the normal untreated animal groups compared to OA groups. Both normal and OA rats were treated with SB203580 (50 mg/kg in 0.25 mL; i.p.) or diluents only (DMSO+saline). On the 30th day of experimental period, western blot with the cartilage homogenate was performed to verify whether that SB203580 inhibits activity of p38 MAPK in the *in vivo* cartilage. The results showed that the induction of p38 MAPK activity was suppressed significantly to a level far below baseline when animals were administered with SB203580 demonstrating the efficacy of the drug to block p38 activity ***(Fig 3A)***. Down-regulated P-p38 expression was associated with increased Mankin score ***(Fig 3B)*** and decrease in the cartilage thickness ***(Fig 3C).*** As expected, the Mankin score was 0 in untreated groups and of interest the degenerative changes were substantially increased in sham and OA animals upon administration of SB203580.

Effect of SB203580 in the normal and OA induced rats: After determining the efficacy of SB203580, both normal and an OA experimental animal model (induced by menisectomy) were used to evaluate the effects of SB203580 treatment on the development of the clinical and pathologic manifestations of the disease.

In the joints of normal (treated with vehicle alone) rats, no macroscopic changes were detected on the articular surfaces of femoral condyles and tibial plateau. Normal untreated animals showed the healthy-appearing cartilage with intact superficial, mild, and deep zones that stained deeply with safranin-O (red) for glycosaminoglycans. The chondrocytes were arranged in columns in untreated animals. However, when the animals were treated with SB203580, a significant loss of matrix staining was observed, which is consistent with decrease of P-p38 immunostaining in the cartilage **(Fig 4A,B&C)**.

When the effect of SB303580 was tested on the OA model, it was found that the menisectomy induced OA knees revealed an extensive degeneration of cartilage, surface roughness, fibrillation, small osteophytes or areas of peripheral fibrous tissue proliferation, both in tibia and femur where the meniscus was removed; however the damage appears much higher when OA models were treated with the SB203580 **(Fig 4A,B&C)**. These results collectively indicate the inhibition of p38 pathway led to more severe damage to the structure of cartilage.

Expression of cartilage markers and hypertrophy markers in the SB203580 treated animals:

We found a strong presence of AGG (78% of total cartilage area) protein expression in articular cartilage of normal rats (treated with vehicle alone), whereas cartilage of the normal knee cartilage treated with SB203580 showed minimal, AGG (40% of total cartilage area) staining ***(Fig 5 A,B&C).*** These data indicated to us that the deprived expression of p38 expression led to phenotypic changes of cartilage even in the normal animals. In contrast to the above findings we found that the expression of COL10 ***(Fig 5 D,E&F)*** and MMP-13 ***(Fig 5 G,H&I)*** were significantly upregulated in normal animals treated with SB203580 compared with corresponding controls, which indicated a transition to hypertrophy phenotype in the absence of P-p38 pathway.

Enhanced expression of TUNEL positive chondocytes in the SB203580 treated animals: Cartilage degradation of the joint is characteristically accompanied by apoptosis related cascades. To test this hypothesis, normal rats that were treated with SB203580 were examined for apoptosis using the TUNEL assay. Very few apoptotic (TUNEL-positive green fluorescent) cells were observed in cartilage from untreated rats. Treatment with SB203580 caused a modest increase in number of apoptotic cells in the cartilage ***(Fig 6 A,B&C)***. These results clearly demonstrate that decrease in the p38 phosphorylation is capable of inducing apoptosis of cartilage cells even in the normal rats.

Animal behavioural studies: No significant differences were found with respect to behavior, assessment of physical appearance, and measurement of body weight in SB203580 treated vs. untreated animals (data not shown).

DISCUSSION

Growing evidence shows that the reversal of structural remodelling represents a key therapeutic target in OA management and treatment. Given the central role of p38 kinase in the regulation of cellular stress response mechanisms, modulation of p38 kinase activity represents an attractive therapeutic approach in the treatment of several diseases. Indeed, small molecule p38 inhibitors have been suggested to have potentially beneficial effects in pulmonary disease [25], septic shock [24], and rheumatoid arthritis disease [26]. The concept of manipulation of the phosphorylation level of p38 as a potential treatment for OA disease is based on the assumption that p38 kinase is activated in these diseases. However, it is surprising to note that the detailed change in p38 kinase activity during OA cartilage has not been previously reported.

Here, we found that phosphorylated p38 is highly expressed in normal human cartilage, which suggests that cartilage must generate significant levels of P-p38 for tissue maintenance and homoeostasis. However, P-p38 is significantly reduced in diseased OA cartilage, confirming and expanding on our previous data for P-p38 levels at cellular level of normal and OA chondrocytes. In line with this evidence, P-p38 expression is significantly down regulated in OA animal model *in vivo*, raising the possibility that those alterations in P-p38 expression is associated with the earliest stages of OA pathogenesis.

To understand the precise role of p38 pathway, normal chondrocytes pellets were incubated with SB203580 to inhibit the phosphorylation of p38 pathway. We found that the lack of p38 expression led to decrease in chondrogenic phenotype and increase in the hypertrophic phenotype. Consistent with our results, Li et al showed regulation of chondrogenesis by p38 pathway with a delicate balance, interacting with the TGF-beta1/Smads signalling pathway [27]. Similarly, Zhang et al, by using the genetic inhibition studies they showed role of p38 in chondrocytes differentiation and suggests that Sox9 is a downstream target of the p38 MAPK pathway [28]. Furthermore, it has been shown previously that complete disruption of p38 is lethal, suggesting that maintenance of normal levels of p38 activity is necessary for the normal embryonic development [29]. The data obtained in this study show that apoptosis in normal rat cartilage (TUNEL-positive chondrocytes) was significantly increased when treated with p38 inhibitor. These observations suggest that a significant proportion of chondrocytes die by apoptosis in the absence of basal p-38 phosphorylation levels.

Under normal conditions mature articular chondrocytes are kept in a state of maturational arrest. In contrast to this phenotype, it has been shown that OA chondrocytes express hypertrophic differentiated markers such as COL10, ALP and RUNX2 [30]. It has been reported that hypertrophy of chondrocytes can lead to the degenerative changes in the cartilage by upregulating the expression of MMP's [31]. These results together indicate that loss of P-p38 activity can lead to phenotypic changes of articular chondrocytes to a hypertrophic phenotype which could be pathological to the adult mature cartilage structure. In line with our findings, Stanton et al showed elevated RUNX2, Osterix and Osteocalcin transcript levels in chondrocyte cultures upon inhibition of p38 activity with the pharmacological inhibitor, suggesting loss of p38 signalling leads to chondrocytes hypertrophy during skeletal maturity [4].

At *in vivo level,* we demonstrated for the first time, an inhibitor for p38 pathway (SB203580) worsened the cartilage degeneration both in normal and OA animal models, evidenced by increase in Mankin score and decrease in the cartilage thickness. The decrease of P-p38 levels was associated with decrease in chondrogenic and increase in hypertrophy phenotype. Consistent with our findings it has been recently demonstrated that the genetic reduction of p38 MAPK activity in cartilage leads to phenotypic changes in bone and/or cartilage in adult mice [32].

Although this study suggests a protective role for p38 kinase both *in vitro* and *in vivo*, a number of studies showed the destructive role of p38 when it is upregulated. It has been reported that upregulated p38 phosphorylation levels can lead to increased cytokine production by several mechanisms such as direct phosphorylation of transcription factors [33]. One study has also found that the attenuation of cartilage degeneration and OA pain in animal models due to upregulated p38 levels and suggests that p38 inhibitors may be a useful approach for the treatment of OA [34]. These results indicate the phosphorylation levels of p38 need to be well-balanced in a physiological level in order to maintain cartilage health and both inhibition and upregulation of the p38 signaling are detrimental to cartilage. Several studies do support a considerable cross-talk between the different MAPKs subtypes, including interactions between inflammatory/stress-activated signal pathways and hormone/growth factor-activated signal pathways [35]. In our previous study we demonstrated the cross-talk between ERK and p38 pathways and showed an upregulation of ERK phosphorylation levels can lead to decrease of P-p38 levels and vice versa, which in turn can lead to cartilage hypertrophy and degradation, indicating the dynamic and balanced interactions of MAPK pathways are required for cartilage homeostasis [7].

Key messages:

1. The present study revealed reduced p38 kinase activity as a causative factor in OA
2. Normalization of p38 kinase activity may represent a new preventive approach against progression of OA.

CONFLICT OF INTEREST: Authors declare there is no conflict of interest.

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FIGURE LEGENDS

Figure 1: Down-regulation of phosphorylated p38 activity in cartilage collected from OA patients. (A) Characterisation of normal and OA ACCs were performed by analysing the gene expression of COL2 and AGG at day 14 chondrocyte pellets. A representative gel image was shown. **(B)** Western blot analysis of chondrocytes showed the decrease of p38 phosphorylated forms in OA patients compared to normal patients. Band density quantification was performed using Image J software for phospho-p38. Each value represents protein bands from three separate experiments. The mean ± SD is shown. * Represents a significant difference ($p<0.05$). **(C, D&E)** Articular cartilage collected from lateral compartment with mild OA cartilage **(D)** contained more P-p38 positive cells compared with medial compartment severe OA cartilage. Arrows point to some of the positive cells **(E)**. No staining was observed in the negative control sections **(C)**. Scale bar: 100µm.

Figure 2: Effects of p38 inhibitor SB203580 on normal ACCs matrix deposition and gene expression (A) Alcian blue staining showed significant decrease in the GAG deposition in ACC pellets treated with SB203580. Similarly, immunostaining for AGG showed a decreased staining pattern in ACCs cultured in the presence of SB203580. On the other hand, the expression of hypertrophic marker COL10 was increased when pellets were cultured in the presence of SB203580. Arrows point to some of the positive cells. Scale bar=100µm. **(B-C)** qPCR showed that chondrogenic markers such as *COL2* and *AGG* were downregulated and hypertrophic markers such as *COL10, RUNX2,* and MMP-13 were significantly upregulated in ACCs pellets cultured with SB203580. mRNA levels were normalized against GAPDH and 18s and the relative gene expression is presented. Results are shown as mean ± SD (n=4). * Represents a significant difference ($p\leq0.05$).

Figure 3: Inactivation of p38 kinase after treating animals with SB203580 and this lead to increased cartilage Mankin score and decreased cartilage thickness. (A) P-p38 activity levels were reduced to a level far below baseline when normal animals were administered with SB203580 demonstrating the efficacy of the drug to block p38 activity. A representative bands obtained from six different animals with similar results were shown. **(B&C)** Graphs showing the increased Mankin score and decreased cartilage thickness both in normal and OA rats that were treated with SB203580, indicating that the lack of P-p38 activity is pathological for the articular cartilage. Results are shown as mean ± SD (n=6). * Represents a significant difference ($p\leq0.05$).

Figure 4: Histological findings demonstrating the detrimental effects on the cartilage of rats treated with SB203580. (A) Gross morphological cartilage changes in the normal and menisectomy (MNX) induced OA rats that were treated with or without SB203580. L: Lateral compartment; M:

medial compartment). Arrows point to damaged surface area. **(B)** Saffranin-O staining demonstrated higher rate of proteoglycan depletion both in normal and OA animal models when treated with SB203580 compared to their respective controls. Scale bars: 100µM. **(C)** Immunostaining showing that p38 phosphorylation was significantly decreased in the animal models treated with SB203580. Scale bar: 50µm.

Figure 5: Reduced chondrogenic phenotype and increased hypertrophic expression in rats treated with SB203580 compared to vehicle alone treated controls. Articular cartilage from SB203580 treated rats **(B)** contained less AGG compared to untreated animals **(A)**. The percentage of AGG positive cells also showed the same trend **(C)**. In contrast, the expression of COL10 in normal rat **(D)** was lower than SB203580 treated rat **(E)**. The percentage of COL10 positive cells were also increased in normal rats treated with SB203580 **(F)**. Similarly, MMP-13 in normal rat **(G)** was lower than SB203580 treated rat **(H)**. The percentage of MMP-13 positive cells showed similar pattern **(I)**. P≤0.05 was considered significant (n=6). Scale bar=200µm.

Figure 6: Enhanced apoptosis in SB203580 treated normal rat. (A&B) TUNEL assay reveals an enhanced number of apoptotic chondrocytes in SB203580 treated normal rats, whereas only few apoptotic chondrocytes are seen in the untreated animals. An overlapped image shows that TUNEL positive cells (green) with nucleus (DAPI, Blue). Arrows point to some of the positive cells. Scale bar= 75 µm **(B)** Percentages of total cell counts that are TUNEL positive. * $P<0.05$, $n=6$ per group.

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