



Hylan G-F 20 attenuates posttraumatic osteoarthritis progression: Association with upregulated expression of the circadian gene *NPAS2*

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ABSTRACT

Aims: The study was to examine the effect of Hylan G-F 20 on the progression of posttraumatic osteoarthritis (PTOA) and the expression of the circadian genes *neuronal PAS domain protein 2* (*NPAS2*) and *period 2* (*Per2*).

Main methods: We used the anterior cruciate ligament transaction and medial meniscectomy (ACLT + MMx) model in Wistar rats. The rats were divided into three groups, the sham-operated group, the Hylan G-F 20-treated group, and the saline-treated group. Rats which underwent ACLT + MMx surgery were injected intraarticularly with, respectively, Hylan G-F 20 or saline once a week for 3 consecutive weeks, starting 7 days after surgery. The gross morphology and histopathology of the experimental knee joints were evaluated at the end of week 6. Expression of the *NPAS2* and *Per2* genes was measured by real-time PCR.

Key findings: Hylan G-F 20 suppressed the articular cartilage destruction and synovitis compared to the saline-treated group. Compared to the sham-operated group, the Hylan G-F 20-treated group showed significantly upregulated expression of *NPAS2* in cartilage (2.53 ± 0.08 -fold higher; $p < 0.05$) and a non-significant increase in *Per2* expression (2.35 ± 1.26 -fold higher $p = 0.28$), while the saline-treated group showed significant down-regulation of *NPAS2* expression and a non-significant decrease in *Per2* expression.

Significance: Our data suggested that early intraarticular injection of Hylan G-F 20 attenuates the progression of PTOA and significantly upregulates *NPAS2* expression. These findings provide a new direction for studying associations between the use of a pharmacological agent, the degenerative process, and circadian gene expression.

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1. Introduction

Joint trauma results in osteoarthritis (OA) within 10–15 years [1]. Compared to age-related OA, posttraumatic osteoarthritis (PTOA) affects younger individuals, who demand greater joint mobility, so joint replacement and joint fusion are not desirable approaches [2]. Current treatments for PTOA include surgical reconstruction and pharmacological interventions, but no single current therapy can completely halt the degenerative process. Symptom relief and postponing joint replacement are the mainstream of therapy [3]. In addition to developing new

pharmacological agents for treating PTOA, early intervention with current drugs might also provide an opportunity to attenuate PTOA progression [3–5].

The circadian rhythm is an intrinsic oscillatory process that regulates the intrinsic homeostasis and allows adaptation changes to the environment, such as the light–dark cycle and temperature change. In mammals, disruption of the circadian rhythm is associated with many diseases, including cancer, metabolic disorder, sleep disorders, and inflammatory joint diseases [6–8]. A recent study demonstrated autonomous molecular circadian rhythms in chondrocytes from mouse cartilage and a human chondrocyte cell line [9]. Circadian clock genes in cartilage are involved in the regulation of cartilage matrix homeostasis and mechanical sensing [10–12] and dysregulation of clock-controlled genes has been observed in cartilage degeneration and inflammatory joint diseases [9,13]. Honda et al. [10] reported that mRNA levels for matrix-related genes (synthesis and degradation) show a circadian oscillation in various types of cartilage, while Gossan et al. [9] found that

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expression of clock genes was disrupted during the development of OA in a mouse model.

Within cells, the circadian rhythm is regulated by an autonomous transcription-translation feedback loop, which involves a core set of genes. The genes *circadian locomotor output cycles kaput* (*CLOCK*) and *brain, muscle Arnt-like 1* (*BMAL1*) encode transcription factors that activate transcription of the *period* (*Per*) and *cryptochrome* (*Cry*) genes, then the PER-CRY heterodimer interacts with the CLOCK-BMAL1 complex to inhibit transcription of the *CLOCK* and *BMAL1* genes [14]. The *CLOCK* gene is a mechanosensitive gene, as its expression is downregulated in cartilage under mechanical stress [12]. Neuronal PAS domain protein 2 (*NPAS2*), a transcription factor belonging to the basic helix-loop-helix-PAS family, is abundantly expressed in the mammalian forebrain [15] and, like *CLOCK*, forms a heterodimer with *BMAL1* to regulate the circadian rhythm [15–17]. In a recent report, *NPAS2* was shown to be associated with cartilage matrix homeostasis [11].

Hylan G-F 20 is a high molecular weight (6–8 MDa), cross-linked hyaluronic acid (HA) derivative. HA is an important component of synovial fluid and cartilage matrix. Intraarticular injection of HA provides effective and persistent pain relief for patients with advanced OA [18]. Hylan G-F 20 improves cartilage integrity and inhibits osteophyte formation in a rabbit OA model by increasing the amount of type II collagen and inhibiting IL-1 β – mediated matrix degradation by decreasing expression of matrix metalloproteinases [19–20]. We previously reported that early intraarticular injection of HA can slow the progression of PTOA in a rat surgical OA model [5]. In the present study, we explored whether early intervention with Hylan G-F 20 can attenuate the progression of arthritis in a rat surgically-induced OA model and alter the expression of the circadian clock gene *NPAS2* and one of its target genes *period 2* (*Per2*).

2. Methods and materials

2.1. Rat PTOA model

Wistar rats (3-months-old), maintained under climate-controlled conditions on a 12 h light–dark cycle at 22–24 °C and 50–55% humidity, were divided into three groups of 10 ($n = 4$ rats/group for mRNA analysis, and $n = 6$ rats/group for histological examination), the sham-operated group and two treatment groups. All three groups underwent arthrotomy under isoflurane anesthesia, then the sham-operated group underwent no further treatment, while the other two groups underwent anterior cruciate ligament transection (ACLT) and medial meniscectomy (MMx) surgery on their right hindlimb knee joint via a medial parapatellar incision to induce PTOA [5,21–23]. In all three groups, the joint cavity was then washed with saline, the joint capsule closed with 4–0 vicryl, and the skin closed with 4–0 nylon sutures, then all groups received intraperitoneal cefazolin (20 mg/kg) for infection prophylaxis. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taiwan.

2.2. Experimental design

The two ACLT-MMx groups were injected intraarticularly in the right hindlimb knee once a week for 3 consecutive weeks starting on day 7 after surgery with 100 μ l of 8 mg/ml of Hylan G-F 20 (Synvisc, Genzyme, NJ, USA) or 100 μ l of saline, then the 3 groups were sacrificed at the end of week 6 after surgery. All intraarticular injections were performed by the lateral midpatellar approach under isoflurane anesthesia (3% isoflurane in an 1:1 oxygen/air mixture at a flow rate of 3 L/min) using a 29G BD insulin syringe [5,24]. During injecting, the soft tissues surrounding the knee were palpated concurrently to check if there was any leakage of fluid. Passive flexion and extension of the experimental knee was performed several times after each injection completed.

2.3. Histopathological examination of the right hindlimb knee joint

The width of the right hindlimb knee joint was measured using calipers (AA847R, Aesculap, AG&CO, KG, German) before surgery (baseline) and at the end of week 6 after surgery, when the rats were sacrificed by exsanguination under isoflurane anesthesia and the knee joints were harvested by cutting 0.5 cm above and 0.5 cm below the joint line, fixed in 4% paraformaldehyde for 2 days, and transferred to Decalcifier II (Surgipath, Richmond, VA, USA) for another day. After decalcification, the joints were paraffin-embedded in an automatic processor (Autotechnicon mono 2, Technicon Co., Chauncey, NY, USA) and 5 μ m serial sections of the central weight-bearing sites of the medial femoral condyles and synovium from the suprapatellar pouch were prepared using a Leica 2065 rotatory microtome (Leica Instruments, Wetzlar, Germany). Ten sections were randomly chosen for each sample, of which 5 were stained with safranin-O/fast green to detect proteoglycan loss from the condyle cartilage and 5 with hematoxylin/eosin to evaluate morphological changes in the synovium. The severity of cartilage destruction was evaluated by Mankin scoring system: cartilage structure (0–6 points), cellularity (0–3 points), matrix staining (0–4 points), and integrity of tidemark (0–1 points), a total 14-point grading system [25].

2.4. Extraction of total RNA and quantitative real-time PCR analysis

All rats were sacrificed between 2 and 4 pm at the end of week 6 after surgery and cartilage was immediately harvested from the whole right hindlimb knee joint and frozen in liquid nitrogen. Total RNA was isolated from homogenized cartilage using TRIzol reagent (Invitrogen, Auckland, New Zealand) following the manufacturer's protocol, then was reverse transcribed into cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). cDNA levels were measured in triplicate by the quantitative real-time polymerase chain reaction (PCR) on a LightCycler® 480 system (Roche Applied Science, Indianapolis, IN, USA). β -actin was used as the housekeeping gene and the relative expression of the *Npas2* and *Per2* genes was calculated using the $2^{-\Delta\Delta Ct}$ method [26]. The primers, designed by ourselves and purchased from Universal ProbeLibrary (Roche, Applied Biosystems, Foster City, CA, USA), were:

Npas2 Forward CAGGCTATGACTACTACCACATTGA and *Npas2* Reverse CTTTGCCAACTGCATCAAG;

Per2 Forward TCCAGGATGTGGACGAAAG and *Per2* Reverse GCACAGGGGTCTCGATCA;

β -actin Forward CCCGCGAGTACAACCTCT and β -actin Reverse CGTCATCCATGGCGAACT.

2.5. Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean. The significance of differences in knee width increase or relative gene expression was examined, respectively, using Student's *t* test or the Mann–Whitney U test. Kruskal–Wallis test was used for the intergroup comparisons of Mankin scores. A *p* value less than 0.05 was considered significant.

3. Results

3.1. Joint swelling and histopathology

All rats recovered from surgery as expected without complications. We measured the width of the right hindlimb knee before surgery and at the end of week 6 after surgery. Knee width in all groups increased over time. At week 6, obvious joint swelling and deformity were observed in the saline- and Hylan G-F 20-treated groups, but not the sham-operated group. As shown in Fig. 1, the increase in knee width over the 6 weeks from before surgery (9.24 ± 0.24 mm) was 0.5 ± 0.08 mm in

the sham-operated rats, 1.16 ± 0.15 mm in the saline-treated group, and 0.87 ± 0.12 mm in the Hylan G-F 20-treated group. The increase in knee width in both the saline- and Hylan G-F 20-treated group was significant compared to that in the sham-operated group ($p < 0.05$), while, although the Hylan G-F 20-treated group showed a smaller increase in knee width than the saline-treated group, the difference was not statistically significant ($p = 0.17$).

Histopathological evaluation of the articular cartilage from the medial femoral condyle in the sham-operated group showed complete integrity of the cartilage surface and all of the cartilage matrix were stained with safranin-O/fast green (Fig. 2A). In contrast, in the saline-treated group, irregularity and fibrillation of the cartilage surface, loss of the superficial zone, clefts in the transitional zone, chondrocyte clusters, and a marked decrease in safranin-O/fast green staining intensity were seen (Fig. 2B), whereas these changes were less pronounced in the Hylan G-F 20-treated group, which showed only a slightly irregular cartilage surface, mild superficial fibrillation, and a lower reduction in safranin-O/fast green staining intensity (Fig. 2C). The Mankin scores of saline-treated group (6.67 ± 1.26) and Hylan G-F 20-treated group (3.17 ± 0.23) were both significantly higher than sham-operated group (0.58 ± 0.15 ; $p < 0.05$). The Mankin score of Hylan G-F 20-treated group was significantly lower than saline-treated group ($p < 0.05$).

Histological examination of the synovium in the sham-operated knees showed a single layer of synoviocytes and no stromal hypertrophy (Fig. 2D). In contrast, the synovium in the saline-treated rats showed significant synovitis, including lining cell hyperplasia, increased cellular density, stromal hypertrophy, and inflammatory cell infiltration (Fig. 2E), whereas these changes were less pronounced in the Hylan G-F 20-treated group (less stromal hypertrophy and inflammatory cell infiltration) (Fig. 2F).

3.2. *Npas2* and *Per2* mRNA levels after ACLT + MMx surgery

We used real-time PCR to measure *NPAS2* and *Per2* mRNA levels in the knee cartilage samples taken at the end of week 6 after surgery, using β -actin mRNA as the endogenous control for normalization. As shown in Fig. 3, compared to the sham-operated group (set as a value of 1), *NPAS2* gene expression was significantly lower (0.16 ± 0.05 -fold; $p < 0.05$) in the saline-treated experimental OA group and *Per2* expression was also reduced (0.54 ± 0.21 -fold; $p = 0.16$). In contrast, in the Hylan G-F 20-treated group, *NPAS2* expression (2.53 ± 0.08 -fold compared to sham; $p < 0.05$) and *Per2* expression (2.35 ± 1.26 -fold compared to sham; $p = 0.28$) were both increased, but only the increase in *NPAS2* expression was significant.

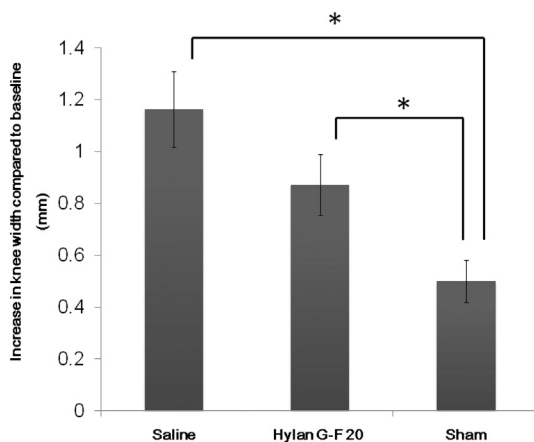


Fig. 1. Increase in right knee width from baseline at the end of week 6 after surgery. The data are the mean $\pm$ S.E.M. for 5 rats per group. * indicates a p value < 0.05 for the difference between the indicated values; other differences are not statistically significant.

4. Discussions

In this study, after ACLT + MMx surgery, significant cartilage damage and synovitis developed and progressed rapidly over 6 weeks. At the end of 6 weeks after surgery, *NPAS2* expression was significantly reduced and *Per2* gene expression reduced, but not significantly, in the articular cartilage in the saline-treated experimental OA rats compared to the sham-operated rats, while intraarticular injection of Hylan G-F 20 had a chondroprotective effect and resulted in upregulation of *NPAS2* and *Per2* gene expression compared to the sham-operated rats. To the best of our knowledge, this is the first study showing that intraarticular injection of Hylan G-F 20 can increase expression of the *NPAS2* and *Per2* genes in OA cartilage.

Compared to rabbits and dogs, the size of rats is relatively small and more easily to facilitate manipulation. In addition, surgery-induced arthritis model progressed faster in rats [22]. Hayami et al. reported that ACLT of rat knee induced OA in a time-dependent manner, and the morphological and histological characteristics were similar to human OA. ACLT + MMx model induced similar pathology to ACLT alone but faster progression of posttraumatic osteoarthritis, and the detectable cartilage damage occurred within 2 weeks after ACLT + MMx surgery [22]. We applied the ACLT + MMx rat model because it provides an useful and efficient experimental model to study the progression of post-traumatic osteoarthritis and evaluate the potential disease-modifying agents [27,28].

It is not known how Hylan G-F 20 has this effect on expression of the *NPAS2* and *Per2* genes in articular cartilage in OA. Bourguignon et al. [29] demonstrated that binding of hyaluronan to the CD44 receptor upregulates p300 expression and promotes β -catenin/NF- κ B signaling in MCF-7 cells. p300 (E1A binding protein p300), a histone acetyltransferase (HAT), acetylates the core histones on target promoters, resulting in chromatin remodeling to enhance assembly of the transcription machinery [30]. p300 increases CLOCK:BMAL1- or NPAS2:BMAL1-mediated transcription by facilitating chromatin remodeling on the promoter E-box of the target genes period (*Per1*, *Per2*) and cryptochrome (*Cry1*, *Cry2*). [30–34]. Curtis et al. [30] showed that p300 interacts with NPAS2 in a circadian time-dependent manner, with the association between the two being maximal at the time of maximal expression of their mRNAs, suggesting that p300 plays an important role in driving circadian rhythmicity through regulation of NPAS2-BMAL1-mediated transcription activation. The *Per2* gene is one of the target genes of the NPAS2 (clock)-BMAL1 heterodimer [34]. In the present study, we observed upregulation of *Per2* expression, but this effect was not statistically significant. We also showed that Hylan G-F 20 up-regulated *Npas2* expression, possibly through CD44-mediated upregulation of p300, which overcomes the *Cry2*-mediated inhibition of *NPAS2* expression.

Mengatto et al. [11] found that *NPAS2* was the most significantly affected gene in a rat osseointegration model and that, in a whole genome microarray analysis of peri-implant bone tissue, the *NPAS2* gene was grouped with the cartilage extracellular matrix genes in the hierarchical cluster analysis, and that siRNA-induced *NPAS2* knockdown resulted in a significant reduction in type II and type X collagen mRNA levels in mouse bone marrow mesenchymal stem cells, suggesting that *NPAS2* might be involved in the homeostasis of the cartilage extracellular matrix. In our present study, we did not examine expression of the gene collagen type II, alpha 1 (*Col2A1*), which is involved in cartilage repair, but a previous study showed that intraarticular injection of Hylan G-F 20 enhances *Col2A1* expression at both the mRNA and protein level in arthritis cartilage from rabbit OA knees [20].

5. Conclusion

The present study shows that Hylan F-20 attenuates the progression of PTOA and upregulates *Npas2* expression possibly via the CD44 and p300 interaction and subsequently modulates the homeostasis of the

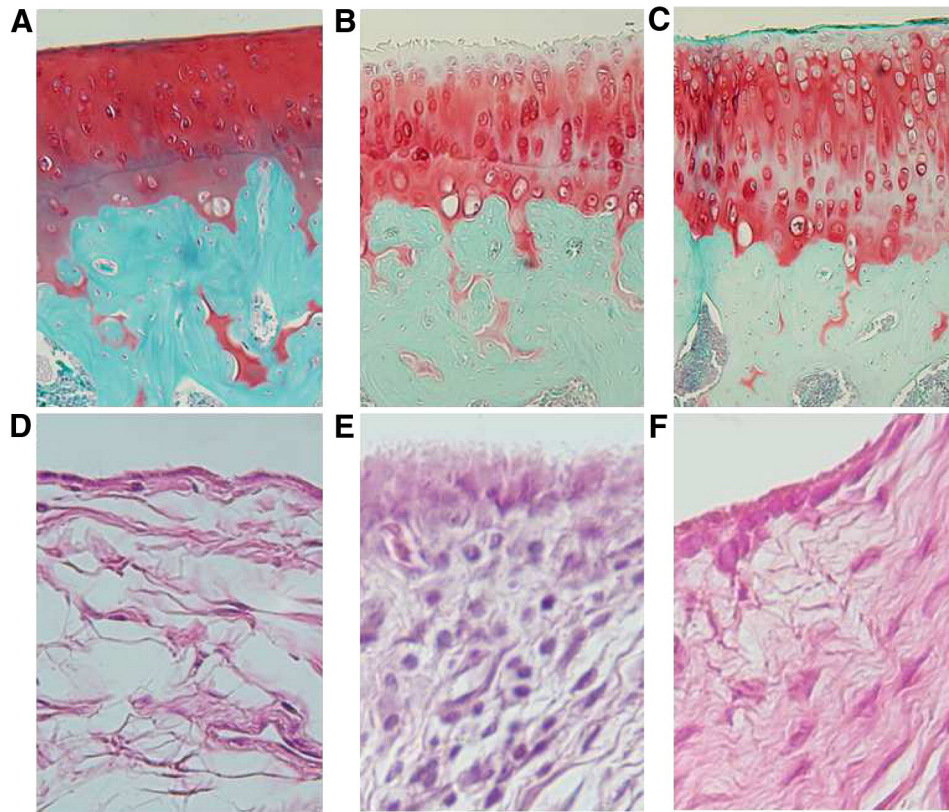


Fig. 2. Histopathology of cartilage from the medial femoral condyle (safranin-O/fast green stain, $\times 100$) (A–C) and synovium (H&E stain, $\times 400$) (D–F).

cartilage extracellular matrix. This suggests a new approach of investigating the regulation of circadian genes in treating PTOA and for studying the use of a frequently used drug, Hylan G-F 20, in the treatment of circadian rhythm-related disorders other than OA.

Conflict of interest statement

The authors report no conflicts of interest.

Acknowledgments

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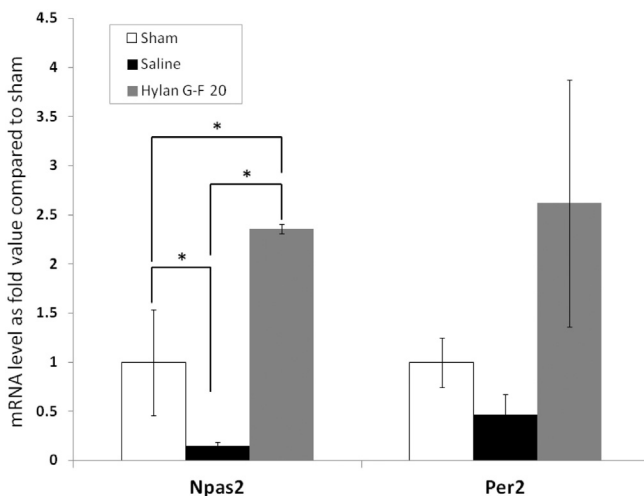


Fig. 3. *Npas2* and *Per2* mRNA levels in cartilage from the Hylan G-F 20-treated and saline-treated groups determined by real-time PCR ($n = 4$ for each group) expressed relative to β -actin mRNA levels and as a fold level compared to the corresponding value for the sham-operated group. * indicates a p value < 0.05 for the difference between the indicated values; other differences are not statistically significant.

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