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Clock mutant promotes osteoarthritis by inhibiting the acetylation of NFκB

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SUMMARY

Objectives: To examine the effect of the circadian gene Clock on posttranscriptional function and pro-inflammatory mechanisms in osteoarthritis (OA).
Methods: The cartilage from Clock mutant mice was assessed using histology, (OA) score, and real-time polymerase chain reaction (PCR) quantification of key pro-inflammatory genes. Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) translocation, posttranslational state and expression levels during day and night conditions were assessed using immunoblot and IP. The regulation of transcription by Clock in cartilage tissue was assessed by using chromatin immunoprecipitation (ChIP) and luciferase assays. Total acetylation level and pattern over 24 h were quantified using immunoblot and real-time PCR. Finally, the effects of exogenous Clock nanoparticle treatment were quantified by histology and immunoblot.
Results: The Clock mutation significantly promoted the degradation of cartilage and the expression of the key pro-inflammatory mediators, IL-1β, IL-6 and MCP-1. The Clock mutation significantly promoted NFκB nuclear translocation. The circadian protein CLOCK positively regulates NFκB at the transcriptional level by binding the E-box domain. The Clock mutation significantly inhibited the total lysine acetylation level in cartilage and inhibited NFκB acetylation at the Lys310 residue but promoted phosphorylation at the Ser276 residue. The forced expression of Clock *in vivo* inhibited NFκB activation by increasing acetylation and decreasing phosphorylation levels and by decreasing cartilage damage and inflammation.
Conclusions: This study demonstrates the mutation of Clock promotes inflammatory activity by mediating the posttranscriptional regulation of NFκB in OA pathogenesis.
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Introduction

The physiological activities of creatures on the earth maintain circadian rhythms of approximately 24 h, as regulated by the core circadian clock gene families. The core circadian clock consists of many regulatory factors such as CLOCK and BMAL1, which form a heterodimer and contribute to the activation of downstream genes. Alternatively, some regulatory factors (such as PER/CRY) regulate the circadian rhythm by inhibiting CLOCK/BMAL1 activity[1]. This transcriptional-translational feedback is mediated by the core circadian molecules and gives rise to circadian rhythm.

In addition to regulating circadian physiology, the CLOCK protein possesses intrinsic histone acetyltransferase (HAT) activity and the ability to acetylate a nonhistone substrate. CLOCK shares homology within the acetyl-coenzyme A binding motifs of HATs and displays high sequence similarity to the SRC proto-oncogene (SRC) family of HATs. Clock mainly acetylates histone H3 at lysine residues 9 and 14 (H3K9 and H3K14) and promotes gene accessibility and transcription[2]. Additionally, BMAL1 is specifically acetylated on the highly conserved Lys537 residue by CLOCK. BMAL1 acetylation

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facilitates the recruitment of CRY1 to CLOCK-BMAL1, thereby promoting transcriptional repression[3]. A recent finding also showed that Clock acetylates argininosuccinate synthase to drive the circadian rhythm of ureagenesis. CLOCK directly acetylates K165 and K176 residues of argininosuccinate synthase (ASS1) to inactivate ASS1, which catalyzes the rate-limiting step of arginine biosynthesis. ASS1 acetylation by CLOCK displays a circadian rhythm that is possibly caused by the rhythmic interaction between CLOCK and ASS1, leading to the circadian regulation of ASS1 and ureagenesis[4]. Thus, changes in protein acetylation caused by CLOCK play an important role in many physiological processes. Our purpose is to determine whether there are other protein substrates that can also be acetylated by CLOCK in addition to the mentioned proteins and their physiological significance.

Acetylation is one of the key mechanisms in many inflammatory diseases. During this process, acetylation opens up the chromatin structure, allowing for gene transcription and synthesis of inflammation proteins[5]. The transcription factor Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a main regulator of the cellular immune response and can be regulated by acetylation of Lys310[6,7]. This posttranscriptional modification activates NFκB, which stimulates the transcription of downstream target genes[8]. In addition, the expression of the NFκB protein level shows a rhythmic pattern, which means that NFκB may be regulated by circadian genes[9]. Constitutive NFκB signaling activation is observed in cryptochrome (Cry) knockout cells, and increased protein kinase A (PKA) signaling activity is a main reason for the increased phosphorylation of NFκB p65 at the S276 residue in Cry knockout cells[10].

Interestingly, circadian rhythms and inflammation have all been linked to the development and progression of osteoarthritis (OA), which is a common cause of disability with a large economic burden[11]. The main characteristic of OA is cartilage destruction with other pathological changes, including osteophyte formation, local inflammation and sclerosis. The degeneration of cartilage is caused by an imbalance in anabolic and catabolic activities. A number of pathophysiological processes contribute to this progressive disease, including injury and aging[12]. These processes lead to the abnormal activation of biochemical pathways in chondrocytes that result in cartilage damage. During cartilage development, the circadian clock is important for driving the cell behavioral changes necessary for skeletogenesis. BMAL1 deficit leads to the disruption of rhythmic expression profiles of downstream genes in the growth plate[13]. Clock mutant mice developed hyperlipidemia and hyperglycemia, liver inflammation and enhanced atherosclerosis[14–16]. Interestingly, Clock mutant mice also exhibited a significant reduction in bone density due to the loss of bone formation as well as increased apoptosis[17]. Additionally, NFκB knockout mice exhibited a marked acceleration of OA through enhanced chondrocyte apoptosis in cartilage[18]. Hence, the circadian clock and NFκB may be extensively involved in the processes of cartilage degradation.

Here, we examined the roles of NFκB and inflammation factors in articular cartilage using Clock mutant mice. During skeletal development, the Clock mutation resulted in impaired cartilage through the enhanced ability of NFκB to translocate and decreased acetylation of NFκB at the Lys310 residue. Our findings provide new insights into the regulation of cartilage degradation and inflammation.

Materials and methods

Animals

The Clock mutant (*Clock*$^{\Delta 19}$) mice and age-matched C57BL/6J (wild type (WT), WT) mice were bred in the Model Animal Research Center of Nanjing University. Mice were fed a chow diet and raised in a clean-grade room with a 12 h light and 12 h dark cycles (lights on at 8:00 am and lights off at 8:00 pm) or in constant darkness. The cartilage of *Clock*$^{\Delta 19}$ and C57BL/6J mice was obtained at the indicated Zeitgeber (ZT) time points (ZT, referring to an objective time scale wherein ZT0 is set as the time that lights are turned on and ZT12 is set as the time that lights are turned off.). C57BL/6J mice ($n = 30$) and *Clock*$^{\Delta 19}$ mice ($n = 30$) were randomly allocated into six experimental groups. For histology, real-time polymerase chain reaction (PCR) and immunoblotting, co-Immunoprecipitation, (Co-IP) and chromatin immunoprecipitation (ChIP), the *Clock*$^{\Delta 19}$ ($n = 5$) and C57BL/6J mice ($n = 5$) were evaluated independently, and the number of animals used per experiment are stated in the figure legends. For the *in vivo* study, the pcDNA3.1-Clock plasmid was injected with EntransterTM nanoparticles (Engreen Biosystem Co, Ltd.) into both knee joint cavities of *Clock*$^{\Delta 19}$ ($n = 5$) and C57BL/6J mice ($n = 5$) every 3 days over the course of 2 weeks. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Histological and histomorphometric analysis

We stained cartilage and meniscus by using Safranin-O staining. To test the presence of glycosaminoglycans, the sections were stained with Alcian blue and with Safranin-O/light green to examine OA. We analyzed the pathologic changes of knee joints in both male and female mice (24 weeks) by histomorphometric scoring. For simple histologic scoring of OA in each mouse, we used the following 0–6 subjective scoring system[19,20]: surface and cartilage morphology intact (0); surface intact (1); surface discontinuity (2); vertical fissures (3); erosion (4); denudation (5); or deformation (6).

Nucleus and cytoplasm extraction and immunoblotting

Proteins were extracted using nuclear and cytoplasmic extraction reagents (Beyotime, China) according to the manufacturer's protocol. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane and then incubated with 5% skim milk overnight. The membrane was then incubated overnight with primary antibodies against IL1, IL6 and MCP1 at 1:1000 (Proteintech, USA), NFκB p65, Acetyl-NFκB p65 (Lys310) and phospho-NFκB p65 (Ser276) at 1:1000 (Cell Signaling Technology, USA), β-actin at 1:5000 (Proteintech, USA), IκB, O-GlcNAc and CLOCK at 1:1000 (Abcam, USA) and acetylated-Lysine and P300 at 1:1000 (Cell Signaling Technology, USA). After incubation with secondary antibodies for 1 h, signals were analyzed using an ECL Western blotting system (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoprecipitation (IP)

HEK-293T cells were transfected with pcDNA3.1-Clock plasmids, and proteins were lysed in NP-40 buffer supplemented with protein inhibitor cocktail (PIC) and phenylmethylsulfonyl fluoride (Sigma). The main methods were performed as described[14]. The lysates of equal amounts of protein were incubated at room temperature with primary antibodies for 1 h and then with protein A/G beads overnight, after which the beads were washed with Phosphate Buffered Saline with Tween® 20 (8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.05% Tween 20, pH 7.4). Bound proteins were solubilized in loading buffer for Western blot analysis.

Quantitative real-time qPCR analysis

Ribonucleic acid (RNA) from mouse cartilage was isolated using Trizol reagent (Life Technologies, USA) according to the manufacturer's instructions. Then, 1 μg of RNA was reversed transcribed into complementary deoxyribonucleic acid (cDNA) using the Reverse Transcription Kit (TOYOBO, Japan). We made the reaction mixture containing primers, the cDNA template, and the

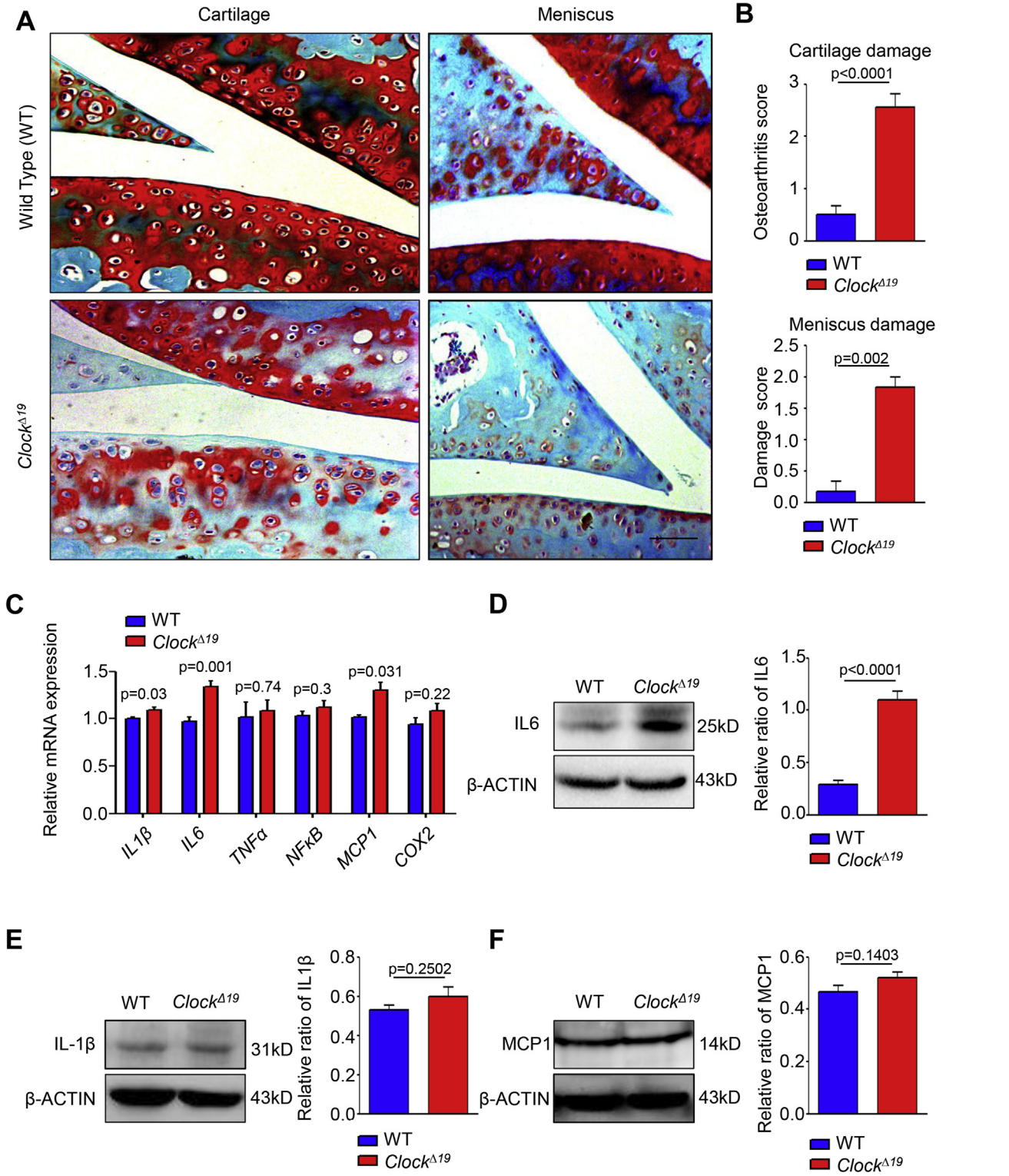

Fig. 1. Clock mutants cause cartilage damage. (A) Representative histological images of wild type (WT) and Clock mutant mouse knees at ZT0. Safranin-O staining revealed cartilage and meniscus. No obvious damage to articular cartilage was observed in WT mice. (B) Cartilage damage and meniscus damage scores based on histology of (A). ($n = 5$ mice per group). (C) Relative expression assessed by qPCR of some inflammation-related genes. Note that the genes, especially *IL6*, were upregulated in Clock mutant mice at ZT0. Data were normalized to *Gapdh* expression. $n = 5$ mice per group. (D) Immunoblots of IL6 from WT and Clock mutant mice at ZT0. Quantification of the immunoblots showed significantly increased expression of IL6. $n = 5$ mice per group. *P*-values less than 0.05 were considered significant.

double strand DNA-specific dye, SYBR® Green PCR Master Mix (Bio-Rad, USA). The sequences of real-time PCR primers are shown in Supplemental Table S1.

Luciferase assays

Luciferase assays were performed according to the procedure described previously. We constructed the pGL3-NFκB plasmid. Then, we transfected 1 μg of pGL3-NFκB plasmid, 1 μg of pRL-TK plasmid and 1 μg each of Clock (or ClockΔ19) and Bmal1 plasmids into HEK293T cells in 6-well plates by using Lipofectamine 3000 reagent (Invitrogen, USA). Cells were harvested 48 h after transfection and firefly and renilla luciferase activity were detected by using the dual luciferase reporter gene assay kit (Beyotime, China).

ChIP

We conducted ChIP assays according the procedure previously described[15]. Cell lysates were initially incubated with anti-CLOCK antibody and magnetic beads (Cell Signaling, USA). ChIP assay results were analyzed by using real-time PCR.

Statistical analysis

The data from experiments were analyzed by several statistical methods. The results are expressed as the mean ± S.E.M. Student's *t* test and one-way ANOVA with Bonferroni *post hoc* tests were used for pairwise comparisons and multicomparisons, respectively. In all cases, significance was accepted at the 0.05 level of probability ($p < 0.05$).

Results

The Clock mutant leads to cartilage degradation and inflammation activation

For simple histologic scoring of OA in mice, we used a 0–6 subjective scoring system[19,20]. Clock mutant mice showed multiple pathologic changes in OA, including cartilage proteoglycan loss and lesions and meniscus damage [Fig. 1(A)]. The OA score increased approximately 5-fold, and meniscus damage increased 6-fold in Clock mutant mice compared with WT mice [Fig. 1(B)]. Because mouse movement may affect the cartilage morphology, we

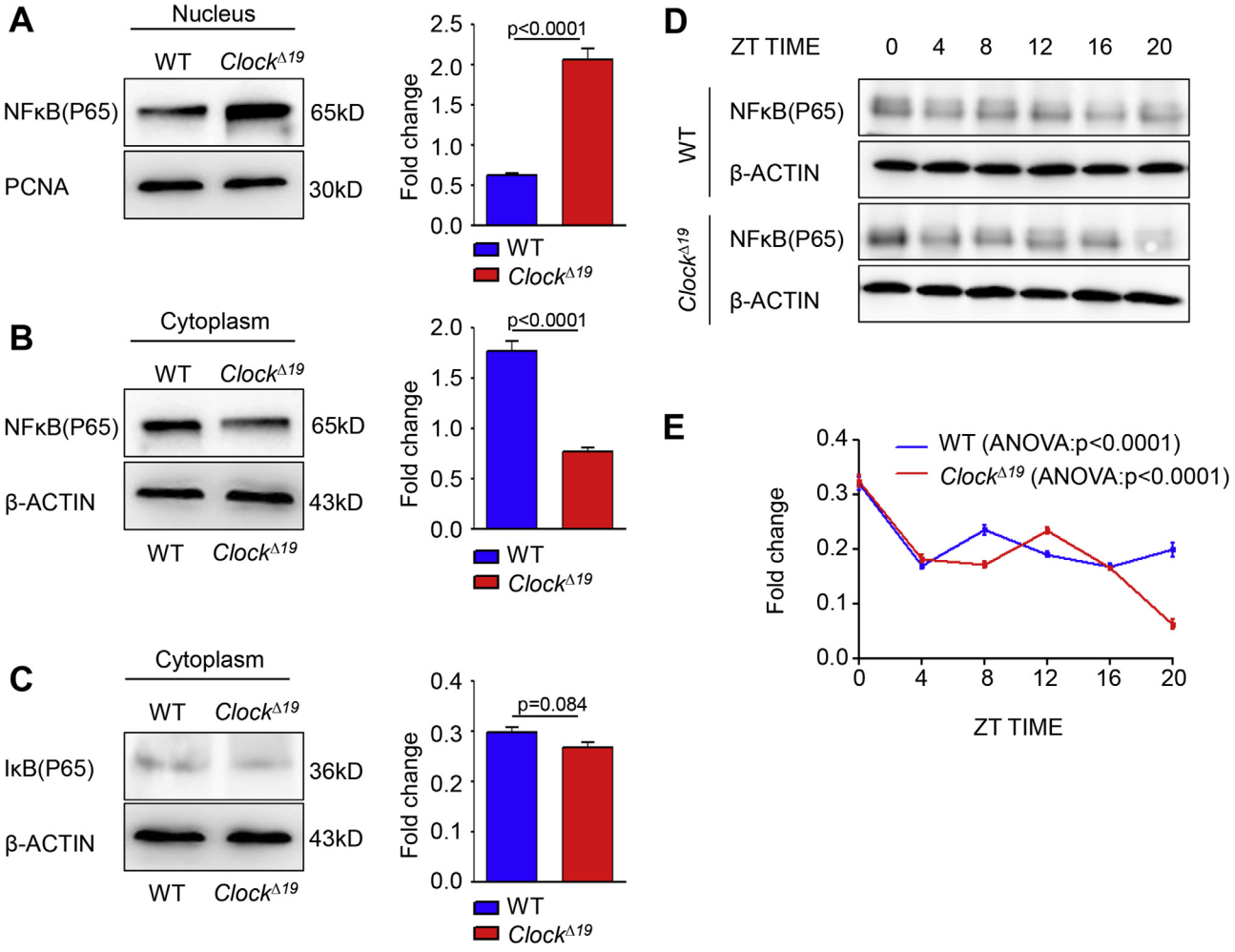

Fig. 2. The Clock mutation promotes NFκB translocation into the nucleus. (A–B) Immunoblots showing the expression of NFκB in the nucleus and cytoplasm of WT and Clock mutant mice at ZT0. $n = 5$ for all groups. (C) Relative cytoplasmic expression of IκB was analyzed in the WT and Clock mutant mice at ZT0. ($n = 5$), (D–E) The cartilages were harvested at the indicated time points (ZT0- ZT12) in WT and Clock mutant mice. The protein expression of NFκB was examined by Western blot, and β-actin was used as a loading control. Note that both WT and Clock mutant mice showed rhythmic gene expression levels of NFκB, but no significant changes were found between the two groups. $n = 5$ for all groups. Data were analyzed by one-way ANOVA and Bonferroni *post hoc* tests and *P*-values less than 0.05 were considered significant.

performed a running wheel experiment to check the movement status in 12:12 light:dark (LD) and constant dark (DD) conditions. We found that there was no significant difference in the total movement activity under LD or DD conditions; only Clock mutant mice showed abnormal activity rhythms under DD conditions [Supplemental Fig. 1(A)]. To confirm whether Clock mutant mice are more susceptible to OA, we performed anterior cruciate ligament transection (ACLT) model and found that the damage in Clock mutant mice was more serious than in WT mice [Supplemental Fig. 1(B)]. Because the increase in O-linked N-acetylglucosamine protein modification stimulates chondrogenic differentiation[21,22], we tested the protein expression and acetylation levels of O-linked N-acetylglucosamine in WT and Clock mutant cartilage tissues. However, we did not find any significant changes in the protein or acetylation levels [Supplemental Fig. 2]. We next detected inflammation-related changes in joint tissue by measuring gene expression in WT and Clock mutant mice. Cartilage inflammation-related molecules showed a tendency to increase, IL1β, MCP1 and IL6 increased significantly [Fig. 1(C)]. Then, we evaluated IL1β, MCP1, and IL6 protein levels by measuring immunology [Fig. 1(D-F)] and found that IL6 increased significantly in Clock mutant mice but not IL1β or MCP1, which was consistent with gene expression [Fig. 1(C)]. Thus, we believe that IL6 may play a key role in Clock mutation-induced OA.

The Clock mutation promotes NFκB translocation but does not affect protein expression

NFκB is a key factor in inflammatory regulation and immune responses, and most pro-inflammation genes are downstream of NFκB, including IL6. Translocation of NFκB p65 into the nucleus is a critical step in which NFκB binds with the promoters of target genes and regulates their translation activity. We extracted proteins from the nucleus and cytoplasm in cartilage tissue and found high levels of NFκB protein in the nucleus, whereas low levels were observed in the cytoplasm of Clock mutant mice strains [Fig. 2(A-B)]. Then, we evaluated IκB content in the cytoplasm, which showed a slight decrease in Clock mutant cartilage [Fig. 2(C)]. Clock is also an important regulator of translation, and whether its mutation could affect the protein expression of NFκB is not clear. We tested the protein expression levels of NFκB over 24 h from ZT0 to ZT12, and there was no significant change in WT and Clock mutant mice [Fig. 2(D-E)].

The Clock mutation inhibits the acetylation of NFκB at Lys310 and promotes phosphorylation at Ser276.

NFκB protein expression showed a rhythmic pattern, which suggests that it may be controlled by a circadian clock through transcriptional or posttranslational regulation [Fig. 2(E)]. First, we evaluated the binding ability of Clock by the ChIP method and

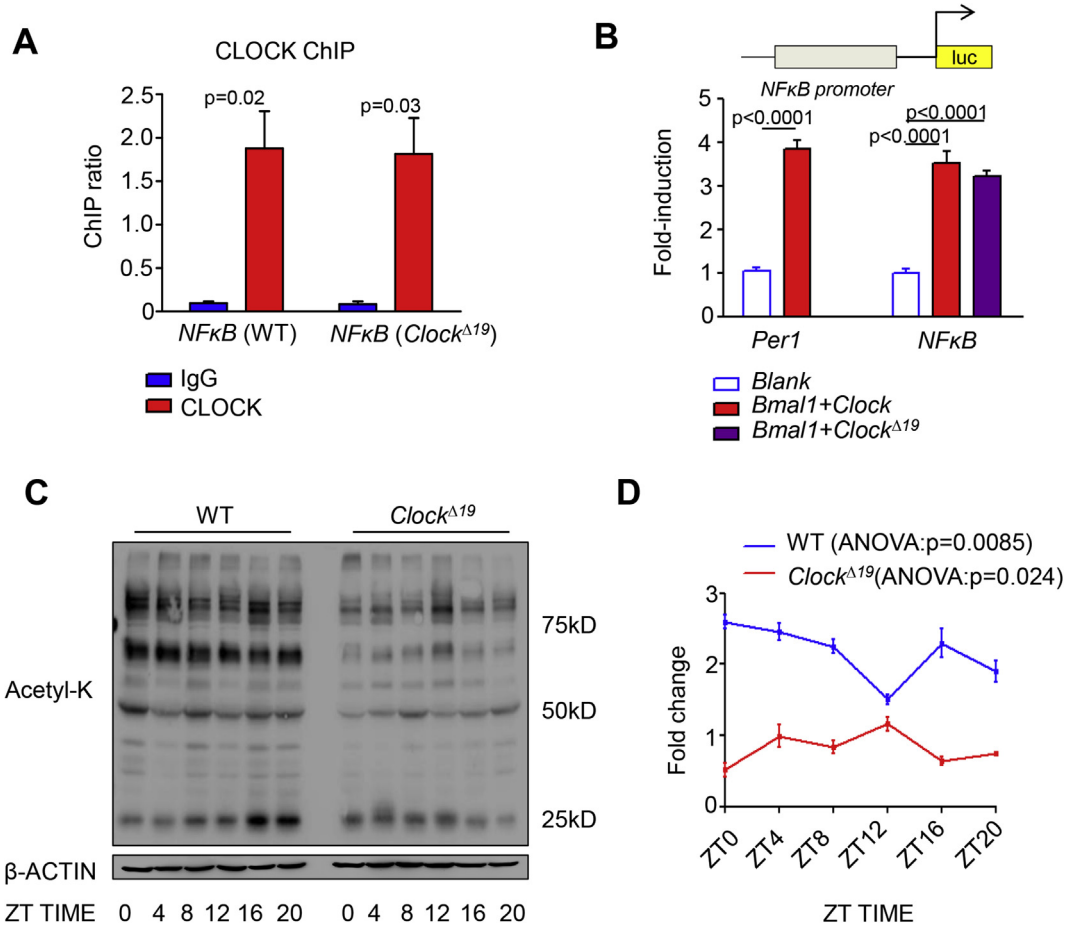

Fig. 3. Clock mediates NFκB by transcription and acetylation regulation, (A) Dual luciferase assay revealed that both Clock and the Clock mutant increased transcriptional activity compared to the control. This suggests that the Clock mutation has little effect on the transcriptional activity of NFκB. Graph represents firefly luciferase expression normalized to renilla luciferase for each condition. Error bars represent standard deviations of biological triplicate experiments. Per1-Luc was used as a positive control ($n = 5$ per group). (B) ChIP analysis of CLOCK binding to the E-box region of NFκB in Clock or Clock mutant cartilage tissue ($n = 5$ per group). (C–E) Immunoblots showing the total acetylation levels in the WT and Clock mutant mouse cartilage tissues. Note that the Clock mutation decreases the total acetylation and disrupts the pattern of acetylation. Data were analyzed by one-way ANOVA and Bonferroni *post hoc* tests and *P*-values less than 0.05 were considered significant.

found that both Clock and the Clock mutant bind with the E-box (CACGTG) motif; however, the binding ability was reduced in Clock mutant cartilage tissue, but not significantly [Fig. 3(A)]. Then, we used the luciferase method to confirm the transcriptional activity. Our findings showed that Clock could activate NFκB transcriptional activity, while there was a decrease in the Clock mutant group [Fig. 3(B)]. These findings suggested that the transcriptional regulation of Clock may not be the only reason for NFκB activation. Thus, we tested the protein acetylation level in cartilage tissue in WT and Clock mutant mice. Interestingly, we found protein acetylation of total lysates showed a rhythmic pattern [Fig. 3(C)], but the acetylation levels decreased in the Clock mutant cartilage tissues [Fig. 3(D)], and the peak protein acetylation level was apparent [Fig. 3(D)]. A study by Knapp S found that the lack of acetylation will induce and exacerbate several diseases[23]. Taken together, we believe that the decreased acetylation level in Clock mutant cartilage may be another cause of inflammation. We tested the acetylation and phosphorylation level of NFκB because both of these posttranslational modifications can activate NFκB. The results showed that the acetylation of NFκB at Lys310 decreased while the phosphorylation of NFκB at Ser276 increased in Clock mutant cartilage tissues compared to control [Fig. 4(A-B)]. In addition to CLOCK, P300 may affect NFκB acetylation. We tested both P300 and CLOCK levels in cartilage tissues but did not find any significant changes in protein levels [Fig. 4(C-D)].

CLOCK binds and promotes NFκB activation via acetylation activity

Because the Clock mutation changes acetylation activity, we performed IP experiments to test whether CLOCK directly binds to acetylated proteins. The results showed that CLOCK binds to NFκB and its posttranslational forms, Ac-NFκB and P-NFκB [Fig. 5(A-C)]. Together with our previous discovery [Fig. 4(A-B)], we deducted that the acetylation and phosphorylation of NFκB affects one another under Clock-induced changes; specifically, the acetylation mediated by Clock inhibits the phosphorylation of NFκB. To further confirm the acetylation function of the CLOCK protein, we knocked down and overexpressed Clock at the gene level. The results showed that the loss of Clock will cause the Ac-NFκB decrease and P-NFκB increase [Fig. 5(D)]. However, overexpression of Clock resulted in the reverse effect, upregulating Ac-NFκB and downregulating P-NFκB [Fig. 5(D)]. Thus, the posttranslational modification of Clock plays a role in balancing acetylation and phosphorylation. Finally, we detected the impact of the posttranslational modification of NFκB on its downstream factor (IL6) under Clock regulation. The results showed that Clock knockdown increased IL6 protein expression, while Clock overexpression caused an IL6 decrease [Fig. 5(E-F)]. Thus, Clock, as an acetyltransferase, regulates NFκB transcription level by protein modification. In summary, these findings suggest that Clock decreases the acetylation of NFκB to induce inflammation.

Exogenous clock promotes acetylation to relieve inflammation in clock mutant mice

To assess whether exogenous clock could alleviate inflammation *in vivo*, we injected nanoparticle-packaged Clock plasmids (*pcDNA3.1-Clock*) into double knee joint cavities of WT and Clock mutant mice for 2 weeks. After injection, we found that the total acetylation level had been rescued, and the acetylation of NFκB was rescued as well [Fig. 6(A-B)]. Moreover, P-NFκB and the pro-inflammation factor IL6 were inhibited after exogenous clock injection [Fig. 6(B–C)]. Importantly, the pathological changes, such as cartilage proteoglycan loss and lesions, were alleviated in the

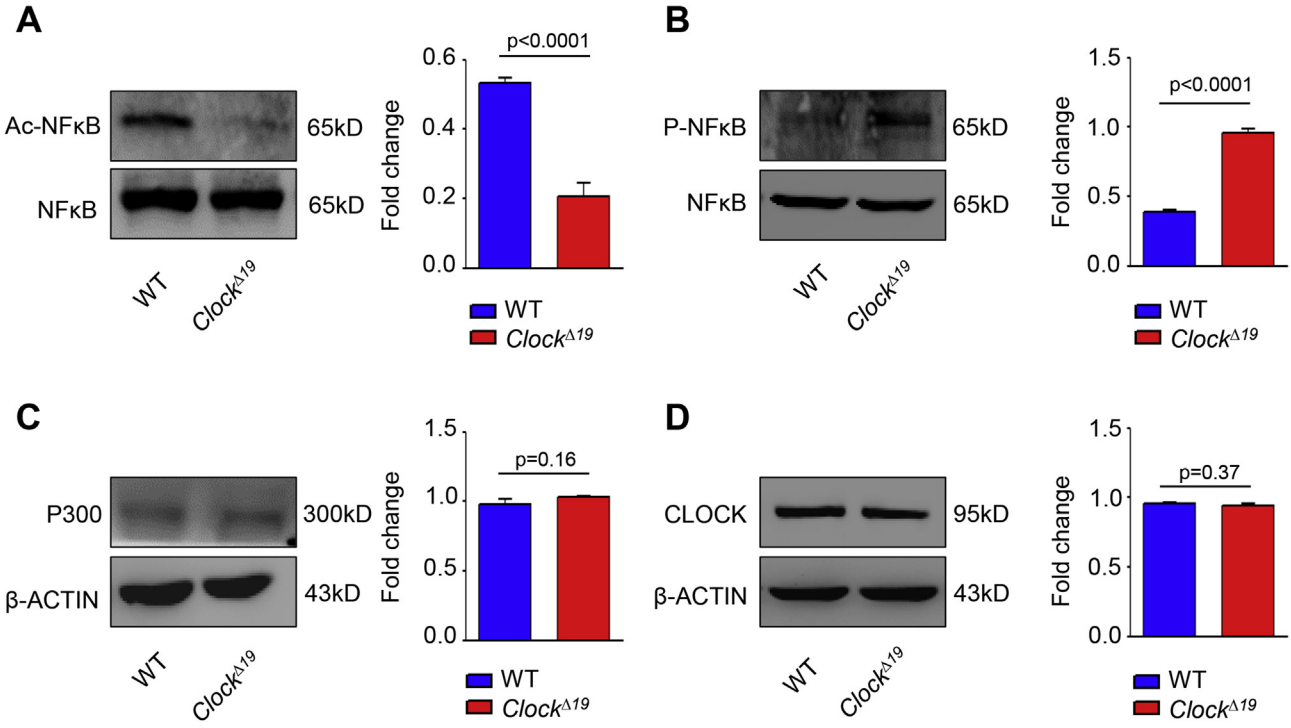

Fig. 4. Clock mutation changes acetylation and phosphorylation of NFκB independent of P300, (A–B) Protein quantification of acetylated-NFκB (Ac-NFκB) and phosphorylated-NFκB (P-NFκB) in the nuclei of WT and Clock mutant mice. Decreased Ac-NFκB and increased P-NFκB are shown in Clock mutant mice. Individual protein changes are not significantly different. NFκB bands were used as an internal control. Columns in graphs show protein normalized to NFκB. $n = 5$ mice per group. (C–D) The protein expression levels of P300 and CLOCK were assessed by Western blotting analysis (expressed as the ratio of P300/Clock to β-actin). Note that there was no significant change in either P300 or CLOCK protein expression. Columns in graphs show protein normalized for β-actin. $n = 5$ mice per group. *P*-values less than 0.05 were considered significant.

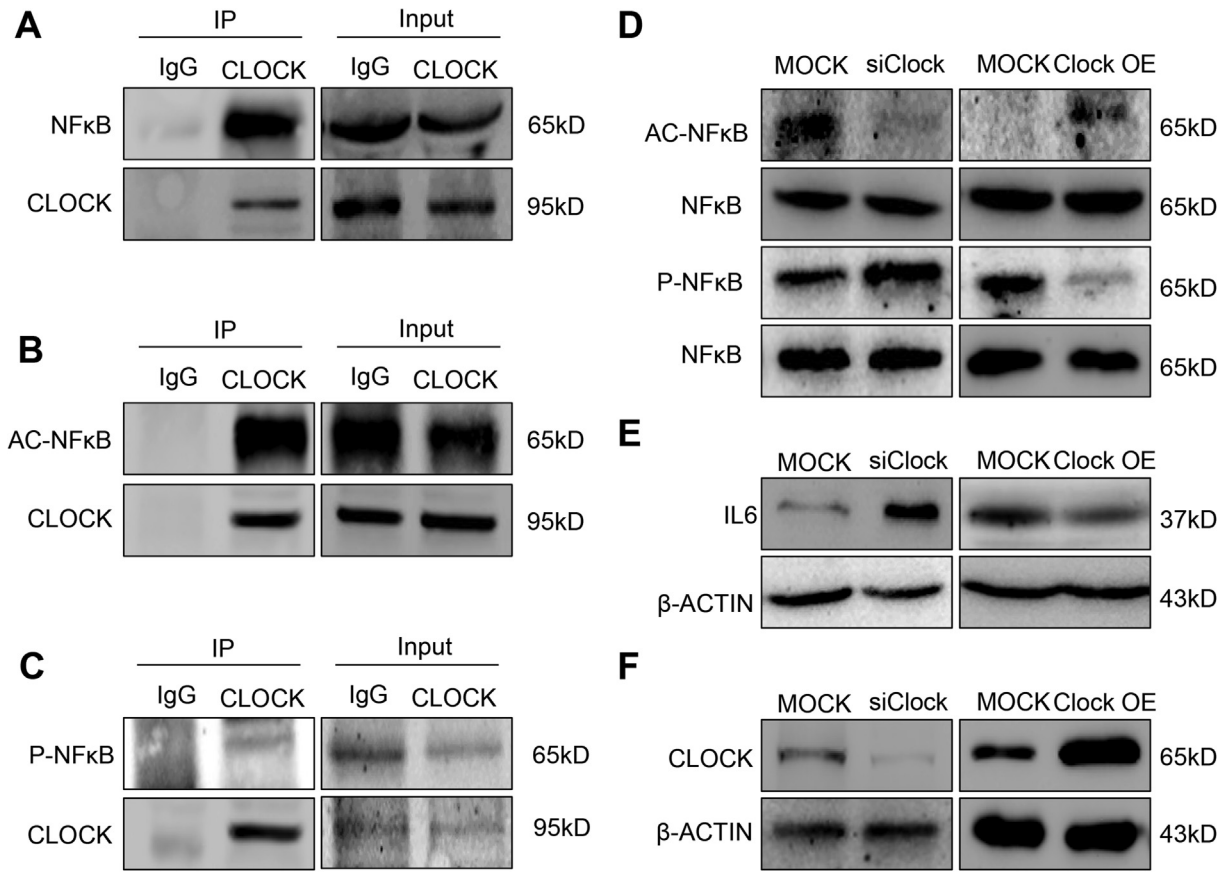

Fig. 5. Clock promotes Ac-NFκB and inhibits P-NFκB. (A–C) Clock binds to NFκB with different modifications. Cartilage tissue was harvested with lysis buffer and immunoprecipitated with normal control IgG or with Clock antibody. The immunoprecipitates were resolved by SDS-PAGE and analyzed for NFκB, Ac-NFκB and P-NFκB levels by Western blot. (D) Clock affects both Ac– and P-NFκB expression. Primary chondrocytes were treated with Control siRNA (MOCK) and siClock or transfected with pcDNA3.1 (MOCK) and pcDNA3.1-Clock plasmids (Clock Overexpression; Clock OE) for 48 h and used to measure Ac-NFκB, P-NFκB and NFκB protein levels by immunoblotting. Note that Clock promotes Ac-NFκB but inhibits P-NFκB protein expression. (E) Primary chondrocytes were treated as described in (D), and the IL6 protein level was detected by Western blot. (F) Clock protein levels were detected by immunoblotting to confirm the knockdown and overexpression effects.

exogenous Clock treatment group [Fig. 6(D)]. In short, our data showed that nanoparticle-packaged Clock plasmids could alleviate inflammation by acetylation regulation.

Discussion

Mice with clock gene disorders can present an abnormal cartilage phenotype[13]. Here, we first demonstrated the post-translational regulation of the circadian gene Clock in OA. Clock controls P-NFκB expression through acetylation in a steady state, but the Clock mutant (lack of exon 19) loses most of its acetylation activity, which causes excessive P-NFκB activation and eventually induces a severe inflammatory response (Fig. 7). Thus, our findings may have a significant impact on the future treatment of OA.

Disruption of circadian genes increases the risk of developing arthritis. Mice subjected to LD cycles or DD show different activity rhythms. Clock mutant mice initially had a long period (approximately 28 h) of rhythmicity and became arrhythmic within 10 days in constant darkness[22]. However, we found no difference in experimental activity under LD conditions, and arrhythmic activity was only observed in the DD condition. In the majority of our study, the mice were kept in LD conditions. Therefore, we believe that rather than behavioral activity, the acetylation activity of Clock plays a role in Clock mutant mice. Given current knowledge of the impact of circadian disruption on cartilage regeneration[24,25], it is possible that the Clock mutant mice showed great differences in arthritic diseases. Human RA patient and mouse studies have shown elevated IL6 levels[26,27], and this phenotype was similarly reflected in the Clock mutant mice. We also demonstrated that the cartilage of Clock mutant mice showed chronic inflammatory conditions (especially the increase of IL6). This finding suggests that IL6 mainly contributes to cytokine signaling in Clock deficient mice.

Pathological environments perturb the circadian clock and increase susceptibility to diseases[28]. The Clock mutation changes the rhythmic activity of mice and leads to impaired glucose tolerance, reduced insulin secretion and defects in the size and proliferation of pancreatic islets that worsen with age. Clock disruption leads to transcriptome-wide alterations in the expression of islet genes involved in growth, survival and synaptic vesicle assembly[29]. Clock mutant mice exhibit a significant reduction in bone formation as well as increased apoptosis[17]. Additionally, the Clock mutation affects the regulation of circulating blood cells and organ senescence[30]. Inder M. Verma found that the absence of the core clock component CRY leads to the constitutive elevation of pro-inflammatory cytokines and activation of NFκB and PKA signaling in Cry knockout cells[10]. Our data showed that the Clock mutation leads to inflammation caused by the activation of NFκB translocation and the disruption of rhythm. Antoch MP also reported that Clock is a positive regulator of NFκB, which binds to the E-box motif of the NFκB promoter and promotes transcription activity[31]. Similarly, we found that both CACGTG of the NFκB promoter and

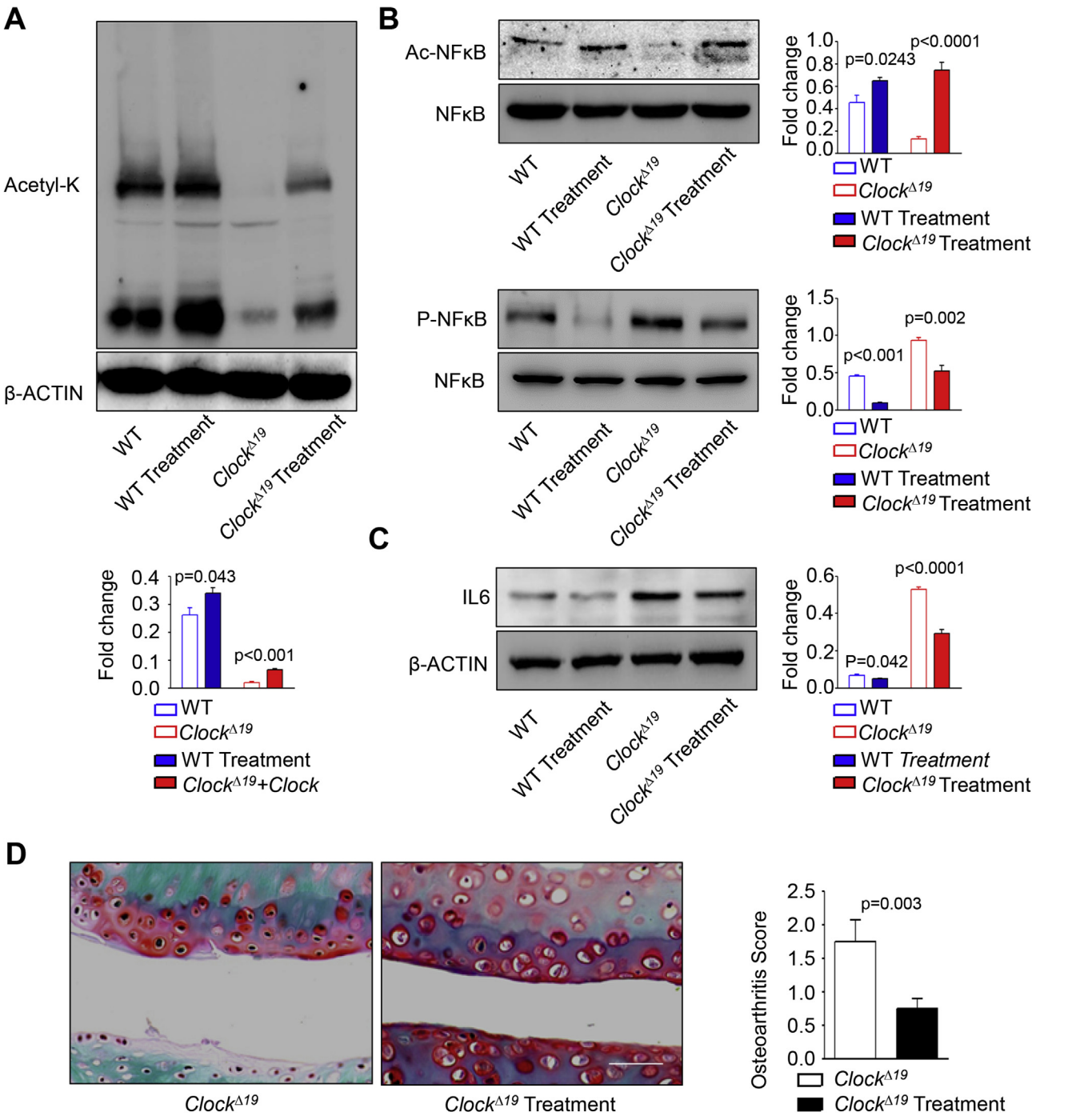

Fig. 6. Clock alleviates the inflammatory response by acetylating NFκB. (A) Nanoparticle-wrapped Clock plasmids were injected into both knee joint cavities to treat osteoarthritis in Clock mutant mice every 3 days for 2 weeks. Cartilage tissues were harvested, and total acetylation protein levels were detected by immunoblots. $n = 5$ mice per group. Note that exogenous Clock treatment promotes the total acetylation level. (B) Mice were treated as described in (A), and Ac-NFκB and P-NFκB were analyzed by Western blot. Columns in the right show protein normalized for NFκB levels in control. $n = 5$ mice per group. *P*-values less than 0.05 were considered significant. (C) Cartilage samples from (A) were used to measure IL6 protein expression, $n = 5$ per group. (D) Histological images of Clock mutant mice and Clock treatment ($Clock^{\Delta 19}$ with Clock nanoparticles) administered to mouse knees. Cartilage damage was scored using histology images. Mice ($n = 5$ per group). Note that Clock treatment alleviates OA. *P*-values less than 0.05 were considered significant.

increase its expression, but the inhibition effect of the Clock mutant is not significant. It is possible that other functions of Clock may play a role in the regulation of NFκB.

In addition to regulating transcription activity, Clock has HAT activity[32]. Previous studies also found that Clock directly mediates the acetylation of Bmal1 or argininosuccinate synthase (ASS1) to control biological function[3,4]. Thus, Clock may control other proteins by regulating acetylation. In our study, we found that the protein acetylation levels changed in Clock mutant mice, which suggests that the Clock mutant not only regulates transcription levels but also protein modification levels. Aberrant acetylation levels have been linked to the development of several diseases, such as diabetes, cancer, inflammation and viral infection. Indeed, the PhosphoSite Plus database shows that there are many lysine

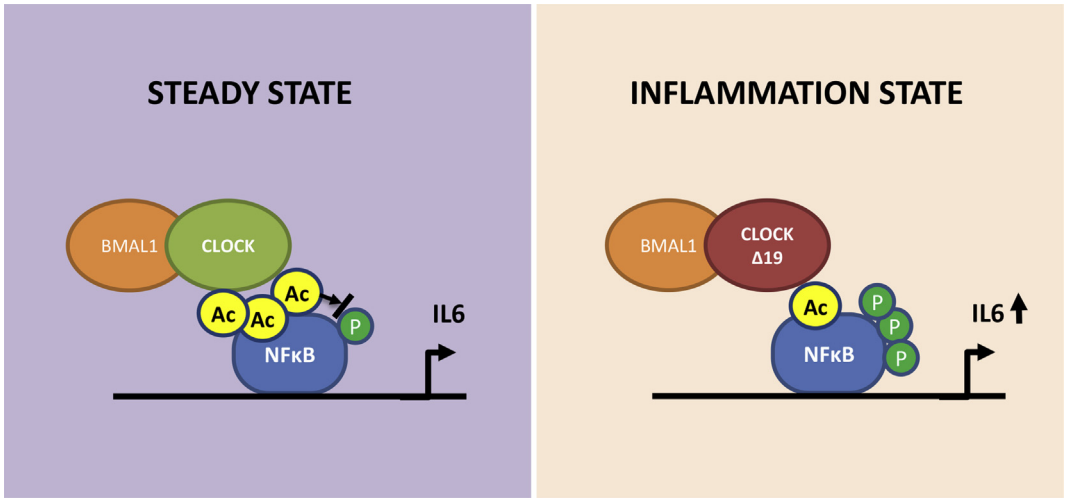

Fig. 7. A schematic diagram suggesting that Clock mediates OA by acetylating NFκB.

acetylation sites in human cells, which suggests that protein acetylation may have a broad role in molecular networks[23]. The significant changes in acetylation in the Clock mutant mice suggests that Clock has an important role in signal transduction. Dysfunctional levels of acetylation are the reasons for oncogenes, aging and metabolic diseases[23,33]. Thus, maintaining the balance of lysine acetylation levels may be a critical therapy for circadian dysfunction diseases. It is worth mentioning that many factors may affect the detection of clinical statistical significance; thus, although we found that Clock has a positive effect on OA, we still have to acknowledge the limitations of this interpretation due to the potential lack of power. We are reporting the results of animal experiments, and more clinical trials are needed to confirm these findings in future studies.

NFκB is acetylated by P300 when translocates into the nucleus[34], which stimulates the transcription of diverse genes, such as IL1 and IL6[35,36]. Some studies have shown that the intracellular proteins released from damaged or necrotic cells induce TLR (toll-like receptor) 4-dependent cartilage catabolism via the upregulation of catabolic mediators such as MMPs and IL-6 with concomitant downregulation of the ECM components aggrecan and type II collagen measured by mRNA expression[37,38]. Here, we found that Clock directly acetylates NFκB at Lys310; however, the P300 levels are not changed in Clock mutant mice, which suggests that NFκB acetylation is not mainly dependent on P300 in this condition. Katrin F. Chua also found that SIRT6 binds to the NFκB subunit RELA and attenuates NFκB signaling by modifying chromatin at NFκB target genes[39]. Therefore, NFκB is affected not only by CBP/P300 but also by other proteins, such as Clock and SIRT6. Interestingly, the level of phosphorylation at the Ser 276 residue of NFκB significantly increases in Clock mutant mice. Vosseller K also reported that overexpression of OGT affects both the acetylation and phosphorylation of NFκB[40]. However, we did not find OGT changed in the Clock mutant mice. Furthermore, we noticed that when NFκB acetylation was changed by the activity of Clock, NFκB phosphorylation was also changed. Therefore, we believe that there may be a balance between acetylation and phosphorylation in the NFκB molecule. Under normal conditions, acetylation maintained by Clock could inhibit phosphorylation, but when Clock is mutated, the lack of acetylation cannot fully inhibit phosphorylation, which then activates NFκB and the inflammatory response.

In summary, our study describes the protective role of Clock. In this work, the Clock mutant causes inflammation and cartilage degradation due to a reduction in acetylation and the abnormal activation of NFκB. This work advances our understanding of how circadian genes increase the risk of OA and suggests that approaches designed to regulate clock acetylation activity may have therapeutic value in the treatment of inflammation.

Author contributions

All authors were involved in drafting the article for important intellectual content, and all authors approved the final version to be published. Study conception and design was performed by Gongsheng Yuan, Chao Lu, and Ruizhe Qian. Data were acquired by Gongsheng Yuan, Tingting Cai, Lirong Xu, Bingxuan Hua, Ning Sun, Zuoqin Yan, Chao Lu, and Ruizhe Qian. The data were analyzed and interpreted by Gongsheng Yuan, Tingting Cai, Chao Lu, and Ruizhe Qian.

Conflict of interest

All authors report no conflicts of interest.

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Supplementary data

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