Disruption of Circadian Rhythm in Mice Increases Susceptibility to Osteoarthritis

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Introduction: The results of a number of recent epidemiological studies have elucidated a correlation between circadian rhythm disruption and prevalence of human diseases, including rheumatoid arthritis. To our knowledge, however, the direct pathophysiological roles of circadian rhythm disruption in articular cartilage homeostasis and degenerative joint disease such as osteoarthritis have yet to be investigated in vivo. This study aims to establish a pathologic link between circadian rhythm disruption and articular cartilage degeneration in mice using a previously established in vivo mouse model of circadian rhythm disruption, and to further identify downstream signaling mediators responsible for these catabolic effects.

Methods: Mice and environmental circadian disruption: Young adult (6-8 week old) male mice were individually housed in cages stored in ventilated, light-tight cabinets and maintained on a 12 hour light:12 hour dark light cycle (12:12 LD) cycle. Environmental disruption of circadian rhythms was achieved using a once weekly 12 hour phase shift in the LD cycle. Mice randomized into the shifted groups were exposed to weekly12 hour phase shifts of the LD cycle for 22 weeks. Mice randomized into the non-shifted groups were maintained on a constant 12:12 LD cycle for the entire experiment.

Genetic circadian disruption: Young adult (6-8 week old) male mice harboring mutations of well-known circadian genes, including Tau and Clock, were housed and handled under stable 12:12 LD conditions.

Locomotor Activity: Mice were individually housed in activity-recording cages (33.0 cm long x 14.0 cm wide x 12.7 cm high), each containing 3 infrared beams with paired sensors across the width of the cage. A movement result in an interruption of a beam, which is recorded as an activity event.

Histology: Following sacrifice, each knee was dissected aseptically and fixed in 4% paraformaldehyde, with decalcification in EDTA, which was changed every 5 days. The decalcified knee was then transsected in the mid-sagittal plane and paraffin-embedded. Serial knee sections of exact 5-μm thickness from the middle part of the knee were obtained to prepare slides. Safranin-Orange (Safranin-O) staining was performed to examine histopathological changes.

Immunohistochemistry Analyses: Immunohistochemical staining was carried out by the standard avidin-biotin-peroxidase complex technique. Sections were probed with primary antibody (anti-p-PKCδ, p-ERK1/2, p-NFκB, Runx2, MMP13, ADAMTS-5, TIMP3, Col2 and SOX-9) diluted in PBS/0.1% BSA followed by incubation for one hour in horse anti-rabbit/mouse biotinylated universal secondary antibody. Sections were then visualized using Vectastain Kit (Vector Laboratories).

Statistical Analysis: Statistical significance was determined by Student’s t-test, or one-way repeated measures ANOVA followed by Sidak post-hoc test. P values less than 0.05 were considered significant.

Results: Histological analyses were performed to evaluate the effects of environmental circadian rhythm disruption in vivo. Both articular cartilage and menisci from knee joints of the experimental “shift” group had significantly increased proteoglycan (PG) loss compared to the control group. In addition, shifted mice exhibited significantly increased infiltration of mast cells along the joint synovial lining tissues, suggesting that disruption of circadian rhythm is associated with an increased inflammatory response in knee joints. Other joints such as glenohumeral joint and lumbar spine intervertebral disc (IVD) also displayed significant decreases in PG accumulation in the shifted mice compared with non-shifted (control) mice, revealing PG matrix loss following environmental circadian disruption. Interestingly, we also found strikingly reduced PG content in the growth plate of shifted mice compared to non-shifted mice.

We investigated whether chronic disruption of the circadian rhythm activates cartilage catabolic factors, thereby leading to the pathological joint changes. We found PKCδ-ERK-RUNX2 regulatory axis was significantly activated in articular cartilage as well as the synovial lining of tissues in shift mice compared to non-shift (control) mice. We next examined production of crucial cartilage degrading enzymes such as MMP-13 and ADAMTS-5 that are transcriptionally regulated by NFκB and RUNX2 in chondrocytes. Immunohistochemical analyses revealed marked overexpression of MMP-13 (p<0.01) and ADAMTS-5 (p<0.05) in mice following circadian disruption compared to control. We also examined whether chronic disruption of the circadian rhythm influences chondroprotective molecules in mice knee joints. Immunohistochemical analyses demonstrated that both SOX-9 and TIMP-3 were significantly (p<0.05) reduced in the joints of mice with a phase shift compared to the control group. Further, the level of type II collagen, a major extracellular matrix protein, was significantly downregulated in joints of shifted animals (p<0.05).

Interestingly, unlike results obtained from the environmentally circadian rhythm disrupted mice, comparative histopathological analyses in genetically circadian disrupted Clock and Tau mutant mice revealed no significant pathological differences in safranin-O staining for PG content in knee articular cartilage/meniscus, shoulder articular cartilage, and spine IVD tissue compared to matched wild-type mice. Further, mutant mice exhibited an intact joint structure with no signs of joint
inflammation (no immune cell infiltration and no synovial lining thickness) following the experimental protocol, suggesting that Clock and Tau mutations may not be a causative factor for joint pathology. Finally, we evaluated whether the mutation of circadian genes such as Clock and Tau maintains the chondroprotective ability of chondrocytes within the joint to prevent cartilage degradation. Immunohistochemical analyses revealed that the mutants and their corresponding littermates display similar expression levels of SOX-9 and TIMP-3.

**Discussion:** This study is the first to identify chronic circadian rhythm disruption via alterations in light/dark cycles as a potentially novel risk factor for the development of OA in mice knee, shoulder, and spine joints. The findings presented here suggest that environmental stimuli rather than endogenous factors may be more important for the development of cartilage degeneration in mice in vivo. In murine joints (ie. knee, shoulder, IVD), disruption of circadian rhythms via chronic shifts in the day/night cycle enhance the development of osteoarthritic changes over time via suppression of PG accumulation, upregulation of matrix-degrading enzyme expression, and downregulation of anabolic mediators. Mechanistically, these effects are mediated, at least in part, by the PKCδ-ERK-RUNX2 and NFκB signaling pathways to induce expression of both MMP-13 and ADAMTS-5, with a simultaneous suppression of the anabolic mediators SOX-9 and TIMP-3 in articular chondrocytes from shifted mice compared to control.

**Significance:** Given the detrimental societal and economic impact of current treatment regimens for symptomatic OA, identification of novel risk factors for OA (i.e. circadian disruption in the form of shift work and "social jet lag") may have a broad impact on translational and cost-effective approaches for musculoskeletal treatment strategies in the future.

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**References:**

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