BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Circadian CLOCK Mediates Activation of Transforming Growth Factor- β Signaling and Renal Fibrosis through Cyclooxygenase 2

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The circadian rhythm regulates blood pressure and maintains fluid and electrolyte homeostasis with central and peripheral clock. However, the role of circadian rhythm in the pathogenesis of tubulointerstitial fibrosis remains unclear. Here, we found that the amplitudes of circadian rhythm oscillation in kidneys significantly increased after unilateral ureteral obstruction. In mice that are deficient in the circadian gene *Clock*, renal fibrosis and renal parenchymal damage were significantly worse after ureteral obstruction. CLOCK-deficient mice showed increased synthesis of collagen, increased oxidative stress, and greater transforming growth factor- β (TGF- β) expression. TGF- β mRNA expression oscillated with the circadian rhythms under the control of CLOCK-BMAL1 heterodimers. The expression of cyclooxygenase 2 was significantly higher in kidneys from CLOCK-deficient mice with ureteral obstruction. Treatment with a cyclooxygenase 2 inhibitor celecoxib significantly improved renal fibrosis in CLOCK-deficient mice. Taken together, these data establish the importance of the circadian rhythm in tubulointerstitial fibrosis and suggest CLOCK/TGF- β signaling as a novel therapeutic target of cyclooxygenase inhibition. (*Am J Pathol* 2015, 185: 3152–3163; <http://dx.doi.org/10.1016/j.ajpath.2015.08.003>)

Most physiological and homeostatic processes, including sleep, heart rhythms, arterial blood pressure, and renal blood flow, have intrinsic circadian rhythms.¹ The circadian rhythm is organized hierarchically and adjusted continuously by environmental clues.² It allows cells and tissues to adapt to metabolic and functional demands as well as diurnal environmental changes.³ In mammals, the central circadian pacemaker is located in the suprachiasmatic nucleus of the hypothalamus and is synchronized to the light and dark cycle through the retinohypothalamic tract.⁴ In addition, evidence of peripheral clocks is growing. Peripheral clocks in individual cells or organs have autonomous and self-sustained intrinsic rhythms with tissue-specific functions.⁵ These peripheral clocks are synchronized with the central clock through neuronal and humoral signaling. The molecular components of the circadian system are autoregulated by transcriptional, translational, and post-translational mechanisms.⁶ One of the important circadian

transcription–translation feedback loops operates as the transcription heterodimers BMAL1 and CLOCK bind to the clock gene promoters and increase the expression of the *period 1/2* (*PER1/2*) and *cryptochrome 1/2* (*CRY 1/2*) genes.⁷ PER 1/2 and CRY 1/2 then translocate back to the nucleus to repress their own transcription by inhibiting the CLOCK–BMAL1 complex.⁸

In humans, a dysregulated circadian rhythm increases the incidence of several diseases. Hypertensive patients with disturbed daily blood pressure rhythms show a higher

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incidence of vascular disease than other patients.⁹ Shift workers have a 40% higher risk of cardiovascular disease than the rest of the population. Every 5 years of night-shift work increases the risk of ischemic stroke by 4% and of breast cancer by 9% in case-control studies.^{10,11} Moreover, disturbed circadian rhythms in shift workers or emergency physicians also are associated with metabolic syndrome or altered telomerase activities.^{12,13}

Several important kidney functions, such as glomerular filtration rate, renal blood flow, urine production, and electrolyte balance, have daily oscillation rhythms.⁹ The amplitude of the melatonin oscillating rhythm decreases as renal disease advances. The disturbed melatonin rhythm in patients with chronic kidney disease is associated with sleep disorders and can be restored by changing dialysis timing.¹⁴ Substantial evidence links aldosterone signaling to disruption of the circadian blood pressure pattern,^{15,16} suggesting the roles of renal function and circadian rhythm. BMAL1 deficiency in the renin-secreting granular cells of the juxtaglomerular apparatus results in altered urine volume, changes in urinary sodium excretion, and lower blood pressure. CLOCK deficiency in mice also causes dramatic changes in the circadian rhythm of renal sodium excretion through 20-hydroxyicosatetraenoic acid.^{17,18} This evidence supports a link between circadian rhythm and renal function. However, whether disturbed circadian rhythm is involved in the progression of renal dysfunction or increases in the incidence of chronic kidney diseases is unknown.

Tubulointerstitial fibrosis is one of the most common histopathologic features of progressive renal disease. Irreversible renal fibrosis leads to deterioration and loss of kidney function. Clock gene *PER2* deficiency or mutation exacerbates liver fibrosis and increases vascular senescence.^{12,19} Based on all of the evidence, we hypothesized that CLOCK-deficient mice would have altered fibrotic responses after unilateral ureteral obstruction.

Materials and Methods

Cells and Antibody

Proximal tubular epithelial cells were isolated from wild-type or CLOCK-deficient mice. The kidneys were de-capsulated and the medulla was removed. The cortices were finely dissected and digested with collagenase. The kidney digests were filtered through a 70- μ m sieve (BD Biosciences, San Jose, CA) and cell pellets were resuspended in a renal cell culture medium (Dulbecco's modified Eagle's medium—F12, 10% fetal bovine serum, 5 μ g/mL insulin, 5 μ g/mL transferrin, 50 nmol/L selenium, 5 nmol/L T3, 50 mmol/L hydrocortisone, and 100 U/mL penicillin/0.1 mg/mL streptomycin). Mouse embryonic fibroblasts (MEFs) were isolated from day 11 to day 13 C57BL/6J mouse embryos using the trypsin method.²⁰ To assess the oscillation of gene expression, cells were synchronized using serum shock. The antibodies that were used for

immunoblotting and immunofluorescence were α -smooth muscle actin (Sigma, St. Louis, MO), glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling, Danvers, MA), 4-hydroxynonenal (R&D Systems), nitrotyrosine (R&D Systems, Minneapolis, MN), cyclooxygenase (COX)1 (Cell Signaling), and COX2 (Cell Signaling).

Mice and Unilateral Ureteral Obstruction

All animal experiments were approved and performed in accordance with the guidelines of Chang Gung University and the Chang Gung Memorial Hospital Institutional Animal Care and Use Committee. CLOCK-deficient mice on a C57BL/6J background were a gift from Drs. Steven M. Reppert and David R. Weaver (University of Massachusetts Medical School, Worcester, MA). The *mPer2^{Luciferase}*—knock-in mice were obtained from Jackson Laboratory (Bar Harbor, ME). Congenic 8- to 12-week-old, male, wild-type, and CLOCK-deficient littermate mice were generated from heterozygote breedings and were used for all studies. The unilateral ureteral obstruction (UO) procedure was performed under general anesthesia with ketamine and xylazine (80/6 μ g/g intraperitoneally, respectively). An incision was made in the left flank area using a scalpel. Ureters then were explored and ligated using 2-0 silk with a double ligature.²¹ After ligation, the surgical fields were rinsed with sterile phosphate-buffered saline to prevent future adhesion. After 7 or 14 days, mice were sacrificed and kidneys were harvested for further analysis. The right kidneys served as controls.

Kidney Pathology

The UO or control kidneys were fixed in 4% paraformaldehyde and paraffin-embedded. Tissue sections with a thickness of 5 μ m were obtained. Paraffin wax was removed using xylene and the sections were rehydrated with ethanol. After washing, the sections were stained with hematoxylin and eosin. To determine the degree of tubulointerstitial collagen deposition, sections were stained using a modified Picrosirius Red Stain Kit (24901; Polysciences, Inc., Warrington, PA). All cortical tubulointerstitial fields were examined at $\times 400$ magnification with Adobe Photoshop CS2 (Adobe, San Jose, CA) and ImageJ version 1.49a (NIH, Bethesda, MD).

Real-Time Bioluminescence Assays

The *mPer2^{Luciferase}* mice underwent UO surgeries within the first 2 hours after lights-on and were sacrificed 7 days later simultaneously within the first 2 hours after lights-on. Kidneys with UO and the contralateral control kidneys from the same animals were dissected and cultured as described previously.²² Organ explants were dissected to approximately 5 mm² and each was placed individually in a culture well with luciferin. Luciferase activities were recorded for 1 minute at 15-minute intervals with an LM2400

luminometer (Hamamatsu, Hamamatsu City, Japan).²³ Experiments with low levels (<6000 cpm) were excluded. Period and phase values were calculated as in previous studies.^{22,24} The raw data were smoothed by an adjacent-averaging method. The peak was measured as the highest point of the smoothed data. The period was computed as the mean between the peaks.

Transcriptional Analysis

Promoter activities were analyzed in renal tubular cells or macrophages from wild-type or CLOCK-deficient mice. Cells (4×10^5) were seeded in 6-well plates and transfected with *mPer2-luc*, transforming growth factor (*TGF*)- $\beta 1$ -*luc*, or *TGF*- $\beta 1$ -*luc* without E-box reporter plasmids with FuGENE HD (Roche, Indianapolis, IN). The pRL-SV40 plasmid that contained *Renilla* luciferase was used to control for differences in transfection efficiency. The cells were lysed 48 hours after transfection and analyzed with a dual-luciferase reporter assay system (Promega, Madison, WI). Chromatin immunoprecipitation (ChIP) was performed with whole kidneys (30 mg per final ChIP) or renal tubular cells that were isolated from the kidneys at each circadian time point with 1% formaldehyde. Cross-linking reactions were stopped by adding 150 mmol/L glycine. The homogenate then was layered on top of 10 mL of 2.05 mol/L sucrose and centrifuged for 30 minutes at $1500 \times g$ at 1°C. The nuclei were resuspended in 1 mL of 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, and sedimented at 1500 g . The nuclei then were lysed with a radio-immunoprecipitation assay buffer and sonicated for 15 seconds on ice 10 times. Duplicate ChIPs were performed using three animals per time point, using an antibody against CLOCK (Abcam, Cambridge, MA). The mouse TGF- $\beta 1$ ChIP primers were as follows: forward 5'-TGCTACTAATCGC-CATCATCC-3', and reverse 5'-CCCTTTCTACCCCG-TAGTTCA-3'. Primers for regions downstream of the *TGF β 1* promoter were used as a negative control. The results were normalized to the input control without immunoprecipitation.

RNA Expression and Protein Analysis

Total RNA was extracted using TRI Reagent (Ambion, Foster City, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed and analyzed using the Applied Biosystems Real-Time PCR System. The relative gene expression method was used for analysis, and the expression of the target genes was normalized to that of 18S rRNA. The assay was repeated independently at least three times. Protein was isolated from homogenized frozen kidneys or cells with a cell lysis buffer (Cell Signaling). The lysates were separated by electrophoresis, transferred to polyvinylidene fluoride membranes, and probed with specific antibodies. The results were normalized to the glyceraldehyde-3-phosphate dehydrogenase band and calculated with ImageJ version 1.49a (NIH).

Lucigenin Assay

The production of O_2^- by renal tubular cells was measured using lucigenin-enhanced chemiluminescence in a microplate luminometer. The lucigenin concentration (5 μ mol/L) was used to minimize background and redox cycling. Proteins were diluted in modified HEPES buffer and loaded (100 μ g/well) onto a 96-well microplate. NADPH (100 μ mol/L) and dark-adapted lucigenin were added to wells just before reading. The production of O_2^- was expressed in arbitrary light units over 20 minutes. Some experiments were performed in the presence of superoxide dismutase (SOD, 200 U/mL) or a cell-permeable superoxide scavenger tiron (10 mmol/L) for control experiments.

Celecoxib Treatment

After undergoing UUO surgery, the CLOCK-deficient mice were divided randomly into a control group ($n = 8$) and a treatment group ($n = 8$). One day after surgery, the mice in the treatment group received 200 mg/kg celecoxib (Pfizer, Taipei, Taiwan) and the mice in the control group received water via oral feeding daily for 6 days at circadian time 1. The mice were sacrificed on day 7 at circadian time 1 and analyzed. The analyzer and feeder were blinded to the treatment of the mice.

Statistical Analysis

Data were evaluated using two-way analysis of variance or the Student's *t*-test. A *P* value < 0.05 was considered statistically significant.

Results

Ureteral Obstruction Results in Increased Amplitude of Circadian Gene Oscillation

To analyze the change in circadian rhythm in the kidney that is caused by ureteral obstruction, we performed UUO in the *mPer2^{Luc}* knock-in mice. The *mPer2^{Luc}* knock-in mice expressed mPERIOD2::LUCIFERASE fusion proteins, which served as a real-time reporter for circadian rhythm oscillation.²⁴ Seven days after UUO surgery, kidneys with ureteral obstruction and contralateral kidneys with sham surgery were isolated and luminescence was measured continuously in real time using an LM-2400 detector (Hamamatsu, Hamamatsu City, Japan).²³ Consistent with a previous report,²³ a robust and sustained circadian rhythm of luminescence was found in kidneys with sham surgery over the 7-day period. In the ureteral-obstructed kidneys, we observed a significantly higher level and oscillatory amplitude of mPERIOD2::LUCIFERASE luminescence than in the kidneys with sham surgery (Figure 1A).

To confirm the increased expression and amplitude of oscillation of the circadian gene in ureteral-obstructed kidneys,

we examined the mRNA expression levels in the kidneys with or without UUO surgery. Mice were sacrificed 7 days after UUO surgery at 4-hour intervals around the clock and kidney RNA was prepared and analyzed. The results showed that the *mPER2*, *mNR1D1*, and *mCRY1* mRNA accumulated in a circadian manner in both the UUO kidneys and the sham kidneys. Consistent with luminescence experiments, *mPER2*, *mNR1D1*, *mBMAL1*, and *mCLOCK* mRNA expression in the UUO kidneys was higher and showed a larger amplitude of oscillation than in the sham kidneys (Figure 1, B and C, and Supplemental Figure S1). Moreover, UUO significantly increased *CLOCK* protein levels (Supplemental Figure S2). No significant difference in *mCRY1* mRNA accumulation was found between UUO and sham kidneys (Figure 1D). These data showed that the circadian gene oscillatory rhythm in kidneys was altered significantly by ureteral obstruction. Therefore, we hypothesized that a deficiency of the circadian gene can affect the process of UUO in mice. Because the UUO significantly increased the level of *CLOCK* protein, *CLOCK*-deficient mice, which had near-normal central circadian clock rhythm and locomotor activities, were chosen to verify our hypothesis.²² Normal locomotor activities in *CLOCK*-deficient mice excluded the influences of altered food intake rhythm or disturbed hormonal rhythm, such as glucocorticoid or interferon.^{25,26}

Renal Fibrosis Is More Severe in *CLOCK*-Deficient Mice with UUO

We performed UUO surgery on wild-type and *CLOCK*-deficient mice and analyzed the extent of renal fibrosis development 7 days after surgery. Consistent with previous studies,²¹ the wild-type mice developed significant hydronephrosis, cortical thinning, and tubular atrophy. Gross examination of the UUO kidneys of *CLOCK*-deficient mice showed extensive fibrosis around perirenal tissues in 8 of the 10 mice, and none in wild-type mice (Figure 2A). Moreover, *CLOCK*-deficient mice showed significantly exacerbated renal injury, as shown by increased hydronephrosis, tubular atrophy, and cortical thinning relative to wild-type mice. Interstitial collagen deposition on picrosirius red staining was significantly higher in *CLOCK*-deficient mice after UUO than in wild-type mice (Figure 2, B and C). There were no significant differences in the numbers of tubular cells, leukocytes, and endothelial cells between wild-type and *CLOCK*-deficient mice after UUO (Supplemental Figure S3).

Figure 1 Circadian gene oscillation amplitudes increase after ureteral obstruction. **A:** Representative records of real-time bioluminescence from kidneys that were isolated from *mPer2^{Luc}* knock-in mice after sham or unilateral ureteral obstruction (UUO) surgery. Relative mRNA levels of *mPER2* (**B**), *mNR1D1* (**C**), and *mCRY1* (**D**) from kidneys that were isolated from wild-type mice after sham or UUO surgery. Data are presented as means $\pm$ SEM. $n = 6$ mice per time point. $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way analysis of variance and Bonferroni *post hoc* test.

Figure 2 Effects of CLOCK (CL) deficiency on tubulointerstitial fibrosis in mice. **A:** Photograph of kidneys from wild-type (WT) and CLOCK-deficient mice after unilateral ureteral obstruction (UUO) surgery. Right obstructed kidney or left nonobstructed kidney were removed 7 days after UUO. **B:** Representative picosirius red staining of WT and CLOCK-deficient kidney sections for assessment of total collagen deposition and extent of fibrosis. **C:** Quantitative analysis of collagen deposition in WT and CLOCK-deficient mice. Representative Western blot images (**D**) and densitometric quantification (**E**) of α -smooth muscle actin (α -SMA) protein levels from mice kidneys. Data are presented as means $\pm$ SEM. Data were analyzed using two-way analysis of variance followed by a Tukey *post hoc* test. $n = 10$. * $P < 0.05$, ** $P < 0.01$ CLOCK UUO versus WT UUO. Original magnification, $\times 100$ (**B**). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Consistent with this observation, α -smooth muscle actin mRNA and protein levels, which are indices of the proliferation of activated myofibroblasts, were higher in CLOCK-deficient mice (Figure 2, D and E, and Figure 3A). Levels of extracellular matrix-related mRNA of Col1A1, Col4A1, and connective tissue growth factor were significantly higher in CLOCK-deficient mice (Figure 3, B–D). UUO reduced the expression of bone morphogenetic protein-7, which plays a protective role in renal fibrosis, but no significant difference was observed between CLOCK-deficient and wild-type mice (Figure 3E). The expression of TGF- β was increased significantly in the CLOCK-deficient UUO kidneys (Figure 3F). These results provide evidence that CLOCK deficiency accelerates renal tubulointerstitial fibrosis in mice after UUO.

TGF- β mRNA Expression Shows Circadian Oscillation and Is Regulated by CLOCK–BMAL1 Heterodimers

The increased TGF- β expression intrigued us. TGF- β is one of the most important factors in the pathogenesis of progressive kidney disease.^{27,28} TGF- β promotes apoptosis or epithelial–mesenchymal transition of tubular epithelial cells, which

lead to abnormal deposition of the ECM and interstitial fibrosis.^{29,30} In light of evidence that CLOCK-deficient kidneys showed increased TGF- β levels after UUO, we hypothesized that TGF- β is a clock-controlled gene. To determine whether TGF- β expression shows circadian rhythms, we first examined the temporal expression of TGF- β mRNA in serum-synchronized cultured MEFs from wild-type and CLOCK-deficient mice. The results thus obtained showed that TGF- β mRNA accumulated in a circadian manner in wild-type MEFs (Figure 4A) and in CLOCK-deficient MEFs, TGF- β mRNA levels were significantly lower and without a circadian rhythmicity (Figure 4A).

The mouse TGF- β gene has a CLOCK–BMAL1 E-box site near the 5' end of the gene, similar to other clock-regulated genes (Figure 4B).³¹ To determine whether CLOCK–BMAL1 heterodimers regulate TGF- β mRNA expression by modulating TGF- β promoter activity, we first tested the TGF- β promoter responses to CLOCK or BMAL1 with luciferase assay. CLOCK or BMAL1 alone increased the TGF- β promoter activity by twofold. Cotransfection of CLOCK and BMAL1 together increased TGF- β promoter activity by approximately threefold (Figure 4, C and D).

Figure 3 Effects of CLOCK (CL) deficiency on gene expressions after tubulointerstitial fibrosis. Relative mRNA levels in kidneys from wild-type (WT) and CLOCK-deficient mice after sham or ureteral obstruction. α -Smooth muscle actin (α -SMA, **A**); type I collagen, $\alpha 1$ (*Col1A1*, **B**); type IV collagen, $\alpha 1$ (*Col4A1*, **C**); connective tissue growth factor (*CTGF*, **D**); bone morphogenetic protein 7 (*BMP7*, **E**); and transforming growth factor- β (*TGF- β* , **F**). Data are presented as means $\pm$ SEM. Data were analyzed using two-way analysis of variance followed by a Tukey *post hoc* test. $n = 6$. ** $P < 0.01$ CLOCK UUO versus WT UUO.

Mutation analysis showed that TGF- β promoter without an E-box failed to respond to the transactivation of CLOCK and BMAL1 (Figure 4E). Whether CLOCK–BMAL1 could bind to the TGF- β promoter *in vivo* then was examined. ChIP assays were performed with nuclear extracts that were obtained from mouse kidney tubular cells

at different circadian times. The binding of CLOCK to the mouse TGF- β promoter was robust and showed a significant circadian rhythm, with its highest peak at circadian time 5 (Figure 4F). These results indicated that TGF- β is a clock-controlled gene and its circadian rhythm is lost in CLOCK-deficient MEFs.

Figure 4 Circadian transactivation of transforming growth factor- β (*Tgfb1*) promoter by CLOCK–BMAL1 heterodimers. **A**: Accumulation of TGF- β mRNAs in serum-shocked mouse embryonic fibroblasts from wild-type (WT) or CLOCK-deficient mice. Circadian rhythmicity was analyzed with JTK_CYCLE. **B**: Schematic diagram of mouse *TGF- β* promoters and E-boxes. **C**: Effect of CLOCK and BMAL1 on the transactivation of mouse TGF (*mTGF- β*) promoter in renal tubular cells and macrophages. **D**: Dose-dependent effect of CLOCK–BMAL1 heterodimers on the transactivation of *mTGF- β* promoter in renal tubular cells and macrophages. **E**: Effects of CLOCK–BMAL1 heterodimers on the transactivation of *mTGF- β* promoter with E-box deletion (Δ Ebox). **F**: Binding of CLOCK to *mTGF- β* promoters in mouse renal tubular cells analyzed by chromatin immunoprecipitation. Data are presented as means $\pm$ SEM. Circadian rhythmicity was analyzed with JTK_CYCLE. Data were analyzed using two-way analysis of variance followed by a Tukey *post hoc* test. $n = 3$ (**A**, **D**, **E**, and **F**); $n = 4$ (**C**). * $P < 0.05$ TGF- β -luc without BMAL1+CLOCK versus TGF- β -luc with BMAL1+CLOCK; ** $P < 0.01$ (WT) and $P = 0.23$ (CLOCK); $^{\dagger}P < 0.05$ BMAL1+CLOCK 300 or 600 ng versus no BMAL1+CLOCK; and $^{\ddagger}P < 0.05$ Δ Ebox versus WT.

Figure 5 Oxidative injury markers increase in CLOCK-deficient mice. Immunohistochemical staining of oxidative stress markers 4-hydroxynonenal (4-HNE) and nitrotyrosine in consecutive kidney sections of wild-type (WT) and CLOCK-deficient mice ($n = 6$). Scale bar = 50 μm . UUO, unilateral ureteral obstruction.

Increased Oxidative Stress in CLOCK-Deficient Mice after UUO

Uncontrolled renal fibrosis is detrimental and may lead to end-stage renal disease. Currently, no effective treatment reverses

renal fibrosis. TGF- β plays a fundamental role in the homeostasis of fibrosis signaling. However, direct TGF- β signaling intervention is not sensible because it is involved in multiple crucial physiological processes.³² Evidence has suggested that reactive oxygen species and related signaling could be critical

Figure 6 Oxidative stress and cyclooxygenase (COX)2 levels increase in CLOCK-deficient mice. **A:** NADPH oxidase activity was measured by lucigenin assay in wild-type (WT) or CLOCK-deficient renal tubular cells with or without transforming growth factor (TGF)- β treatment (2 ng/mL, 8 hours). **B and C:** COX1 and COX2 protein and mRNA levels in WT and CLOCK-deficient renal tubular cells with or without TGF- β treatment. **D:** COX1 and COX2 protein and mRNA levels in wild-type and CLOCK-deficient renal tubular cells with or without preincubation with 1 mmol/L n-acetyl cysteine (NAC) for 1 hour and treatment with TGF- β , as indicated. Data are presented as means $\pm$ SEM. Data were analyzed using two-way analysis of variance followed by a Tukey *post hoc* test. $n = 4$ (**B–D**). * $P < 0.05$ CLOCK versus WT. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RLU, relative light unit.

Figure 7 Cyclooxygenase 2 inhibition rescues the tubulointerstitial fibrosis after unilateral ureteral obstruction (UUO) surgery in CLOCK-deficient mice. **A:** Accumulation of *COX1* and *COX2* mRNAs in the kidneys from wild-type (WT) or CLOCK-deficient mice. **B:** Representative Western blot images and analysis of *COX1*, *COX2*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in kidneys from wild-type or CLOCK-deficient mice after sham or UUO surgery. **C:** Representative picrosirius red staining. **D:** Quantitative analysis of CLOCK-deficient kidney sections for assessment of total collagen deposition and extent of fibrosis after UUO surgery and treatment with H₂O or celecoxib. **E:** Relative α -smooth muscle actin (α -SMA) mRNA levels in CLOCK-deficient kidneys after sham or UUO surgery and treatment with H₂O or celecoxib. **F:** Analysis of α -SMA protein levels in CLOCK-deficient kidneys after sham or UUO surgery and treatment with H₂O or celecoxib. Data are presented as means $\pm$ SEM. Data were analyzed using two-way analysis of variance followed by a Tukey *post hoc* test. $n = 3$ (**A** and **B**); $n = 4$ (**F**); $n = 6$ (**E**); $n = 8$ (**D**). * $P < 0.05$ CLOCK versus WT. Scale bar = 50 μ m. Celec, celecoxib.

downstream mediators in renal fibrosis.^{21,33} Accordingly, we hypothesized that the exacerbation of fibrosis in CLOCK-deficient mice may be a result of the increased formation of reactive oxygen species. To evaluate oxidative stress in the kidney after UUO, we performed immunohistochemical staining of nitrotyrosine. In CLOCK-deficient mice, UUO significantly increased the number of nitrotyrosine-positive tubules (positive tubules/field: wild-type UUO 7.9 ± 1.2 versus CLOCK UUO 39.6 ± 11.1 ; $n = 6$; $P < 0.05$) (Figure 5) and the amount of another oxidative stress marker, 4-hydroxynonenal (positive tubules/field: wild-type UUO 5.1 ± 1.2 versus CLOCK UUO 19.2 ± 7.3 ; $n = 6$; $P < 0.05$) (Figure 5). These data supported increased oxidative stress in CLOCK-deficient mice.

We further studied the oxidative stress in CLOCK-deficient renal tubular cells. TGF- β treatment increased the lucigenin activity and oxidative stress in wild-type renal

tubular cells. CLOCK-deficient renal tubular cells showed higher lucigenin activity without TGF- β treatment than wild-type renal tubular cells. TGF- β treatment significantly increased lucigenin activity in CLOCK-deficient renal tubular cells (Figure 6A). COX1 and COX2 convert arachidonic acid to prostaglandin.³⁴ COX1 is responsible for the control of renal hemodynamics and glomerular filtration rate.³⁵ COX2 is inducible in response to inflammation or injury in kidney and affects salt and water handling.³⁶ Selective inhibition of COX2 reduces proteinuria after 5/6 renal ablations in mice, inhibits nephritis in rats, and reduces both renal fibrosis and TGF- β levels in rats after UUO.^{37,38} Based on the observation that CLOCK deficiency increased oxidative stress, we hypothesized that CLOCK deficiency would affect COX1 and COX2 levels in renal tubular cells. After treatment with TGF- β , CLOCK-deficient renal tubular cells had significantly

Figure 8 A model illustrating the circadian rhythm control of transforming growth factor (TGF)- β and cyclooxygenase (COX)2 signaling axis. Loss of CLOCK leads to circadian disruption of TGF- β oscillation and increased oxidative stress in kidneys. Increased COX2 after oxidative stress results in enhanced renal fibrosis and chronic kidney diseases.

higher COX2 protein and mRNA levels than wild-type cells (Figure 6, B and C).

To test the mechanism that links CLOCK deficiency to increased oxidative stress and increased COX2, we assessed whether the preincubation of CLOCK-deficient renal tubular cells with 1 mmol/L *N*-acetyl–cysteine, which is an antioxidant that scavenges reactive oxygen species, suffices to affect COX2 levels. After incubation with *N*-acetyl cysteine, CLOCK-deficient renal tubular cells had COX2 levels that were similar to those of wild-type cells (Figure 6D). Altogether, these data showed that CLOCK-deficient renal tubular cells accumulate excess reactive oxygen species, causing the up-regulation of COX2.

Increased Expression of Cyclooxygenase 2 in CLOCK-Deficient Mice after UUO and Celecoxib Treatment Attenuation of Renal Fibrosis in CLOCK-Deficient Mice

Based on the evidence that CLOCK deficiency increases TGF- β levels, oxidative stress, and up-regulates COX2, we explored whether the selective inhibition of COX2 can ameliorate exacerbated renal fibrosis after UUO in CLOCK-deficient mice. First, we studied whether COX1 or COX2 showed circadian oscillation in kidneys. The *COX1* and *COX2* transcripts showed no circadian oscillation in wild-type mice. However, in CLOCK-deficient mice, *COX1* and *COX2* transcripts showed paradoxical oscillation with higher levels than in wild-type mice (Figure 7A). Although COX1 protein levels in wild-type mice with and without UUO were similar, COX1 protein levels in CLOCK-deficient mice were increased (Figure 7B). COX2 protein levels were nearly absent in wild-type mice and were increased significantly in CLOCK UUO kidneys (Figure 7B). These results support the possibility that the inhibition of COX2, a downstream effector of TGF- β , is a potential therapeutic target of renal

fibrosis that is caused by CLOCK deficiency. We then treated CLOCK-deficient mice with the oral selective COX2 inhibitor, celecoxib, after UUO and analyzed the severity of renal fibrosis. Picrosirius red staining showed that celecoxib effectively inhibited interstitial fibrosis in CLOCK-deficient mice (Figure 7, C and D). Consistent with the results of picrosirius staining, celecoxib treatment significantly reduced the α -smooth muscle actin mRNA and protein levels in CLOCK-deficient kidneys after UUO (Figure 7, E and F). These results support the fact that the selective inhibition of COX2 effectively reduced the renal fibrosis that was caused by CLOCK deficiency after UUO.

Discussion

Our results provide evidence that tubulointerstitial fibrosis after UUO was controlled by the circadian gene *Clock*. After UUO, the circadian oscillatory amplitude increased as a protective mechanism. The CLOCK-deficient mice, which did not have this circadian protective mechanism, showed exacerbated fibrosis after UUO. The enhanced fibrotic responses were mediated by clock-controlled TGF- β and its downstream target COX2. Moreover, the selective inhibition of COX2 reduced fibrosis after UUO in CLOCK-deficient mice. These observations provided a new signaling insight into the mechanisms of interstitial fibrosis after UUO and the pathogenesis of chronic kidney diseases.

The interaction between TGF- β signaling and circadian rhythm is complex. Our data have shown that the control of TGF- β expression by CLOCK–BMAL1 heterodimers is through TGF- β promoter E-box. TGF- β 2 has been shown to inhibit the expression of *PER1*, *PER2*, *NR1D1*, and *DBP* in neurons.³⁹ Consistent with our data, in the rabbit aqueous humor, TGF- β 2 shows endogenous circadian rhythms.⁴⁰ Activation of TGF- β signaling resets the circadian clock.⁴¹ Moreover, an exploration of the publicly available circadian expression profiles database showed that TGF- β 1 is a clock-controlled gene.^{42,43} These results support the finding that TGF- β signaling is an interacting molecular clock pathway.

The finding that CLOCK-deficient kidneys showed increased oxidative stress markers is consistent with previous studies.⁴⁴ BMAL1-deficient fibroblasts showed an increased sensitivity to hydrogen peroxide and a disrupted regulation of reactive oxygen species homeostasis.⁴⁵ CLOCK mutant mice also respond to low-dose irradiation with accelerating aging phenotypes.⁴⁶ Indeed, the reactive oxygen species, when induced, have their own circadian rhythms.⁴⁷ Our study and previous evidence has suggested that circadian disruption can result in deregulated oxidative stress, which is responsible for increasing fibrosis, accelerated aging,⁴⁸ and carcinogenesis. We also found that SOD1, an antioxidant enzyme, was decreased significantly in CLOCK-deficient mice with or without UUO surgery (Supplemental Figure S4A). Moreover, SOD1 mRNA levels

had robust circadian oscillation (Supplemental Figure S4B). In *CLOCK*-deficient mice, the *SOD1* mRNA levels lost circadian oscillation and were significantly lower than in wild-type mice (Supplemental Figure S4B). Wild-type and *CLOCK*-deficient mice showed no difference in *SOD2* mRNA levels and no circadian oscillation. These findings suggest that decreased *SOD1* may be responsible for the increased oxidative stress in *CLOCK*-deficient kidneys.⁴⁹

The effects of selective COX2 inhibition in chronic kidney diseases are controversial. In elderly patients with impaired renal function, COX2 inhibition may reduce glomerular filtration rates.⁵⁰ Selective inhibitors of COX2 can prevent the progression of glomerular sclerosis in mice and reduce tubulointerstitial injury after UO and diabetes.^{51,52} These observations imply that the COX2 inhibitor can be effective only in certain contexts, such as enhanced TGF- β signaling or deregulated oxidative stress. The significant effects of COX2 inhibition in our study imply that circadian disruption indeed enhances oxidative stress and increases inflammatory responses. The effectiveness of treatment with a COX2 inhibitor in *CLOCK*-deficient mice with UO herein provided evidence that enhanced fibrosis in these mice was caused by an enhanced TGF- β –COX axis. However, this treatment cannot be used to treat all other chronic kidney disease because circadian disruption is only one of the many mechanisms that underlie the development of chronic kidney disease.

Consistent with our results, *PER2*-deficient mice also showed markedly increased cholestatic liver injury and fibrosis. The expression of fibrosis-related genes such as *Tnf*, *Tgfb1*, and *Timp1* also was increased in *PER2*-deficient mice.¹⁹ These data suggest that circadian rhythm is critical in TGF- β signaling and fibrotic homeostasis. However, we have performed UO in *PER2* mutant mice but failed to observe different fibrotic responses or kidney changes between *PER2* mutant mice and wild-type mice (C.Y. Wang and S.L. Lin, unpublished data). Different clock genes may have different peripheral clock functions or impacts on organ functions, such as angiogenesis, aging, or oncogenesis.

CLOCK-deficient mice have lower blood pressure than wild-type mice.⁵³ Blood pressure plays an important role in renal fibrosis and the UO model.⁵⁴ High blood pressure causes interstitial fibrosis, proteinuria, and glomerulosclerosis. Blood pressure reduction reduces glomerular-capillary pressure, inhibits interstitial fibrosis, and prevents the development of chronic kidney diseases.^{55,56} In mice, blood pressure lowering with angiotensin-receptor antagonists attenuates renal interstitial fibrosis in a UO model.⁵⁷ Based on evidence from clinical studies of blood pressure and related mice experiments, we assume that *CLOCK*-deficient mice with reduced blood pressure would have less renal fibrosis. However, in our studies, *CLOCK*-deficient mice had increased renal fibrosis despite having lower blood pressure. *CLOCK* may have multiple physiological effects in mice, including on blood pressure and renal fibrosis.

In conclusion, this study established that the circadian gene *CLOCK* mediates the oscillation of TGF- β signaling

and COX2 activation, which promote kidney interstitial fibrosis (Figure 8). Targeting the *CLOCK*–TGF- β –COX axis with celecoxib has effective therapeutic effects on renal fibrosis in *Clock* knockout mice after UO. These data imply that the circadian rhythm in the kidney is a potential therapeutic target in the treatment of chronic kidney disease.

This study had some limitations. First, significantly increased TGF- β mRNA levels were observed in *CLOCK*-deficient mice. However, in *CLOCK*-deficient MEFs, TGF- β mRNA levels were decreased. Because the TGF- β promoter is controlled by the circadian heterodimer *CLOCK*–*BMAL1*, a lower TGF- β mRNA level in *CLOCK*-deficient context is reasonably expected. This paradoxical difference between circadian-deficient cells and mice also was observed in *Per2* mutant mice.⁵⁸ The exact mechanisms that underlie this difference are not known and presumably involve the circadian regulation of RNA processing⁵⁹ or the dependence on the accuracy of *in vivo* mRNA accumulation on circadian rhythm. Future studies must be performed to investigate these possibilities. Second, we have found that *SOD1* expression showed a robust circadian oscillation. *CLOCK*-deficient mice lost the circadian rhythm of *SOD1* expression and showed lower *SOD1* levels. The lower *SOD1* levels in *CLOCK*-deficient mice may have been responsible for the increased oxidative stress. However, a further study using *Sod1* knockout mice will be needed to determine the mechanism.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.08.003>.

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