



Research Paper

Inhibition of G₀/G₁ Switch 2 Ameliorates Renal Inflammation in Chronic Kidney Disease



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ABSTRACT

Chronic kidney disease (CKD) is a global health problem, and novel therapies to treat CKD are urgently needed. Here, we show that inhibition of G₀/G₁ switch 2 (G0s2) ameliorates renal inflammation in a mouse model of CKD. Renal expression of chemokine (C-C motif) ligand 2 (Ccl2) was increased in response to p65 activation in the kidneys of wild-type 5/6 nephrectomy (5/6Nx) mice. Moreover, 5/6Nx *Clk/Clk* mice, which carry homozygous mutations in the gene encoding circadian locomotor output cycles kaput (CLOCK), did not exhibit aggravation of apoptosis or induction of F4/80-positive cells. The renal expression of G0s2 in wild-type 5/6Nx mice was important for the transactivation of Ccl2 by p65. These pathologies were ameliorated by G0s2 knockdown. Furthermore, a novel small-molecule inhibitor of G0s2 expression was identified by high-throughput chemical screening, and the inhibitor suppressed renal inflammation in 5/6Nx mice. These findings indicated that G0s2 inhibitors may have applications in the treatment of CKD.

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1. Introduction

Chronic kidney disease (CKD) is a global health problem (Barsoum, 2006; Hedayati et al., 2008; Murray, 2008; Enomoto et al., 2008; Krishnan and Kiernan, 2007). Therapeutic strategies for the treatment of CKD involve attenuation of progressive renal dysfunction, blood pressure control, reduction of proteinuria, and exclusion of uremic toxins (Ruggenenti et al., 1998, 1999; Niwa and Ise, 1994; Ruggenenti et al., 2012; Codreanu et al., 2005). Although inhibition of the renin-angiotensin-aldosterone pathway and elimination of uremic toxins confer

renal protection, there are no adequate treatments available for alleviation of all the symptoms of CKD.

Most living organisms exhibit behavioral and physiological rhythms with a periodicity close to 24 h (Vitaterna et al., 1994; Kume et al., 1999; Jin et al., 1999; Preitner et al., 2002). Mammalian clock genes regulate biological functions in the central and/or peripheral tissues (Ueda et al., 2002; Siepka et al., 2007; Isojima et al., 2005). Molecular dissection of the circadian biological clock has revealed links between genetic mutations and/or alteration of clock genes and diseases, including cancer, metabolic syndrome, and diabetes, in humans and animal models (Filipski et al., 2005; Ohdo et al., 2011). In patients with CKD, the rhythms of sleep and serum hormone levels are dysregulated (Niemczyk et al., 2006). Moreover, many mediators of inflammation contribute the pathology of CKD (Yu et al., 2014), and the activities of these inflammatory mediators in various inflammatory disorders reveal the circadian characteristics of the diseased state (Hashiramoto et al.,

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2010; Gibbs et al., 2014). In a recent study, the core molecular clock protein circadian locomotor output cycles kaput (CLOCK) was shown to be important for nuclear factor- κ B (NF- κ B)-mediated transcription of various pro-inflammatory cytokines (Spengler et al., 2012; Narasimamurthy et al., 2012). Indeed, although it is difficult to discern causes from effects, these insights have suggested that there may be a relationship between the molecular clock and CKD pathology. Thus, elucidation of these relationships may facilitate the development of new therapies for treating CKD.

The use of high-throughput screening (HTS) techniques has long been employed by the pharmaceutical industry to increase discovery rates for new drugs that could be useful for disease treatment (Brey et al., 2011; Patel et al., 2012). Additionally, chemical screening has emerged as a powerful tool to investigate biological mechanisms and other processes (Hirota and Kay, 2009; Hirota et al., 2010; Isojima et al., 2005). Inflammation is a risk factor for various pathological conditions, such as renal disease, hepatitis, diabetes, and cardiovascular diseases (Meijer et al., 2015). Chronic inflammation is affected by activation of the NF- κ B pathway, a prototypical pro-inflammatory signaling pathway; NF- κ B induces the expression of various pro-inflammatory genes, including cytokines, chemokines, and adhesion molecules (Toby, 2009). Because NF- κ B has various biological functions, targeting NF- κ B in therapeutic strategies for inflammatory diseases is complicated (Toby, 2009). Therefore, identification of novel factors regulating inflammation in disorders such as CKD is urgently needed to facilitate the discovery of new therapeutic agents.

In this study, we aimed to identify the novel molecular mechanisms that mediate renal dysfunction in mice with CKD by examining the relationships between the circadian clock and CKD aggravation. Our data showed that the expression of the gene encoding G₀/G₁ switch 2 (G0s2), controlled by the molecular clock pathway (Turek et al., 2005), increased transcriptional activation of *Ccl2* and that G0s2 knock-down or inhibition by a novel small-molecule inhibitor ameliorated renal inflammation in CKD. Thus, our data suggested that molecular clock-dependent changes in G0s2 expression aggravated renal inflammation in CKD mice.

2. Results

2.1. Renal CLOCK Expression Was Altered in Wild-Type 5/6Nx Mice

First, we sought to elucidate the association between the molecular clock and CKD pathology. We found that 24-h locomotor activities were altered in mice that underwent nephrectomy (hereafter referred to as 5/6Nx mice) at 7–9 weeks after the second operation (Fig. S1A). To evaluate the renal clock genes involved in various renal functions, we quantified the temporal expression profiles of renal clock genes in 5/6Nx mice. Renal *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, *Rev-erba*, *Dbp*, and *Ppar α* mRNA expression oscillated in wild-type sham-operated mice at 8 weeks after operation (Fig. 1A). The expression of clock genes containing *cis*-element E-boxes increased. Additionally, wild-type 5/6Nx mice exhibited increased expression of CLOCK protein (Fig. 1B), a critical regulator of renal function (Zuber et al., 2009; Nikolaeva et al., 2012). These results suggested that there may be a relationship between increased CLOCK expression and CKD pathology.

Thus, we further examined the role of CLOCK in 5/6Nx mice using *Clock* mutant mice (*Clk/Clk*), which exhibit more severe behavioral and molecular phenotypes than *Clock*-knockout mice (Debruyne et al., 2006). The Δ 19 *Clock* mouse, which carries a deletion of exon 19 in the *Clk* locus, produces a protein that has been characterized as dominant negative by some researchers, but as functionally null by others (Gekakis et al., 1998). After the second operation at Zeitgeber time (ZT) 6, serum creatinine and serum urea nitrogen (SUN) levels were increased at 8 weeks in wild-type 5/6Nx mice, but decreased at 8 weeks in *Clk/Clk* 5/6Nx mice (Fig. 2A). The decrease in glomerular filtration rates (GFRs) in wild-type 5/6Nx mice at 8 weeks was ameliorated in *Clk/Clk*

mutant mice (Fig. S1C). The region of renal fibrosis, indicated by blue staining of histological sections subjected to Masson's trichrome staining, decreased markedly in *Clk/Clk* 5/6Nx mice (Fig. 2B). The area of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells, as a marker of apoptosis, and the activity of caspase 3/7 were suppressed in *Clk/Clk* 5/6Nx mice compared with those in wild-type mice (Fig. 2C). Functional microarray analysis of renal genes in *Clk/Clk* 5/6Nx versus wild-type mice showed that the biological pathways related to the immune system were altered (Fig. 2D; NCBI accession no. GSE35135). Importantly, immune system function is correlated with inflammation and apoptosis (Sanz et al., 2008). The area of F4/80-positive cells was decreased in *Clk/Clk* 5/6Nx mice compared with that in wild-type 5/6Nx mice (Fig. 2E). These results suggested that renal inflammation in *Clk/Clk* 5/6Nx mice was less than that in WT 5/6Nx mice.

2.2. Transcriptional Control of Renal *Ccl2* Expression by p65

Increased tubular expression of chemokine (C-C motif) ligand 2 (*Ccl2*) is observed in human progressive renal disease, and interstitial inflammatory infiltrates express CC chemokine receptor 2 (CCR2) (Eardley et al., 2006). Thus, we next examined the expression of *Ccl2* mRNA and protein in wild-type 5/6Nx mice (Fig. 3A). Renal *Ccl2* transcript levels were increased in wild-type 5/6Nx mice, peaking at ZT6 and exhibiting a trough at ZT18 (Fig. 3B). We then investigated the consensus sequences within the promoter region of the *Ccl2* gene. Cotransfection with mouse *Ccl2* luciferase reporters and p65 expression constructs led to significant increases in transcriptional activity (Fig. 3C). In vivo binding of the p65 protein to the p65 binding site in the *Ccl2* promoter at ZT6 was greater in lysates from 5/6Nx mice than in lysates from *Clk/Clk* 5/6Nx mice (Fig. 3D). The levels of phosphorylated p65 were decreased in *Clk/Clk* 5/6Nx mice compared with those in wild-type 5/6Nx mice (Fig. 3E). These results suggested that induction of *Ccl2* by p65 in *Clk/Clk* 5/6Nx mice was lower than that in wild-type 5/6Nx mice.

2.3. G0s2 Functioned as a Transcriptional Co-activator in Response to p65 Activation

Next, we examined the mechanism responsible for reduced the binding of p65 to the p65 binding site in the *ccl2* promoter in *Clk/Clk* 5/6Nx mice. In previous studies, the G0s2 has the relationship between pathophysiology of cancer, diabetes and inflammation (Welch et al., 2009; Zhang et al., 2014; Cristillo et al., 1997). And, cytosolic G0s2 is important for transactivation by p65 in inflammation. We demonstrated that the microarray analysis in hepatic knockdown of G0s2 in culture cells or mice induced the alteration of the cytokine and chemokine signaling genes expression (Table S1A, B). Thus, we examined this interaction in 5/6Nx or *Clk/Clk* 5/6Nx kidney cell lysates by immunoprecipitation. The binding of G0s2 was increased in wild-type 5/6Nx renal cell lysates compared with that in *Clk/Clk* 5/6Nx renal cell lysates (Fig. 4A). Next, to clarify the interactions between p65 and G0s2 proteins, we performed mammalian two-hybrid assays in Hepa1-6 cells (Fig. 4B). Co-transfection with ACT-p65 and BIND-G0s2 expression plasmids significantly increased the interactions of these proteins, suggesting that there may be an interaction of between p65 and G0s2 proteins. The transcriptional activity of p65 was decreased by G0s2 knock-down (Fig. 4C). Additionally, p65-dependent *Ccl2* promoter activity was suppressed by G0s2 knockdown (Fig. 4D). Next, to clarify the effects of G0s2 on function of p65 protein, we investigated the localization of p65 in G0s2-knockdown cells. In intact cells, G0s2 protein accumulated in the cytosol, and p65 protein accumulated in the nucleus (Fig. 4E). In contrast, nuclear accumulation of p65 protein and *Ccl2* mRNA expression were suppressed in G0s2-knockdown cells (Fig. 4E). Moreover, the expression of G0s2 mRNA was suppressed in the kidneys of *Clk/Clk* sham-operated or 5/6Nx mice compared with that in wild-type mice

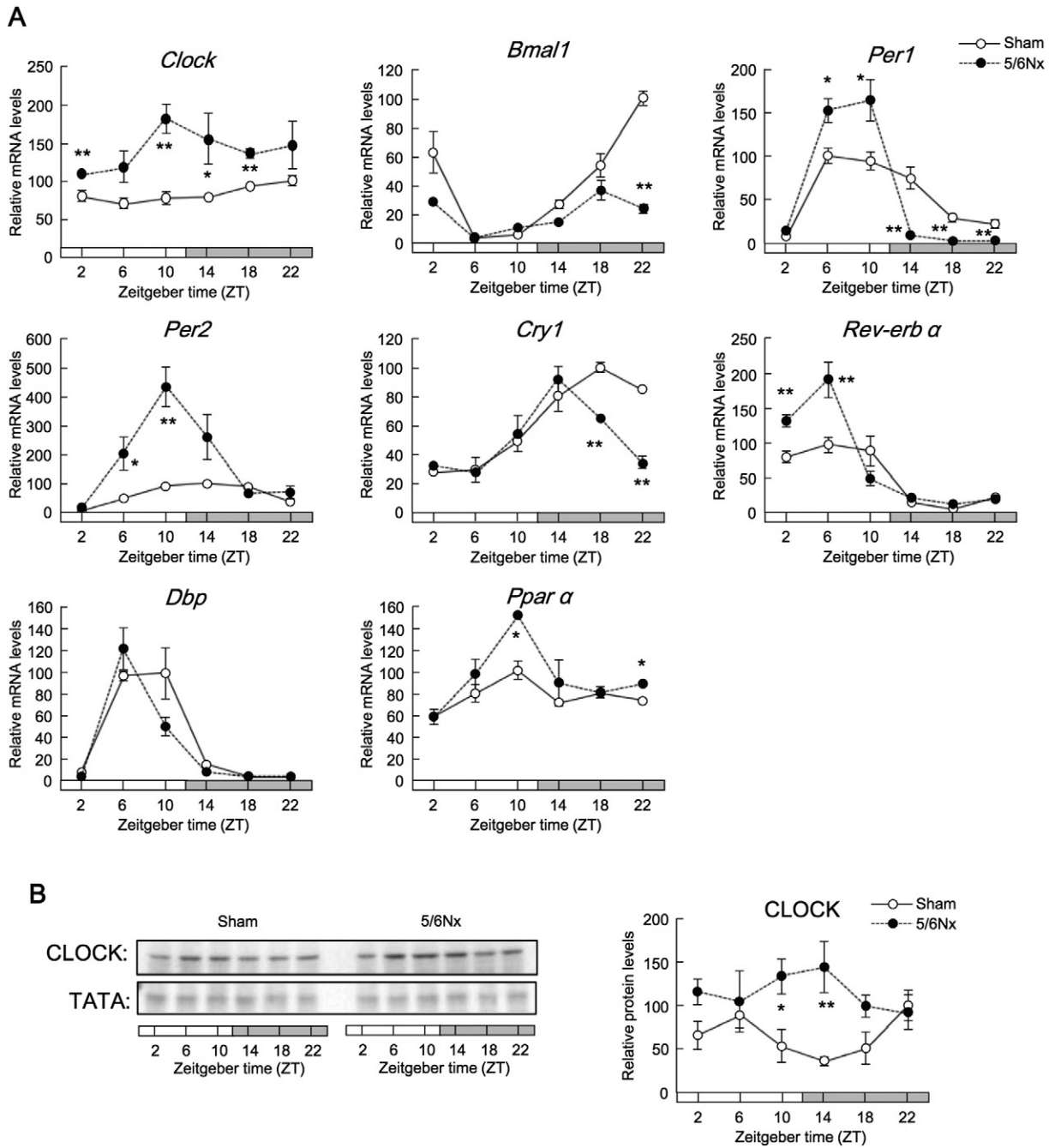
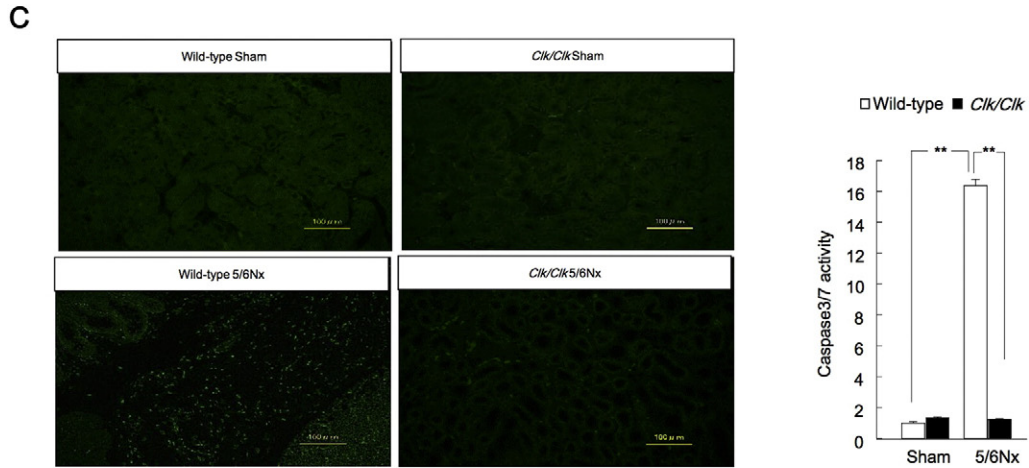
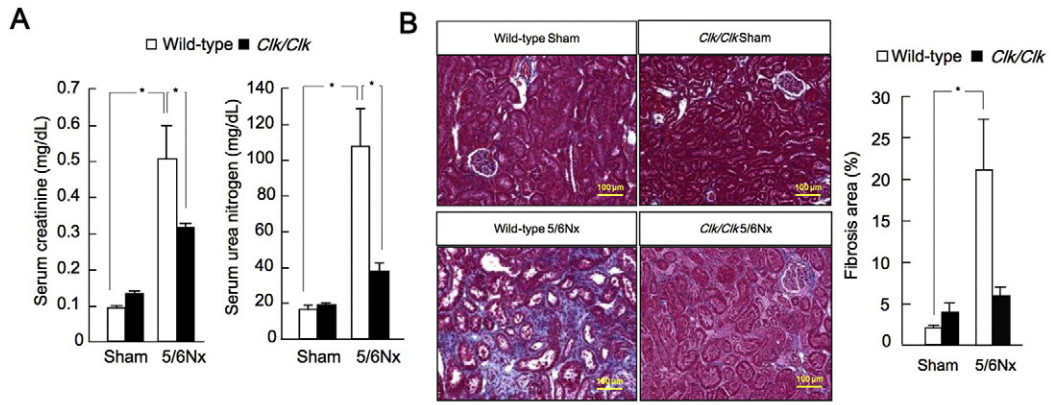


Fig. 1. Renal CLOCK expression was increased in wild-type 5/6Nx mice. (A) Temporal expression profiles of *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, *Nr1d1* (*Rev-erba*), *Dbp*, and *Ppara* mRNA in the kidneys of wild-type 5/6Nx and sham-operated mice. (B) Representative temporal CLOCK protein expression profiles. Values are the means \pm SEMs for triplicate experiments ($n = 3-6$). All experiments were performed three times. * $P < 0.05$, ** $P < 0.01$ vs. sham-operated mice at the same time point by Bonferroni/Dunn test.

(Fig. 4F). G0s2 may be involved in immunoregulation in autoimmune diseases, cancer, and metabolic syndrome (Nakamura et al., 2006; Yim et al., 2016; Yang et al., 2010; Zhang et al., 2014; Cristillo et al., 1997;

Russell and Forsdyke, 1991). Thus, these results suggested that G0s2 expression may be important for the p65-dependent transcription of inflammation-related genes in wild-type 5/6Nx mice.

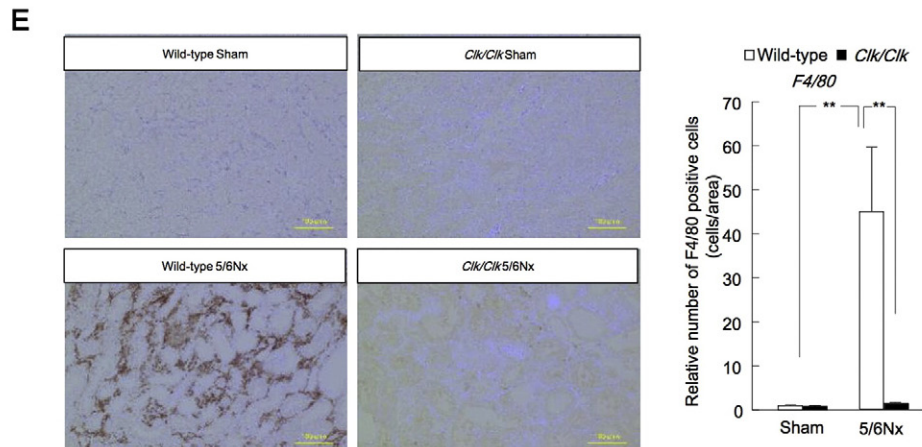
Fig. 2. The progression of renal fibrosis was suppressed in *Clk/Clk* 5/6Nx mice. (A) Serum creatinine and serum urea nitrogen (SUN) production in sham-operated and 5/6Nx wild-type or *Clk/Clk* mice. (B) Left: Masson's trichrome staining of tissue fibrosis (blue). Right: quantitative analysis of interstitial fibrosis by light microscopy in 5/6Nx and sham-operated mouse kidney tissues 8 weeks after the second operation in wild-type or *Clk/Clk* mice. (C) Left: apoptotic cells are identified by TUNEL staining (green). Right: Caspase-3/7 activity in sham-operated and 5/6Nx wild-type or *Clk/Clk* mouse kidneys 8 weeks after the second operation. (D) Functional analysis of gene expression in sham-operated and 5/6Nx wild-type and *Clk/Clk* mice based on functional annotation clustering by the Database for Annotation, Visualization, and Integrated Discovery (DAVID). (E) Left: F4/80 immunostaining (brown). Right: F4/80 protein expression profiles in the kidneys of sham-operated and 5/6Nx wild-type or *Clk/Clk* mice at Zeitgeber time (ZT) 6 after the second operation. Quantitative analysis of F4/80 staining in tissues from sham-operated and 5/6Nx wild-type or *Clk/Clk* mice by light microscopy 8 weeks after the second operation. Values are the means \pm SEMs for triplicate experiments ($n = 3-6$). * $P < 0.05$, ** $P < 0.01$ by Bonferroni/Dunn test.



D

Term	Count	<i>P-Value</i>	Benjamini
Immune system process	151	1.30E-30	2.80E-29
Response to stimulus	268	3.60E-15	3.90E-14
Biological adhesion	70	4.10E-06	3.00E-05
Locomotion	46	1.60E-04	9.00E-04
Multi-organism process	41	2.60E-03	1.10E-02
Localization	215	1.60E-02	5.70E-02
Death	49	3.30E-02	1.00E-01
Establishment of localization	185	6.90E-02	1.80E-01

*Wild-type 5/6Nx mice vs *C1k/C1k* 5/6Nx mice



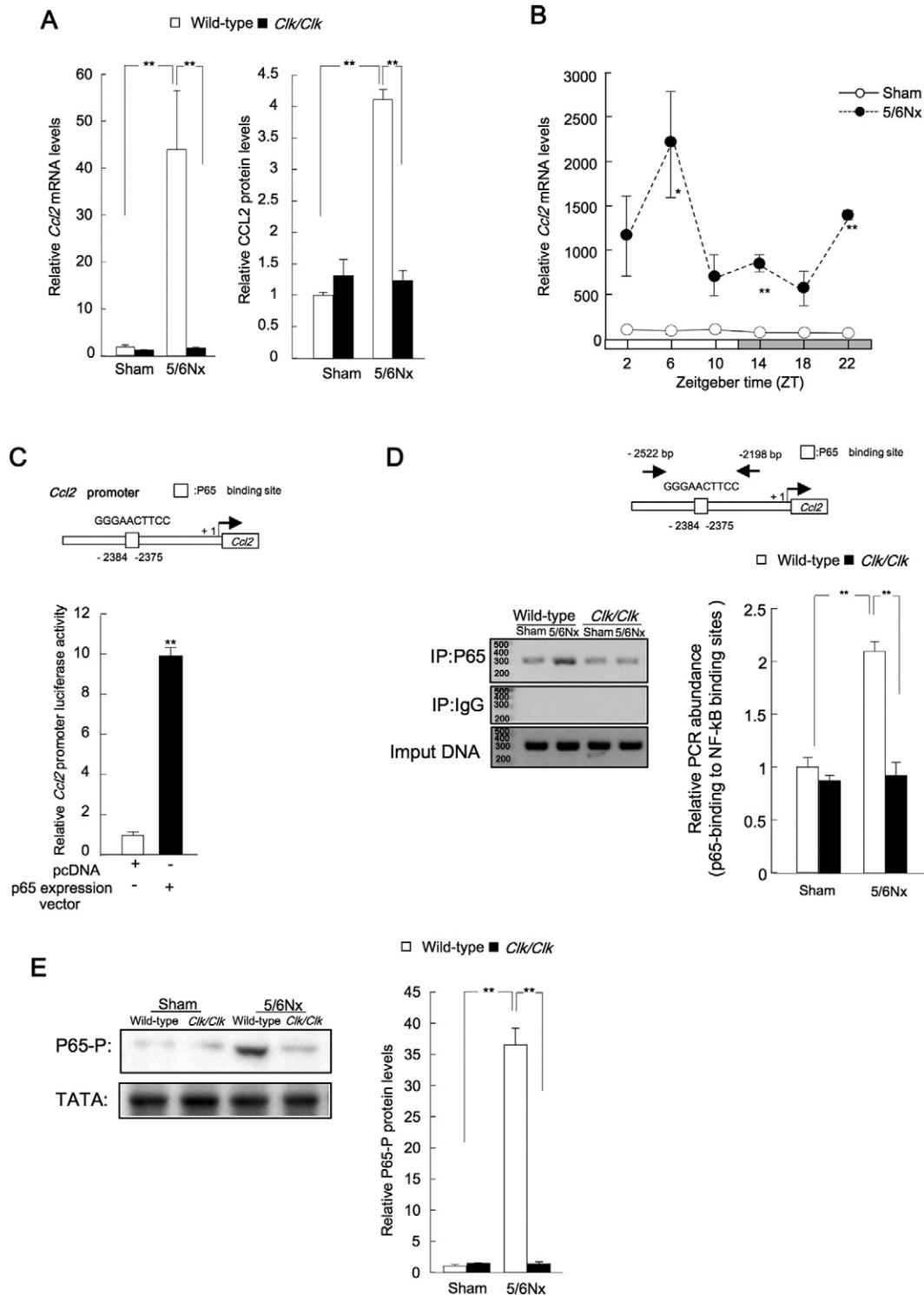


Fig. 3. Transcriptional control of renal *Ccl2* expression by p65. (A) Left: *Ccl2* mRNA expression in the kidneys of sham-operated and 5/6Nx wild-type or *Ctk/Ctk* mice. Right: *Ccl2* protein expression in the kidneys of sham-operated and 5/6Nx wild-type or *Ctk/Ctk* mice. (B) Temporal profiles of *Ccl2* mRNA expression in sham-operated and 5/6Nx mice. (C) Effects of p65 transfection on mouse *Ccl2*-luciferase reporter activity in NIH3T3 cells. ** $P < 0.01$ compared with the pcDNA3.1 group by Student's *t*-test. (D) Left: endogenous p65 protein binding to the *Ccl2* promoter in sham-operated or 5/6Nx kidney cells. (E) Renal p65 phosphorylation (Ser-536) in sham-operated and 5/6Nx wild-type or *Ctk/Ctk* mice at ZT6. Values are the means \pm SEMs for triplicate experiments ($n = 3-6$). ** $P < 0.01$, * $P < 0.05$ vs. sham controls at the corresponding time points by Bonferroni/Dunn test.

2.4. *G0s2* Expression Was Stimulated by the Signal Transducer and Activator of Transcription 5 (*Stat5*) Pathway in Wild-type 5/6Nx Mice

Our results described above showed that the temporal expression of *G0s2* mRNA and protein peaked during the light phase in 5/6Nx mice (Fig. 5A). The circadian oscillation of *G0s2* mRNA in sham mice was

not detected. Moreover, CKD has been shown to induce significant changes in the plasma levels of a myriad biomolecules, including increased levels of serum retinol (Gibbs et al., 2014, Spengler et al., 2012, Narasimamurthy et al., 2012). Transcription of the *G0s2* gene is controlled by the retinoic acid response element (Kitareewan et al., 2008), and *G0s2* expression exhibits a 24-h rhythm in the livers of

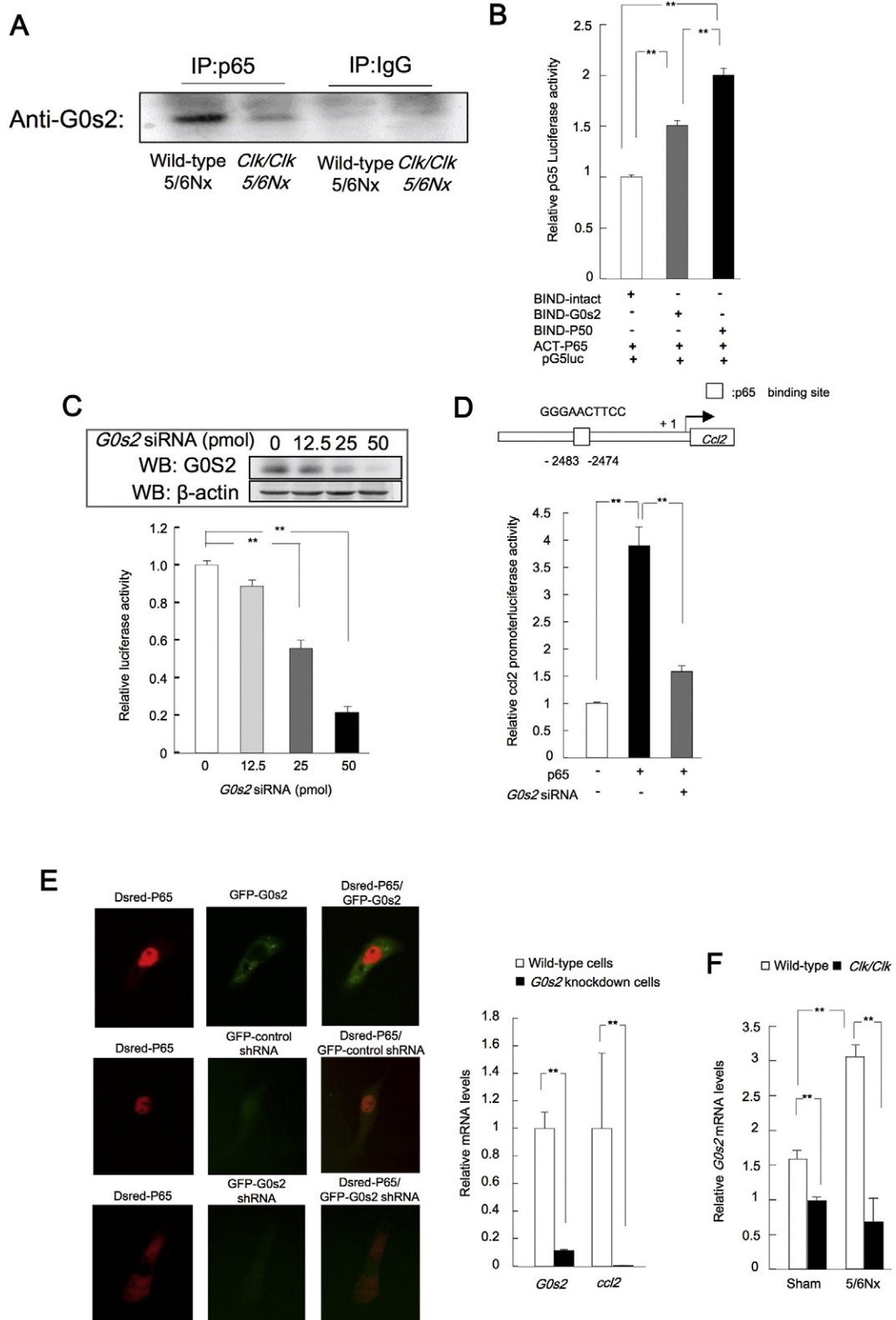


Fig. 4. G0s2 interacts with p65 to regulate *Ccl2* expression. (A) Immunoprecipitation was performed using anti-p65 antibodies in renal cell lysates from 5/6Nx wild-type or *Clk/Clk* mice. G0s2 expression in the immunoprecipitated samples was then analyzed by Western blotting. (B) The G0s2 and p65 interaction was assessed in NIH3T3 cells using mammalian two-hybrid assays. Cells cotransfected with the BIND (GAL4)-p50 and ACT (VP16)-p65 expression vectors and pG5luc (GAL4 binding site promoter) luciferase reporter plasmid. (C) Upper panels show G0s2 expression in knockdown cells. (D) Relative p65 binding element (NRE)-luciferase activity in NIH3T3 cells cotransfected with G0s2 siRNA and p65 binding element (NRE)-luciferase plasmids. (E) Relative luciferase activity in NIH3T3 cells cotransfected with the p65 expression and *Ccl2*-promoter luciferase reporter plasmids and G0s2 siRNA. (F) G0s2 protein and p65 protein localization in Hepa1-6 cells. Left: cells at 48 h after GFP-G0s2 and Dsred-p65 expression vector transfection. Green: G0s2 protein; red: p65 protein. Center: the influence of G0s2 knockdown on p65 protein localization. *G0s2* mRNA-GFP, green; p65 protein (red); colocalization (orange). Right bar graph: levels of *G0s2* and *Ccl2* mRNAs in *G0s2*-knockdown cells following transfection with the *G0s2* mRNA-GFP expression vector. (F) Renal *G0s2* expression in sham-operated and 5/6Nx wild-type or *Clk/Clk* mice at ZT6 after the second operation. Values are the means \pm SEMs for triplicate experiments ($n = 3-6$). ** $P < 0.01$, * $P < 0.05$ vs. controls at the corresponding time points by Bonferroni/Dunn test.

wild-type mice, in which *G0s2* expression is elevated with that in other organs (Jiang et al., 2014). Thus, we next examined the influence of retinol production on *G0s2* expression.

Our results showed that the rhythmic expression of *G0s2* mRNA was suppressed in livers from *Clk/Clk* mice (Fig. S3A). Moreover, transcription of the *G0s2* gene was regulated by retinoic acid receptor α

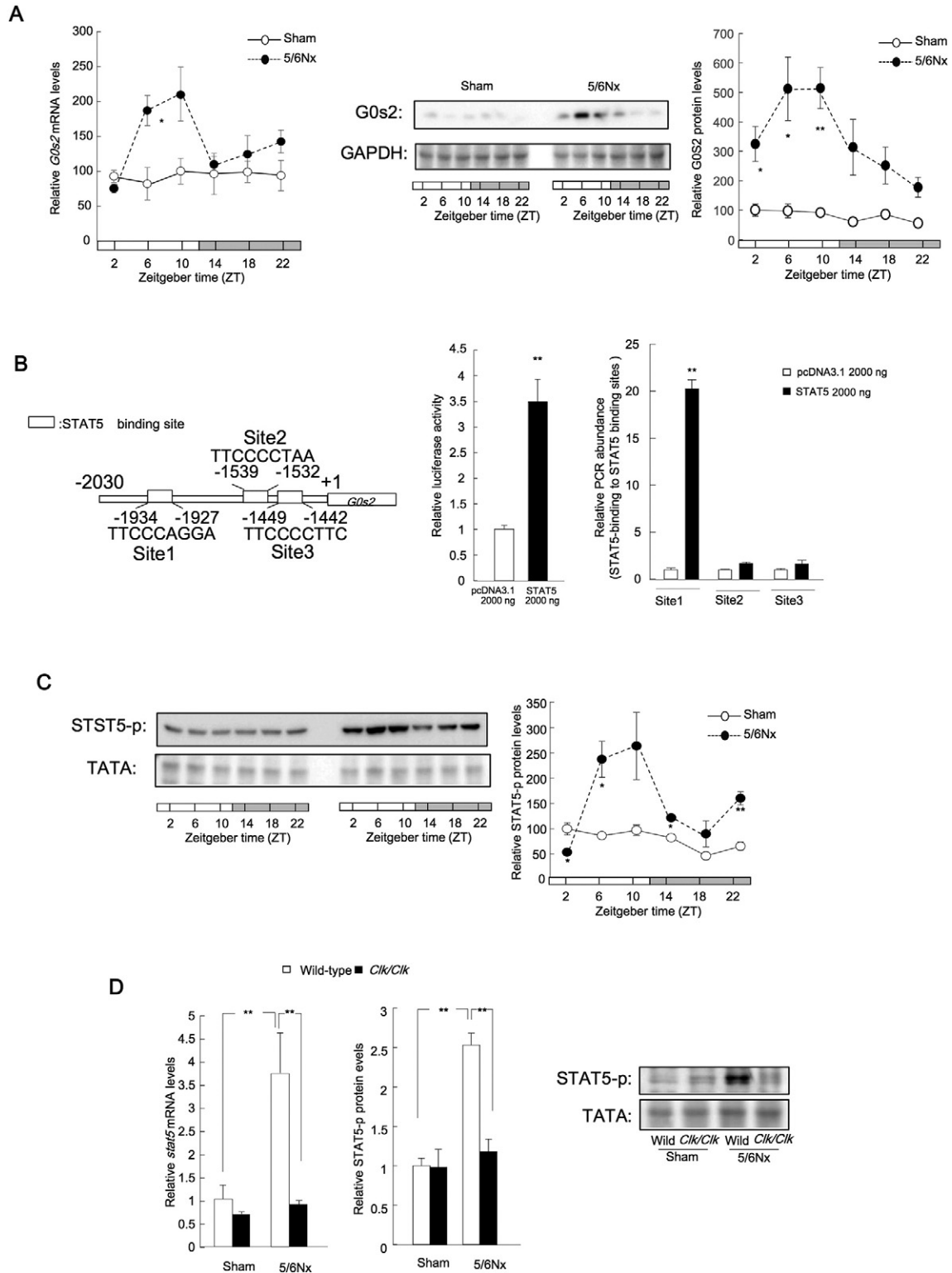


Fig. 5. Stat5 expression and activation in 5/6Nx mouse kidneys. (A) *G0s2* temporal mRNA (left) and protein expression profiles. Center: Representative protein expression data. Right: temporal Stat5 protein phosphorylation in the kidneys of wild-type sham-operated and 5/6Nx mice. (B) Right: *G0s2* promoter activity in response to Stat5 overexpression. $**P < 0.01$ compared with the pcDNA3.1 vector control group by Student's *t*-test. Left: Stat5 protein binding to the *G0s2* promoter in Stat5-transfected NIH3T3 cells. $**P < 0.01$ compared with pcDNA3.1 in site1, site2, and site3 groups by Bonferroni/Dunn test. (C) Left: representative protein data. Right: temporal Stat5 phosphorylation (Tyr694/699) profiles in the kidneys of wild-type sham-operated and 5/6Nx mice. (D) Left: *Stat5a* mRNA expression in sham-operated and 5/6Nx wild-type or *Clk/Clk* mice. Right: Stat5 phosphorylation (Tyr694/699) in sham-operated and 5/6Nx wild-type or *Clk/Clk* mice. $**P < 0.01$, $*P < 0.05$ vs. controls at the corresponding time points by Bonferroni/Dunn test. Values are the means \pm SEMs for triplicate experiments ($n = 3-6$).

(*Rarα*) and retinoic acid in our mouse model (Fig. S3B–G). Serum retinol levels in wild-type mice were also markedly increased 8 weeks after the 5/6Nx operation (Fig. S1F). Notably, renal *Rarα* mRNA levels were also markedly decreased in wild-type 5/6Nx mice at 8 weeks after the 5/6Nx operation (Fig. S1G).

The *G0s2* 5'-flanking region (from –2030 bp to –1057 bp) was important for promoter activity (Fig. S3D). Moreover, this region appeared to contain a binding site for Stat5 (Fig. 5B), which has various roles in inflammation and immunity (Yu et al., 2009). Consistent with this, cotransfection with the mouse *G0s2* luciferase reporter and *Stat5* expression construct led to significantly increased transcriptional activity (Fig. 5B). Chromatin immunoprecipitation revealed that Stat5 may bind to Stat5 binding site 1 in the *G0s2* 5'-flanking region (Fig. 5B). The phosphorylation of Stat5 (Stat5-p) was markedly increased in the kidneys of wild-type 5/6Nx mice (Fig. 5C), and the expressions of *Stat5* mRNA and levels of Stat5-p protein were lower in the kidneys of *Clk/Clk* 5/6Nx mice than in those of wild-type 5/6Nx mice (Fig. 5D).

2.5. Treatment With *G0s2* siRNA or a Novel Transcriptional Inhibitor of *G0s2* Ameliorated Renal Dysfunction in Wild-type 5/6Nx Mice

Renal *G0s2* expression may be important for renal inflammation mediated by p65 in mice with CKD. Thus, we carried out knockdown of *G0s2* in the kidneys of 5/6Nx mice kidney (Fig. 6). Treatment of wild-type 5/6Nx mice with control or *G0s2* miRNA expression plasmids resulted in decreased levels of *G0s2* and *Ccl2* mRNA in the kidneys

(Fig. 6A). The phosphorylation of nuclear p65 protein was decreased in wild-type 5/6Nx mice treated with *G0s2* miRNA (Fig. 6A), and SUN concentrations decreased in 5/6Nx mice treated with *G0s2* miRNA (Fig. 6B). Finally, the F4/80-positive area and F4/80 protein levels were decreased in 5/6Nx mice treated with *G0s2* miRNA (Fig. 6C). These results revealed that knockdown of *G0s2* ameliorated renal dysfunction in our mouse model of CKD.

In addition, we carried out high-throughput screening of 9600 small compounds to identify novel transcriptional inhibitors of *G0s2* (Figs. S4, S5). NS-3-008 inhibited the transcription of *G0s2* with a half-maximal inhibitory concentration (IC₅₀) of 2.25 μM (Fig. S6). Moreover, treatment of wild-type 5/6Nx mice with NS-3-008 (5 mg/kg, P.O.) resulted in decreased levels of *G0s2* and *Ccl2* mRNA in the kidneys (Fig. 7A). The phosphorylation of Stat5 and p65 protein was decreased in wild-type 5/6Nx mice treated with NS-3-008 (Fig. 7B), and SUN concentrations and renal caspase 3/7 activity decreased in 5/6Nx mice treated with NS-03-08 (Fig. 7). Finally, the F4/80-positive area and F4/80 protein levels were decreased in 5/6Nx mice treated with NS-3-008 (Fig. 7D). Interestingly, NS-3-008 bound to Hsd17b4 (Fig. S7A–D); knockdown of Hsd17b4 decreased *G0s2* mRNA levels, whereas overexpression of Hsd17b4 induced *G0s2* mRNA expression (Fig. S8A, 8B). The inhibitory effects of NS-3-008 were blocked by knockdown of Hsd17b4 or deletion of the Stat5 binding site in the *G0s2* promoter (Fig. S8A, 8C). NS-3-008 decreased the nuclear phosphorylation of Stat5 (Fig. S8D). Thus, these results revealed that *G0s2* inhibition by the novel compound NS-3-008 ameliorated renal dysfunction in our mouse model of CKD.

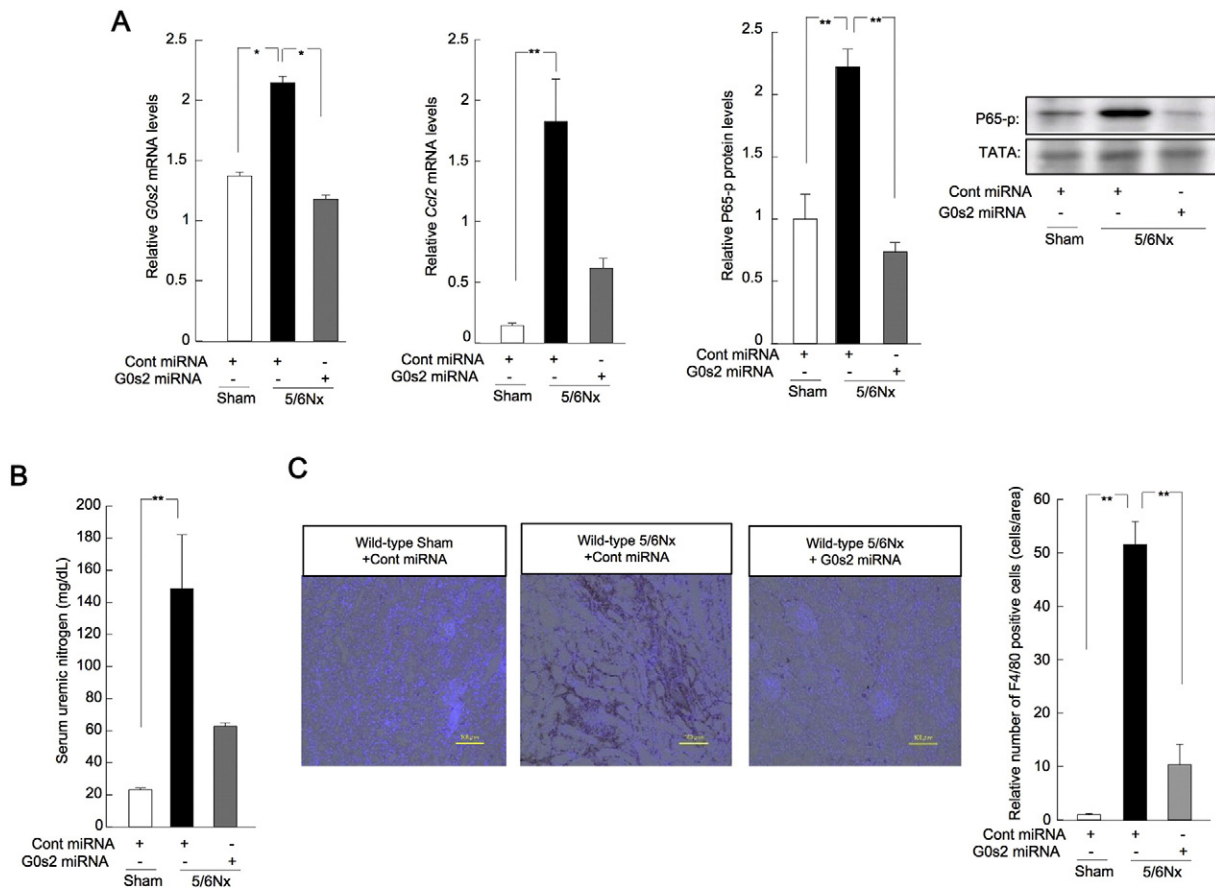


Fig. 6. *G0s2* knockdown ameliorated renal inflammation in CKD. (A) Renal expression profiles for *G0s2* and *Ccl2* mRNA and p65 protein phosphorylation in wild-type sham-operated and 5/6Nx mice treated with control or *G0s2* miRNA expression plasmids (50 μg/μL/animal, two times/week, i.v.) from 4 to 8 weeks after the second operation. **P* < 0.05, ***P* < 0.01 by Bonferroni/Dunn test. (B) SUN levels in wild-type sham-operated and 5/6Nx mice treated with *G0s2* or control miRNA expression plasmids (50 μg/μL/animal, two times/week, i.v.) from 4 to 8 weeks after the second operation. (C) Left: F4/80 immunostaining (brown) in wild-type sham-operated and 5/6Nx mice treated with *G0s2* or control miRNA expression plasmids (50 μg/μL/animal, two times/week, i.v.) from 4 to 8 weeks after the second operation. Right: quantitative analysis of F4/80 staining by light microscopy. **P* < 0.05, ***P* < 0.01 by Bonferroni/Dunn test. Values are the means ± SEMs for triplicate experiments (*n* = 3–6).

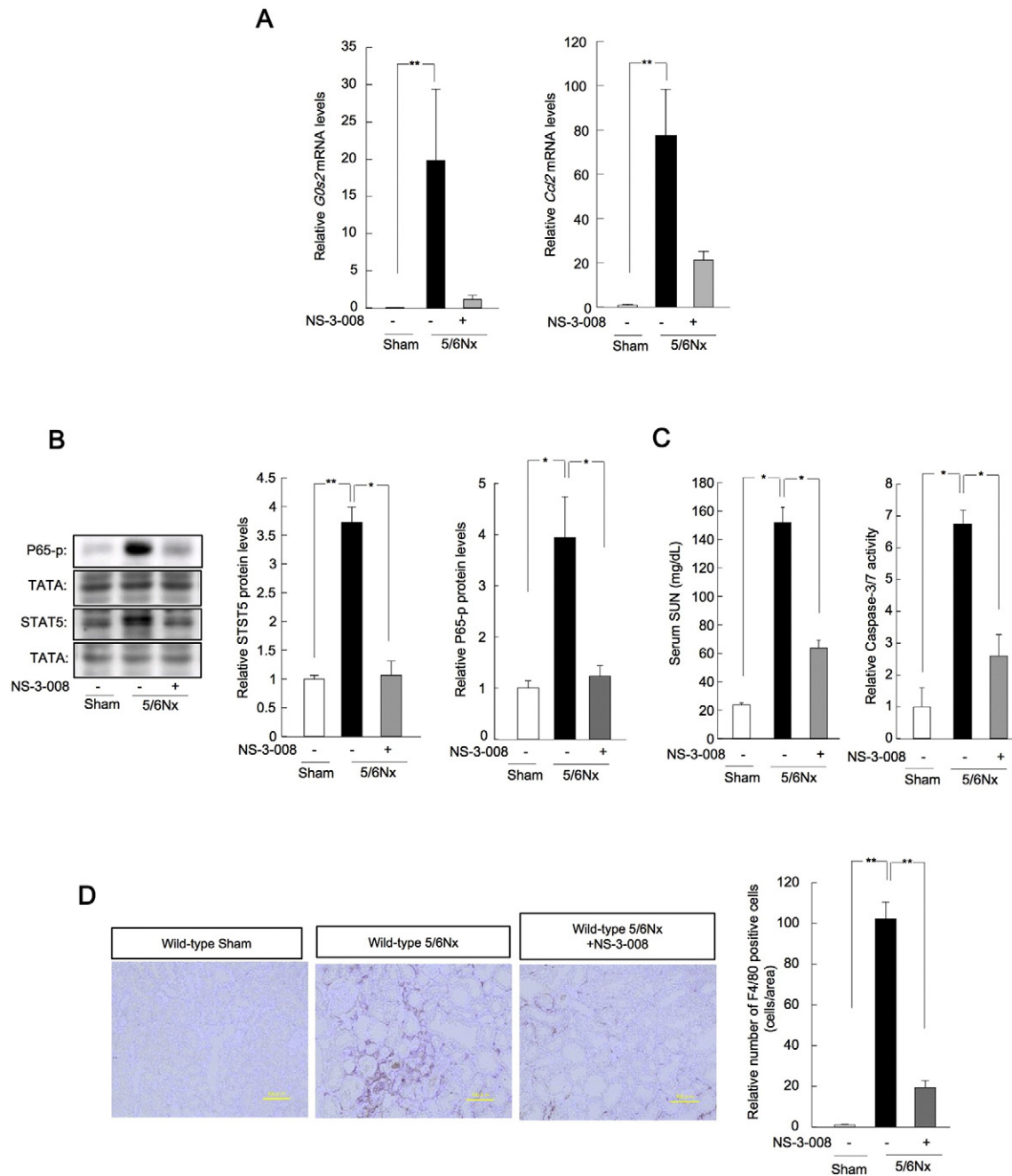


Fig. 7. Treatment with the G0s2 inhibitor NS-3-008 ameliorated renal inflammation in CKD. (A) Renal expression profiles of *G0s2* and *Ccl2* mRNA in wild-type sham-operated and 5/6Nx mice treated with NS-3-008 (5 mg/kg oral [PO]) from 4 to 8 weeks after the second operation. (B) Profiles of Stat5 phosphorylation (left) and p65 phosphorylation (right) in the kidneys of wild-type sham-operated and 5/6Nx mice treated with NS-3-008 (5 mg/kg, PO) 4 to 8 weeks after the second operation. (C) SUN (left) and renal caspase 3/7 activity (right) in wild-type sham-operated and 5/6Nx mice treated with NS-3-008 (5 mg/kg, PO) 4 to 8 weeks after the second operation. (D) Left: F4/80 immunostaining (brown) in wild-type sham-operated and 5/6Nx mice treated with NS-3-008 (5 mg/kg, PO) from 4 to 8 weeks after the second operation. Right: quantitative analysis of F4/80 staining by light microscopy. * $P < 0.05$, ** $P < 0.01$ by Bonferroni/Dunn test. Values are the means \pm SEMs for triplicate experiments ($n = 3-6$).

3. Discussion

In patients with CKD, the circadian rhythm and serum hormone levels are dysregulated (Niemczyk et al., 2006). Moreover, inflammation, which can be mediated by the molecular clock (Hashiramoto et al., 2010), is related to the pathology of CKD (Yu et al., 2014). Thus, in this study, we aimed to examine the relationships between circadian rhythms and CKD pathology. To this end, we investigated the role of G0s2 in CKD. Our data indicated that G0s2 may aggravate renal dysfunction in the context of CKD through mechanisms involving increased renal expression of the *Clock* gene. Subsequent induction of G0s2

expression by Stat5 in the kidney may further exacerbate renal dysfunction by inducing inflammation. These data, combined with our identification of a novel G0s2 inhibitor, suggest that G0s2 may be a promising therapeutic target in the treatment of CKD.

The molecular clock system is important for renal function in wild-type mice (Zuber et al., 2009; Nikolaeva et al., 2012). In this study, we found that the expression of renal *clock* genes was altered in kidneys from 5/6Nx mice, suggesting that increased expression of CLOCK, which is important for the core loop in the circadian clock system and is involved in inflammation, may affect the expression of other clock genes. Interestingly, we also found that mutations in the *Clock* gene

ameliorated renal fibrosis, apoptosis, and inflammatory reactions in mice with CKD. Circadian proteins are known to regulate both blood pressure and kidney function (Curtis et al., 2007; Stow et al., 2012; Doi et al., 2010). In this study, we found that AT II levels in *Clk/Clk* mice were higher than those in wild-type mice (Fig. S1D). In addition, food intake in mice affects AT II levels, which are also highly dependent on salt intake and renal perfusion pressure. No significant changes were observed in the amount of food and water intake between wild-type and *Clk/Clk* mice in this study (Fig. S1E). In addition, angiotensin-converting enzyme (ACE) levels in the lungs at ZT8 were higher in intact *Clk/Clk* mice than in intact wild-type mice (Fig. S1E). In addition, there were no differences in mean blood pressure (MBP) increases between 5/6Nx wild-type mice and 5/6Nx *Clk/Clk* mice (Fig. S1E). This may have contributed to the clock-related pathology observed in our model. Renal fibrosis, apoptosis, and inflammation were ameliorated in *Clk/Clk* mice following the 5/6Nx procedure. These data provided insights into the mechanisms of renal inflammatory reactions induced by *Clock*, directing our further studies.

Ccl2 is expressed at sites of injury and inflammation to direct macrophage recruitment; it binds with CCR2 to promote macrophage adhesion and chemotaxis to disease sites. Increased tubular expression of CCL2 is present in human progressive renal disease, and interstitial inflammatory infiltrates express CCR2 (Eardley et al., 2006). Although Ccl2 expression was increased in wild-type 5/6Nx mice, this phenotype was not observed in *Clk/Clk* mice. Moreover, *Ccl2* mRNA expression varied in a time-dependent manner in wild-type 5/6Nx mice and was regulated by p65. The expression of *Ccl2* is activated by binding of transcription factors to NF- κ B elements in the *Ccl2* promoter (Deng et al., 2013). Although the CLOCK protein enhances transcription mediated by NF- κ B, CLOCK Δ 19 protein does not affect NF- κ B-dependent transcription (Spengler et al., 2012), and the mechanism by which NF- κ B regulates transcription in *Clk/Clk* mice remains unclear. In our study, we showed that G0s2, which is involved in the transition from G₀ phase to G₁ phase and is mediated by cyclosporine A (Cristillo et al., 1997), interacted with p65 protein in the kidneys of wild-type 5/6Nx mice. Nuclear accumulation of p65 protein was suppressed in G0s2-knockdown cells. Therefore, G0s2 protein may promote the nuclear localization of p65 protein. Moreover, knockdown of *G0s2* blocked the transactivation of renal *Ccl2* expression and the progression of renal dysfunction. Knockdown of *G0s2* also reduced the number of F4/80-positive cells. These effects were also identified in a diethylnitrosamine (DEN) hepatitis mouse model (Fig. S2A–C). *G0s2* mRNA expression was also decreased in the kidneys of *Clk/Clk* 5/6Nx mice, suggesting that G0s2 may be a novel activator of inflammatory reactions via p65 in 5/6Nx mice. Further analysis showed that repression of G0s2 expression in *Clk/Clk* 5/6Nx mice may be related to the lack of induction of Stat5, which has been shown to be directly regulated by the molecular clock in humans and rodents (Mavroudis et al., 2013). Thus, these data provided important insights into the regulation of inflammation and CKD by G0s2 and related pathways.

Chemical screening is a powerful tool to investigate various biological pathways (Pieper et al., 2010). Several recent studies have employed cell-based chemical screening strategies to identify small molecules with biological activities (Chen et al., 2012), providing evidence supporting the use of chemical screening approaches. In this study, we performed a high-throughput chemical screen of 9600 synthetic small molecules from a chemical library (Open Innovation Centre for Drug Discovery, The University of Tokyo) to identify transcriptional inhibitors of G0s2. Administration of NS-3-008 inhibited the expression of G0s2 in healthy mice liver. In contrast, administration of NS-3-008 did not affect the MBP in wild-type sham and 5/6Nx mice. We identified NS-3-008 as a novel G0s2 inhibitor and showed that administration of this inhibitor ameliorated renal dysfunction in wild-type 5/6Nx mice. In addition, the lipopolysaccharide (LPS)- or tumor necrosis factor alpha (TNF α)-dependent transactivation of *G0s2* and *Ccl2* was inhibited by administration of NS-3-008 (Fig. S10A–E). Moreover, NS-3-008 bound to

Hsd17b4 to block G0s2 expression. Similar studies have shown that chroceceptin affects the transcription by Stats via binding to Hsd17b4 (Choi et al., 2006). Thus, our experiments identified a novel inhibitor of G0s2 and provided evidence for the mechanism of inhibition by this novel inhibitor, supporting the potential utility of G0s2 inhibition in the treatment of CKD.

In conclusion, our results showed that altered expression of molecular components of the clock signaling machinery was associated with the degree of CKD pathology. Environmental factors, such as light exposure, feeding habits, and working conditions, in human societies are complex; although these environmental conditions may also contribute to the observed effects, our data clearly indicated that inhibition of G0s2 function may have therapeutic benefits in the treatment of CKD.

4. Experimental Procedures

4.1. Mouse Experiments

Male ICR mice (Charles River Laboratory Japan, Inc.; Yokohama, Japan) were housed in a light-controlled room (lights on from ZT0 to ZT12) at 24 \pm 1 °C and 60% \pm 10% humidity, with food and water available ad libitum. Mice were synchronized to the light/dark cycle for 2 weeks before surgery. *Clock* mutant mice (C57BL/6J-Clock^{ml/jt/j}) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and backcrossed to wild-type Jcl: ICR mice for more than eight generations to enhance breeding and offspring care. Experiments were performed using wild-type ICR, homogeneous, and *Clock*-mutant mice. CKD was induced in 6-week-old mice by 5/6Nx two-stage surgery under isoflurane anesthesia (Baxter, Deerfield, IL, USA). During the first procedure, two-thirds of the left kidney was removed by cutting off both poles. Seven days later, the right kidney was completely removed. After the operations, mice were housed for 8 weeks to allow CKD development. Sham mice were subjected to laparotomy on the same days as the 5/6Nx mouse surgeries. For G0s2 inhibitor NS-3-008 treatment, mice were administered NS-3-008 (5 mg/kg body weight) or vehicle control in the drinking water from weeks 4 to 8 following the second 5/6Nx operation. All animals used in this study were treated in accordance with the guidelines stipulated by the Animal Care and Use Committee of Kyushu University.

4.2. Cell Culture

NIH3T3 cells (supplied by the Cell Resource Center for Biochemical Research, Tohoku University) were grown at 37 °C in a humidified environment (5% CO₂, 95% air) in Dulbecco's modified Eagle's medium (Sigma-Aldrich; St. Louis, MO, USA) containing 10% fetal bovine serum (SAFC Biosciences; Kansas City, MO, USA), 0.1 μ M insulin, 0.1 μ M dexamethasone, and 2% penicillin/streptomycin.

4.3. Statistical Analysis

All data represent the mean \pm standard error of the mean (SEM) of at least three individual experiments. Differences were assessed using Student's *t* or analysis of variance (ANOVA) with Bonferroni/Dunn post hoc testing as indicated. *P* > 0.05 was considered statistically significant.

Author Contributions

N. M. and S.O. participated in the design of the study, conducted the experiments, analyzed the data, and drafted the manuscript. E. I., K.K., M.W., N.S., T.Y., K.H., T.Y., Y.Y., M.M., T.O., K.H., A.T., H.K., H.I., K.T., K.I., H.I., Y.F., T.A., H.A., M.T., K.I., T.N., A.O. and S.K. conducted the experiments A.D. and K.Y. analyzed microarray data. All authors have read and approved the final manuscript.

Competing Financial Interests

The authors have no competing interests to declare.

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Appendix A. Supplementary Data

Additional methods and reagents are described in the supplementary methods available at <http://www.ebiomedicine.com/>. Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.ebiom.2016.10.008>.

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