

THE ROLE OF PER1 AND EDN1-AS IN MINERALOCORTICOID AND SALT-
SENSITIVE HYPERTENSION

By

KRISTEN SOLOCINSKI

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2017

© 2017 Kristen Solocinski

To My Family and Friends

ACKNOWLEDGMENTS

First and foremost, I have to thank my mentor, Dr. Michelle Gumz, for all of her support and help. When I first came to UF, I was so nervous about only having three rotations' time to choose a mentor but after my first one with Michelle, I was ready to skip the others. Over the years, Michelle has pushed me to write articles, present posters and submit abstracts. Indubitably, I would not have accomplished half of what is on my CV without her guidance and the least aggressive push to write imaginable. I also thank Michelle for her wonderfully bubbly personality which makes it very hard for people to have anything bad to say about her at all. It is not only great to work for someone like that, but it is great for me in terms of networking because everyone is happy to talk to her. It is also a big bonus to have a boss who likes the same great TV shows as you. I thank Michelle for being not only a mentor but a mentor/friend/mom hybrid over the years.

In a similar vein, I have to thank Dr. Brian Cain for his mentorship and role as my lab grandpa as well. I have lost count of the times he has explained the antisense of the antisense of the reverse compliment to me and I'm still constantly confused by it. Dr. Cain has always been such a welcoming, generous person, going so far as to invite me to his home for Thanksgiving and Christmas. He has also given up so much time in order to offer thoughtful criticisms of countless talks, posters and papers. I thank both Dr. Gumz and Dr. Cain for always having an open door whenever I needed to talk or troubleshoot. I am also extremely grateful for the glowing letters of recommendation you have written for me.

I also need to express my gratitude to my other committee members. I thank Dr. Peter Sayeski for always responding to my emails and writing me great letters of

recommendation. He has always listened thoughtfully to my presentations and given me helpful critiques and recommendations. I also thank Dr. Debbie Scheuer for her assistance on my committee. I apologize for never doing as much brain-related work as I had hoped (and possibly promised). Nevertheless, Dr. Scheuer was always helpful in her thoughtful critiques and suggestions for future experiments. I thank Dr. Jorg Bungert for his role on my committee. He has always kept his thoughts and critiques short and sweet which I appreciate. He has also offered some very insightful experiments to confirm that what I have seen with my antisense is real. This is greatly valuable to me because I always want to be sure of my results. Finally, I thank Dr. Kevin Brown as an honorary committee member. In addition to an incredible amount of help with my CRISPR/Cas9 project, Dr. Brown has given me countless hours of time discussing anything from job opportunities to science to politics. To all of my committee members, I cannot thank you enough for your valuable time and input over the past four years.

In addition to my committee mentors, I have had a number of non-official mentors I would like to thank as well. I thank Dr. Charles Wingo for his helpful input at lab meetings as well as the thoughtful conversations we have shared about science. I owe him many thanks for his assistance with mice and his allowance to use his facilities at the VA. I also have to thank him for his wonderful fashion sense so I have a new bow tie to look forward to at our meetings. Finally, I have to thank Dr. Wingo for his excellent taste in food, especially Cubans (sandwiches, not cigars), which I have been treated to numerous times. I also have to give great thanks to I. Jeanette Lynch for the countless hours she has spent teaching and explaining any aspect of physiology which I was curious about. Any time I would drop in to her lab, she always had time to talk or help

me with whatever I needed. She has also been invaluable with respect to our mouse studies, as all of the telemetry surgeries on which this dissertation relies so heavily were done by her. Lastly, I thank Jeanette for being a good friend and person to talk to. Although she was not with the Wingo lab for a very long time, I also thank Dr. Luciana Morla for her time and contributions to all of our labs. She always had a funny story to brighten the day and made some good macaroons.

For her guidance with immunohistochemistry and perfusion-fixation, I thank Dr. Jill Verlander. Her protocols have been the most reliable ones I have used in this lab and her attention to detail is no doubt what gives us such great results. Her tough yet accurate criticisms have given me extreme faith in the immunohistochemistry work I have done here. I also thank Dr. Rajesh Mohandas for his thoughtfulness about my work and his great personality. He literally always has something funny to say to brighten my day and I could not have picked a better person to have in the lab. I also have to thank the entire Nephrology Department for having such great people to talk to – about science or other topics. I cannot thank Shiyu Li enough, whose sweet smile and simple wave brightens the day with more force than our big centrifuge generates.

I could not have been half as successful without the guidance of Dr. Jacob Richards and Dr. Sarah Barilovits. Jacob started me off in the lab and showed me the importance of maintaining a lab notebook and clearly labeled samples throughout one's tenure in a lab. We also shared many insightful conversations as well as laughs, for which I am most indebted. While I did not spend much time with Sarah, she started off the EDN1-AS project for me and really helped me to get on my way with it. She also produced the first evidence of the antisense in multiple cell lines, which I use as opening

data in all of my presentations. Dr. Lauren Douma has also been a great source of knowledge and laughter, for which I thank her. She has been invaluable when it comes to RNA isolation and has golden hands which produce the most beautiful results. I also cannot overstate how much I appreciate her help with the undergraduate students. Additionally, I thank Thomas Cheng for his help in the lab with mice, comedy and foreign goodies from his many travels. When Thomas left for his many trips, he always made sure that all the orders were in and things were in place before he left. He also made sure to send post cards which decorate the lab.

Undergraduate students all deserve my gratitude as well. I thank Sean All and Alex Diaz who were always willing to help with anything at all and made great contributions to the lab in the form of data and laughter. I thank Kareme Alder for his professional attitude and dedication to work at all times. I also thank him for immersing me in the Jamaican culture, broadening my knowledge of duppies, Tevin and rolling calf. I thank Ravi Patel for his countless efforts to produce the perfect Western blot and aid in troubleshooting the ECL reagents. I thank Amber Miller for her continuation of Ravi's work on Western blots and her willingness to help with anything I needed. I thank Sarah Masten for continuing to return to lab after being given hundreds of tubes of urine to analyze. I don't think many people would handle that much urine without monetary compensation. Finally, although technically not an undergraduate in the lab anymore, I thank Maggie Holzworth for her obsessive efforts to clone an EDN1-AS plasmid and continual effort where most others would have given up. I thank her for her enthusiasm to continue my work on the EDN1-AS project and give it just as much love as I did. I

also thank her for being a great friend outside of lab as well and watching my dogs which ultimately brought even more urine into her life.

Finally, I have to thank all of the people outside of lab who have helped me. I thank all of my ex-boyfriends for showing me just how much I can put up with and still be successful. I thank Natalie Ross for always being there for me even though we are over a thousand miles apart. I thank my cousins, Mike Lawrence, Laura Bush and Billy Bush for being there when I need it and making time to see me during the two times that I came home during my studies. I am so glad to be part of the CCC. I thank Jason “Ratface” Solocinski for always being so encouraging and never having a single mean thing to say to me. He is the best baby brother a girl could ask for. I thank my mom for raising me as a parent, not a friend, so I became independent and able to handle stressful situations, which is graduate school in a nut shell. I also thank her for her continual support, be it monetary, emotional or otherwise.

I don’t consider myself an overly emotionally expressive person, so I hope this has done justice to just how grateful I am to all of you who have helped me along the way.

TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS.....	4
LIST OF TABLES.....	12
LIST OF FIGURES.....	13
LIST OF ABBREVIATIONS.....	15
ABSTRACT.....	18
CHAPTER	
1 INTRODUCTION.....	20
Background.....	20
The Kidney.....	23
Hormones and the Kidney Clock.....	25
The Kidney Clock.....	29
PER1 Action in the Kidney.....	34
Development.....	38
Regulation of the Kidney Clock.....	41
Potassium.....	43
Clinical Implications.....	45
2 TRANSCRIPTIONAL REGULATION OF NHE3 AND SGLT1 BY THE CIRCADIAN CLOCK PROTEIN PER1 IN PT CELLS.....	51
Background.....	51
Methods.....	53
Animals.....	53
Cell Culture.....	54
RNA Isolation and quantitative real-time PCR.....	54
Membrane Protein Analysis, Nuclear Protein Isolation, and Western Blot Analysis.....	54
hnRNA Analysis.....	55
Chromatin Immunoprecipitation (ChIP).....	56
Ouabain-sensitive ⁸⁶ Rb uptake.....	56
Statistical Analysis.....	57
Results.....	57
Discussion.....	60
3 DOCP-SALT TREATMENT LEADS TO NONDIPPING HYPERTENSION IN PER1 KNOCKOUT MICE.....	73

Background.....	73
Methods.....	75
Mice.....	75
Tissue dissection and quantitative real time RT-PCR (qPCR).....	76
Flame Photometry.....	76
Aldosterone ELISA.....	76
Statistics.....	76
Results.....	76
Discussion.....	80
4 LOSS OF THE CIRCADIAN CLOCK PROTEIN PER1 DOES NOT LEAD TO NONDIPPING IN FEMALE C57BL/6 MICE.....	91
Background.....	91
Methods.....	93
Mice.....	93
Tissue dissection and Real-Time PCR.....	93
Flame Photometry.....	93
Aldosterone ELISA.....	93
Statistics.....	94
Results.....	94
Discussion.....	96
5 IDENTIFICATION AND INITIAL CHARACTERIZATION OF A lncRNA ANTISENSE TO THE EDN1 GENE: EDN1-AS.....	105
Background.....	105
Methods.....	108
Exploration of EDN1 Chromatin State and Regulation with the UCSC	
Genome Browser.....	108
Strand-Specific Primer Design.....	109
RNA Isolation and DNase Treatment.....	109
cDNA Conversion and PCR.....	109
Cell Culture.....	110
Aldosterone Treatment.....	110
Dexamethasone Treatment.....	110
Chromatin Immunoprecipitation.....	111
Mice.....	111
CRISPR/Cas9.....	111
gRNA Design.....	111
Creation of the gRNA Plasmid.....	112
Puromycin Kill Curve.....	112
Creation of Virus and Infection of Cells.....	112
96 well cloning.....	113
10 cm dish cloning.....	113
Nomenclature.....	114
CRISPR Genotyping Primer Design.....	114

Sequencing	114
Mycoplasma Decontamination and Detection	114
ET-1 ELISA	115
Results.....	115
Discussion	118
6 CONCLUSIONS AND FUTURE DIRECTIONS	136
Conclusions	136
Future Directions	138
LIST OF REFERENCES	141
BIOGRAPHICAL SKETCH.....	168

LIST OF TABLES

<u>Table</u>		<u>page</u>
2-1	Sequences and exon numbers.....	64
2-2	Primer sets for each gene.	64
3-1	Baseline characteristics of WT and <i>Per1</i> KO mice.	89
3-2	Cosinor analysis of HS/DOCP effects on MAP in WT and <i>Per1</i> KO mice.	90
3-3	Analysis of MAP by JTK Cycle.	90
3-4	Analysis of MAP by Cosinor Analysis.	90
4-1	Cosinor analysis of mean arterial BP on different diets.	104
4-2	Baseline Characteristics of WT and KO Mice.	104
5-1	Cut size and expected amplicon size for all gRNA combinations.	135

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
1-1 Diagram of Two Nephrons and Representative Cell Types.....	48
1-2 Circadian Expression of Core Clock Genes in the Kidney.....	50
2-1 Pharmacological blockade of Per1 nuclear entry <i>in vivo</i> results in decreased NHE3 and SGLT1 expression, but not SGLT2 expression in the renal cortex ...	65
2-2 Per1 knockdown results in decreased NHE3 and SGLT1 expression, but not SGLT2 expression in HK-2 cells <i>in vitro</i>	65
2-3 Pharmacological blockade of Per1 nuclear entry results in decreased NHE3 and SGLT1 expression, but not SGLT2 expression <i>in vitro</i>	66
2-4 Pharmacological blockade of Per1 nuclear entry results in decreased nuclear Per1 <i>in vitro</i>	67
2-5 Pharmacological blockade of Per1 nuclear entry results in decreased transcription of NHE3 and SGLT1	68
2-6 Pharmacological blockade of Per1 nuclear entry leads to decreased occupancy of CLOCK and Per1 on the NHE3 promoter in HK-2 cells.....	69
2-7 Pharmacological blockade of Per1 nuclear entry leads to decreased occupancy of CLOCK and Per1 on the SGLT1 promoter in HK-2 cells.....	70
2-8 Pharmacological blockade of Per1 nuclear entry results in decreased membrane and intracellular protein expression of NHE3 and SGLT1 in HK-2 cells	71
2-9 Na ⁺ /K ⁺ ATPase activity is reduced in HK-2 and HKC11 cells following pharmacological blockade of CK1δ/ε.....	72
3-1 Differences in renal gene expression between WT and <i>Per1</i> KO mice at noon and midnight on control diet.....	85
3-2 There are no significant differences in expression of Pcsk9 or ETA in WT or <i>Per1</i> KO mice at noon and midnight.....	86
3-3 Inactive/active averages and 72 hour telemetry recordings of MAP in WT and <i>Per1</i> KO mice on control diet.....	86
3-4 Inactive/active averages and 72 hour telemetry recordings of MAP in WT and <i>Per1</i> KO mice on HS/DOCP diet	87
3-5 <i>Per1</i> KO mice lose the inactive/active difference in MAP on HS/DOCP diet.\ ...	87

3-6	Parallel changes in DBP, SBP and MAP	88
3-7	WT and <i>Per1</i> KO mice maintain inactive/active activity patterns on control and HS/DOCP diet	89
4-1	Circadian Rhythm of BP on Control Diet.	100
4-2	Mean Arterial Pressure Averaged Over Multiple Days of Diets.	100
4-3	Average MAP for All Diets	101
4-4	Changes in DBP and SBP on Control and HS/DOCP Diets	101
4-5	Real Time PCR Expression of Selected Genes in Kidney Cortex and Medulla	102
4-6	Additional Real Time PCR Expression of Selected Genes in Kidney Cortex and Medulla.....	103
5-1	The UCSC genome browser predicted chromatin state of EDN1 in HUVEC (human umbilical vein endothelial cells)	123
5-2	Transcription Factor Binding Sites at the EDN1-AS Predicted Promoter Region	123
5-3	Diagram of strand-specific primer placement	124
5-4	Dexamethasone Treatment of HK-2 Cells and RNA Isolation	124
5-5	Rationalization for gRNA Design in Human EDN1	125
5-6	96-well Cloning Method for CRISPR/Cas9 Knockout Cells	126
5-7	EDN1-AS is expressed in several human cell lines and in human kidney.	127
5-8	EDN1-AS expression is increased in the murine renal outer medulla	128
5-9	EDN1-AS Expression in Mouse Aorta	129
5-10	EDN1-AS expression may exhibit sex differences in murine renal cortex	130
5-11	EDN1-AS expression is regulated by aldosterone in mmIMCD3 cells.....	131
5-12	EDN1-AS is regulated by the circadian clock	132
5-13	RNA polymerase II, GR and <i>Per1</i> are present at the EDN1-AS promoter.....	133
5-14	Representative Genotyping Gel for CRISPR/Cas9 Knockout.....	134
5-15	ET-1 ELISA of Media from CRISPR Cell Lines.....	134

LIST OF ABBREVIATIONS

20-HETE	20-hydroxyeicosatetraenoic acid
3 β -HSD	3-beta dehydrogenase isomerase
ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
ADH	antidiuretic hormone
Ang II	Angiotensin II
ANP	atrial natriuretic peptide
ARB	Ang II receptor blocker
BP	BP
Cav-1	Caveolin-1
ChIP	chromatin immunoprecipitation
CK1 δ/ϵ	casein kinase 1 isoform delta/epsilon
CKD	chronic kidney disease
CLOCK	circadian locomotor output cycles kaput
Cry	cryptochrome
CVD	cardiovascular disease
DBP	diastolic BP
DCT	DCT
DNA	deoxyribonucleic acid
DOCP	desoxycorticosterone pivalate
ECF	extracellular fluid
ENaC	ENaC
eRNA	enhancer-derived RNA
ET-1	Endothelin-1

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFR	glomerular filtration rate
HK-2	human kidney PT cells
HLF	hepatic leukemia factor
hnRNA	heterogeneous nuclear RNA
HPA	hypothalamic-pituitary-adrenal
HR	heart rate
HS/DOCP	high salt + DOCP
mIMCD3	mouse inner medullary CD cells
K	potassium
KO	knockout
lncRNA	long, non-coding RNA
MAP	mean arterial pressure
mpkCCDc14	mouse principal cortical CD cells
Na	sodium
NCC	sodium chloride cotransporter
NHE3	sodium hydrogen exchanger isoform 3
NKCC2	sodium-potassium-2 chloride co-transporter
Per	period
PT	PT
RAAS	Renin-Angiotensin-Aldosterone System
RNA	ribonucleic acid
ROR	retinoid-related orphan receptor
SBP	systolic BP
SCN	suprachiasmatic nucleus

SGLT1	sodium glucose transporter isoform 1
SHR	spontaneously hypertensive rats
TAL	thick ascending Loop of Henle
TEF	thyrotroph embryonic factor
Ube2e3	Ubiquitin-Conjugating Enzyme E2E3
WNK	with no lysine kinase
WT	WT
ZT	Zeitgeber time

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

THE ROLE OF PER1 AND EDN1-AS IN MINERALOCORTICOID AND SALT-
SENSITIVE HYPERTENSION

By

Kristen Solocinski

December 2017

Chair: Michelle L. Gumz

Major: Medical Sciences – Biochemistry and Molecular Biology

Cardiovascular disease is responsible for 1/3 of the total American deaths every year. Hypertension is one of the main risk factors for developing cardiovascular disease and thus is a large target for treatment to prevent cardiovascular disease as well as other associated morbidities such as chronic kidney disease and renal injury. While many treatments can be extremely effective for some patients, about half of the hypertensive population has uncontrolled hypertension with about 10% of the hypertensive population being resistant to treatment with multiple drugs including diuretics. Even with the best medications we have, around 8 million Americans still do not have their blood pressure (BP) under control. Clearly, better medications are needed and to accomplish this, we need a better understanding of the progression of hypertension.

The Gumz lab and others have clearly demonstrated the importance of the circadian clock to BP as every clock gene knockout animal exhibits a BP phenotype. Period 1 (Per1) is one of four core clock proteins and coordinately regulates many aspects of renal sodium reabsorption. Per1 has been implicated in the pathogenesis of hypertension, including salt-sensitive hypertension. We have shown that male mice

lacking the *Per1* gene display a harmful non-dipping phenotype while on a high salt and mineralocorticoid diet. However, female *Per1* knockout mice on the same diet do not show any significant phenotype compared to WT (WT) mice, in line with reported sex disparities with respect to cardiovascular and kidney disease.

Per1 regulates Endothelin-1 (ET-1) which is itself an important contributor to hypertension as it is one of the most potent endogenous vasoconstrictors. Our lab has discovered a novel long non-coding RNA (lncRNA) antisense to the Endothelin-1 gene (EDN1), named EDN1-AS. It appears to be regulated by the circadian clock, which suggests the possibility that both *Per1* and EDN1-AS are novel, potential targets for the treatment of hypertension.

CHAPTER 1 INTRODUCTION

Background

Many aspects of behavior and physiology cycle over the course of ~24 hours, being synchronized to the day/night cycle, and are accordingly called circadian (latin for “around a day”) rhythms*. Circadian rhythms in physiological function are critical to maintaining health and the biochemical pathways mediating these effects are highly conserved and are present in species from archaebacteria to humans (reviewed in²). The pacemaker of the circadian clock is located in the suprachiasmatic nucleus (SCN) of the brain and is entrained by light^{3,4}. For this central clock, light is a dominant Zeitgeber, literally translated as “time giver,” a signal for entrainment. The central clock synchronizes the peripheral clocks, located in other areas of the brain and in tissues throughout the body, via neuronal and humoral signaling. While light is the dominant Zeitgeber for central clock entrainment, metabolic cues likely act as an additional Zeitgeber for peripheral clocks such as those located in the liver and kidneys.

While the circadian clock is composed of many genes, the core molecular clock mechanism is comprised of four key circadian genes: *Clock*, *Bmal1*, *Period* (*Per* homologs 1, 2 and 3) and *Cryptochrome* (*Cry* homologs 1 and 2). These genes encode proteins that function in a feedback loop to regulate transcription of clock-controlled genes (for an excellent review on this mechanism, see⁴). Briefly, circadian proteins CLOCK and BMAL1 form a heterodimer which binds to E-box elements of clock-

*Reprinted with Permission from: ¹ Solocinski K, Gumz ML. The circadian clock in the regulation of renal rhythms. *J Biol Rhythms*. 2015;30:470-486

controlled genes. This BMAL1/CLOCK heterodimer also binds to E-box elements in the promoters of the *Cry* and *Per* genes to activate transcription, thus forming the positive arm of the feedback loop. In the negative arm of the loop, PER and CRY then act on BMAL1/CLOCK to inhibit their own transcription. Two nuclear receptors involved in the molecular clock regulatory loops are the retinoid-related orphan receptor (ROR) and REV-ERB α . ROR acts positively on *Bmal1* whereas REV-ERB α mediates opposing action on *Bmal1*. Kinase and phosphatase-mediated post-translational modifications provide an additional layer of regulation for clock proteins. For example, nuclear entry of PER and CRY is regulated via phosphorylation by Casein Kinase 1 isoforms δ/ϵ (CK1 δ/ϵ)^{5, 6}. The molecular circadian clock mediates the regulation of rhythmic physiological function via transcriptional control and post-transcriptional control of downstream clock target genes and this regulation occurs in a tissue-specific manner. In an elegant genome-wide study in the liver, Koike et al. demonstrated that the core circadian clock proteins interact with regulatory sequence elements in several thousand genes⁷. Studies such as these are increasing our understanding of circadian clock function and shedding light on the tissue-specific effects of the clock. A recent landmark genomics study characterized circadian gene expression over a 48 hour period in wild-type mice in a variety of tissues, including the kidney⁸. This work demonstrated that more than 40% of all expressed genes in the tissues tested exhibited circadian variations in expression. In terms of the absolute numbers of cycling genes, the kidney was second only to the liver with several thousand genes exhibiting circadian expression.

In his 1985 Bowditch Lecture, Moore-Ede discussed the concept of physiological homeostasis, or equilibrium⁹. In the 19th century, Claude Bernard popularized the notion that the body is in a constant, fixed state. It wasn't until 1925 that Cannon and other physiologists developed the concept of homeostasis: that the body makes constant changes to maintain a steady state. It was thought that the body reacted to daily activities in order to maintain homeostasis. For instance, in response to sodium intake, the body would then react to excrete excess sodium from the body. In his lecture, Moore-Ede talked about predictive homeostasis in which the body starts to enact changes to counter activities it can anticipate because they occur in a predictable, cyclical manner. Thus, if meals are frequently eaten at the same time each day, the body can start to prepare for this predicted meal before it occurs by inducing expression of necessary solute transporters and metabolic enzymes in the liver, gut and kidney, for example. Discovery of the molecular clock components provided evidence that this predictive homeostasis occurs due to the activity of the circadian clock; the clock allows the body to keep track of the time using multiple cues such as light and food intake. While laying down at night suppresses urinary sodium, potassium and water excretion, excretion is not suppressed by laying down during the day¹⁰. The body predicts we are going to bed at night and prevents loss of precious nutrients. The kidney provides a textbook illustration of predictive homeostasis. Here, we review the evidence for circadian control of rhythms in renal function and present the current state of knowledge regarding the regulation and function of the clock in the kidney.

The Kidney

The kidneys function to filter the blood, maintaining fluid and ion homeostasis¹¹. This is crucial and tightly regulated because even slight changes in filtrate reabsorption can lead to large changes in blood chemistry and blood pressure (BP). Sustained hypertension (high BP) can lead to a number of diseases and is a main risk factor for chronic kidney disease (CKD) and cardiovascular disease, the main cause of about 1/3 of American deaths every year¹². The kidney achieves this fine level of regulation via the many specialized cell types located along the segmented nephron, the functional unit of the kidney (Figure 1-1). An adult kidney contains about 1 million nephrons on average and each one is tightly controlled to avoid large fluctuations in BP. Blood enters every nephron and is filtered through the glomerulus. The filtrate flows through the nephron and the many specialized cell types in each segment of the nephron reabsorb or secrete solutes according to the needs of the body. The final filtrate flows into the ureter to eventually become the urine.

The kidney carefully maintains homeostasis for sodium, potassium and several other key solutes in addition to maintaining acid/base balance. Each segment of the nephron has specific channels and transporters dedicated to the transport of sodium, potassium, hydrogen and bicarbonate, in addition to other important ions and solutes. Each is regulated differently, allowing for fine-tuned control of each segment in order to maintain homeostasis. Because much of the work on the circadian clock in the kidney has focused on sodium balance and effects on BP, this review will focus on regulation of renal sodium handling by the molecular clock.

After passing through the glomerulus, the filtrate from the blood enters the proximal tubule (PT) where about 60% of sodium reabsorption takes place (reviewed

in¹¹). Cells in this segment of the nephron express an apical (facing the lumen of the tubule) sodium-hydrogen exchanger (NHE3) and sodium glucose transporter (SGLT1); expression of these transporters appears to be regulated by the circadian clock¹³ (CircaDB¹⁴ <http://circadb.hogeneschlab.org/>). Filtrate then moves to the Loop of Henle where around 25% of sodium reabsorption occurs in the thick ascending portion (TAL). In this segment, the clock appears to regulate expression of the sodium-potassium-2 chloride co-transporter (NKCC2) and the estrogen-related receptor beta (ERR β) (CircaDB¹⁴)¹⁵. The next segment, the distal convoluted tubule (DCT), accounts for about 10% of sodium reabsorption and this occurs mainly through the sodium chloride co-transporter (NCC). NCC is regulated by a kinase cascade involving members of the with-no-lysine (WNK) family. Increasing evidence suggests that NCC and the WNK pathway are subject to regulation by the circadian clock^{16, 17}. The final segment the filtrate enters is the collecting duct (CD) where only about 5% of sodium reabsorption occurs. However, the distal nephron is the most highly regulated segment of the nephron. In addition to transporters and exchangers, ion channels are also integral to sodium reabsorption. In principal cells of the CD, sodium entry occurs through the apical epithelial sodium channel (ENaC). The alpha subunit of ENaC (α ENaC) and other key regulators of sodium transport in this segment have all been linked to the clock¹⁸⁻²⁰.

Early evidence for the existence of a “kidney clock” comes from a 1952 study of five human subjects in which activity, light, temperature, diet and other variables which may affect circadian rhythms were carefully controlled and monitored. Rhythms in urine flow and pH were observed in addition to sodium (Na⁺) and potassium output (K⁺), both peaking shortly after waking²¹. In 1975, the circadian rhythm of potassium excretion

was observed in three males who maintained a constant supine position and subsisted on a liquid diet administered every three hours²². These studies provided the first evidence for a kidney-specific clock.

Subsequent work continued to demonstrate that renal function appears to vary with a circadian rhythm. For example, many aspects of kidney function exhibit circadian fluctuations, such as glomerular filtration rate (GFR)²³, sodium excretion and renal blood flow²⁴. At a transcriptional level, expression of clock genes including *Clock*, *Bmal1*, *Cry1*, *Cry2*, *Per1* and *Per2* oscillate with a ~24 hour rhythm²⁵. Urinary sodium excretion exhibits a circadian pattern in rodents, primates and humans (reviewed in²⁶) and this may be explained by the clock-mediated regulation of a number of renal sodium transport genes^{13, 18, 20}. As illustrated in Figure 1-1, circadian clock-mediated regulation of a several key transport genes in the kidney has been established. In the following sections, the evidence for this regulation is considered in detail.

Hormones and the Kidney Clock

The kidney is both an important target and a source of hormones critical for maintaining ion homeostasis and BP control. The circadian rhythm of plasma sodium is in phase with many hormones involved in BP homeostasis including angiotensin II, aldosterone^{27, 28} and vasopressin²⁹ as well as angiotensin converting enzyme (ACE)³⁰ and plasma renin activity^{27, 28}, detailed below. Thus, proper regulation of these hormones is critical for BP homeostasis.

All forms of Mendelian (inherited) hypertension are due to a defect in the Renin-Angiotensin-Aldosterone System (RAAS)³¹. The RAAS acts to maintain BP homeostasis. When low BP is sensed, juxtaglomerular cells in the nephron release the enzyme renin. Renin then converts angiotensinogen to angiotensin I. Angiotensin I then

is further converted to Angiotensin II (Ang II) by ACE. Ang II acts on the vessels as a vasoconstrictor but can also stimulate the adrenal glands to secrete aldosterone. Aldosterone further stimulates sodium transporters in the aldosterone-sensitive distal nephron, leading to an increase in sodium reabsorption and BP. An intrarenal RAAS exists in addition to the well-characterized systemic RAAS³²⁻³⁴, further solidifying a role for the in the kidneys in the development of high BP.

In a human study, Ang II levels were examined from 15 patients around 14 years of age who had monosymptomatic nocturnal enuresis (MNE) and 10 normal patients of a similar age³⁵. They found that within the MNE group, Ang II was expressed in a time-dependent manner with nighttime levels being more than twice as high as daytime levels in patients without polyuria (excess urination). The MNE group with polyuria did not show any significant variation in Ang II levels between day and night, suggesting that disrupted circadian rhythms of Ang II may play a part in the pathogenesis of MNE.

The peptide hormone atrial natriuretic peptide (ANP) is made in the heart and is a vasodilator. Its actions on the kidney can lead to water and electrolyte excretion which, combined with vasodilatory properties, lead to an overall decrease in BP. Reports of daily, rhythmic changes in levels of this peptide in humans are conflicting and often have small subject numbers. In one study, ANP levels in humans (10 normotensive patients and 10 hypertensive patients), display a rhythm that appears to be antiphase to BP and HR rhythms³⁶. Furthermore, in normotensive subjects, there was a correlation between ANP, renin and aldosterone levels while this was not observed in the hypertensive group. In another study of 12 males, the authors state no rhythm of ANP but a 15% increase at night³⁷. In 21 healthy, middle aged men, ANP has

a significant rhythmic expression, with the peak of expression occurring at night³⁸. These results are similar to those obtained for 20 year old men and women³⁹. While it is definitely possible that daily variation of ANP can vary between humans and other animals, more studies would be helpful to confirm these findings.

Urodilatin is encoded by the same gene as ANP but has four extra amino acids on its N-terminus⁴⁰. Unlike ANP, urodilatin is only secreted in the kidney and is not secreted into the systemic circulation. It causes inhibition of sodium and water reabsorption when high BP is sensed. In 1991, ten years after ANP was discovered, Drummer et al. described the time-dependent rhythm of urodilatin⁴¹. In six healthy men, a rhythm of urodilatin was shown with a maximal expression during the day. However, in this study, a significant rhythm for ANP immunoreactivity was not observed.

Dopamine is released from nerves in the kidney and it is synthesized by cells in the PT (reviewed in⁴²). In the kidney, dopamine inhibits sodium and water reabsorption when extracellular fluid (ECF) volume is increased. Under constant light and under normal 12:12 LD, there were time-dependent changes in dopamine expression in the striatum and nucleus accumbens of rats⁴³. There was no circadian rhythm of dopamine expression under constant darkness, however, demonstration that variations in dopamine levels are likely dependent on time cues. While the circadian expression of dopamine in the kidney has not yet been investigated, it does appear to have a circadian rhythm in mouse adrenal glands and skeletal muscle (CircaDB¹⁴). It would be interesting to investigate the expression of dopamine in the kidney as well, given that it regulates water and sodium handling and in turn, BP.

Vasopressin, also called antidiuretic hormone (ADH), is released when there is a decrease in extracellular fluid volume. It causes an increase in water reabsorption in the CD, thus decreasing diuresis as the name implies. Challet et al. looked at the rhythm of vasopressin in mice lacking folate, vitamin B₉⁴⁴. It has previously been shown that folate may be a cofactor for cryptochromes⁴⁵. It has also been shown that rats lacking folate have decreased amplitude of melatonin secretion⁴⁶, which is important for maintaining the sleep-wake cycle. This group found that their folate-deficient mice had decreased rhythms of vasopressin expression compared to normal mice⁴⁴. The authors further point out that folate reduction is commonly seen with aging. Decrease or loss of circadian rhythms has also been associated with increasing age⁴⁷, although whether it is a cause or result of the aging process is unclear. It also leads to the possibility that these disruptions in circadian rhythms may be implicated in pathogenesis of many diseases associated with aging.

The kidney is mainly responsible for excretion of cortisol and its metabolites and this process is impaired in chronic kidney disease patients⁴⁸ and reviewed in⁴⁹. In healthy subjects, aldosterone and cortisol exhibit time-dependent increases in the early morning. In anephric patients, the correlation between plasma aldosterone and plasma cortisol is lost: daily variation in plasma aldosterone is not present whereas plasma cortisol rhythms remain⁵⁰. Rhythmic changes in urinary cortisol and aldosterone excretion are disrupted in kidney transplant patients as well⁵¹. Renal handling of cortisol and aldosterone may affect adrenal hormone production through disruption of known negative feedback loops. For example, synthesis of the pituitary hormone adrenocorticotrophic hormone (ACTH), which stimulates cortisol production by the

adrenals, is inhibited by high levels of cortisol in the circulation. Thus, decreased renal function, or perhaps, disruption of the kidney clock, negatively affects cortisol handling and may subsequently alter glucocorticoid production inappropriately. Although central and peripheral circadian clocks certainly contribute to regulation of the hypothalamic-pituitary-adrenal (HPA) axis^{52, 53}, the role of the molecular kidney clock in these processes remains poorly understood.

The Kidney Clock

The core circadian clock proteins regulate almost half of all expressed genes and do so in a tissue-specific manner⁸. As illustrated in Figure 1-2, *Bmal1*, *Clock*, *Per1*, and *Cry2* exhibit clear circadian variation in expression in the whole kidney over a 48 hr period (CircaDB¹⁴). Rodent models have proven invaluable to our understanding of the mechanism of the renal circadian clock and its impact on BP regulation. An animal model of CKD can be produced by removing one entire kidney and 2/3 of the other kidney, removing about 5/6 of the total kidney mass and hence providing the name for this model, the 5/6 nephrectomy. In mice, this causes an increase in MAP of about 30 mmHg two weeks after the procedure⁵⁴. In rats, this caused peak *Bmal1* expression in the remnant kidney to shift 4 hours earlier than sham operated rats⁵⁵. Using immunohistochemistry to determine localization of protein, D-site-binding protein (DBP) localization changed from mainly glomerular in the sham controls to much more widely dispersed in the CKD model rats. PER2 protein expression was high in the corticomedullary junction in controls and decreased in CKD rats. Conversely, expression was highly increased in the cortex of CKD rats compared to controls. Thus, not only can circadian proteins mediate changes in BP, changes in BP can affect localization of circadian proteins in the kidney.

The link between the kidney and maintenance of normal BP was further illustrated using spontaneously hypertensive rats (SHRs). Kidneys transplanted from normotensive rats into SHRs lead to a decrease in mean arterial pressure of over 50 mmHg compared to sham-operated controls⁵⁶. Conversely, when kidneys from SHRs were transplanted into normotensive rats, the MAP increased by around 40 mmHg. The fact that a hypertensive phenotype tracks with the kidney to such a large degree highlights the magnitude of the renal contribution to BP homeostasis. While the SHRs display daily rhythms in plasma sodium concentration⁵⁷, closer investigation into the contribution of the kidney clock to this phenotype remains to be done.

Animal models with disruption of circadian genes of interest also provide evidence for the roles of specific clock genes. However, it is important to keep in mind that most of these models have disruption of clock genes systemically, so while the phenotypes observed certainly support heavy renal involvement, participation from other systems cannot be ruled out. Global knockout (KO) of *Bmal1* results in complete loss of circadian rhythmicity measured via wheel running activity⁵⁸. These mice are also sterile⁵⁹ and have shorter lifespans than wild type (WT) mice⁶⁰. They have a loss of circadian rhythmicity in HR and BP and are also hypotensive compared to the WT mice⁶¹. Whether a renal phenotype exists in global *Bmal1* KO mice has yet to be determined.

The circadian proteins are important for BP control through mechanisms involving multiple tissues. Loss of *Bmal1* specifically in the forebrain leads to mice with a loss of a circadian pattern of activity in constant light or constant dark conditions but

normal rhythms in LD (12 hours light, 12 hours dark) and otherwise healthy, fertile mice⁶².

In the first cell-type specific KO of a clock gene in the kidney, Firsov and colleagues generated mice lacking BMAL1 in renin-producing cells⁶³. Expression of BMAL1 was lost in cells of the juxtaglomerular apparatus (see Figure 1F) and in some cells of the CD. These kidney-specific *Bmal1* KO mice have decreased plasma aldosterone compared to control mice and also have significantly lower BP compared to controls. Thus, the loss of BMAL1 only in certain cells of the kidney was profound enough to cause systemic changes in aldosterone and BP.

Clock KO mice are hypotensive as well, yet they retain the normal 24 hour rhythmic variation in BP¹⁸. Expression of 20-HETE (20-hydroxyeicosatetraenoic acid), which is a regulator of BP, is also altered in these mice⁶⁴. If acting on preglomerular arterioles in the kidney, 20-HETE leads to an increase in BP through constriction of the arterioles⁶⁴. However, 20-HETE also inhibits some of the sodium transporters found in the PT and TAL of the nephron which leads to less sodium reabsorption and a consequent decrease in BP. Therefore, the dysregulation of 20-HETE has been proposed as a contributory mechanism to the hypotensive phenotype shown in *Clock* KO mice. While these mice have similar mean levels of plasma aldosterone compared to WTs, they have significantly lower levels at ZT12⁶⁴, which stands for Zeitgeber Time 12 – 12 hours after the lights have been turned on and on a 12:12 LD cycle, the onset of darkness. Interestingly, ZT12 is the time that *Per1* mRNA expression peaks in mouse kidney (CircaDB¹⁴).

In 2005, the first evidence for circadian control of a renal gene was provided by Okamura and colleagues¹³. They showed that NHE3 (encoded by the gene *Slc9a3*) mRNA expression is circadian and furthermore is regulated by CLOCK:BMAL1 heterodimer binding to an E-box element on *Slc9a3*. The circadian expression of NHE3 mRNA is severely blunted in *Cry1/2* KO mice¹³, further supporting the circadian control of this gene. These mice also exhibit salt-sensitive hypertension due to increased levels of aldosterone⁶⁵. Sglt1 (encoded by *Slc5a1*) and its expression is also circadian: SGLT1 mRNA expression peaks around ZT12 in the mouse kidney while NHE3 mRNA expression peaks about two hours earlier (data derived from CircaDB, see¹⁴).

Dbp/Hlf/Tef triple KO mice lack three circadian clock regulated genes: DBP, hepatic leukemia factor (HLF), and thyrotroph embryonic factor (TEF)⁶⁶. Mice lacking one or two of these factors have mild phenotypes which may be due to the fact that DBP, TEF and HLF have well conserved amino acid sequences and thus they likely compensate for each other in the single or double KO mice. Mice lacking all three usually do not live longer than 1 year, with symptoms such as seizures occurring during the first 3 months of life⁶⁷. In the kidney of the triple KO mice, Northern blot analysis revealed significantly less mRNA from potential target genes involved in detoxification and drug metabolism. This may be related to the mechanism of variable drug effectiveness depending on the time of administration. Later, this group showed that the triple KOs had low BP, decreased aldosterone levels and cardiac hypertrophy⁶⁸.

Another useful model of circadian gene disruption is the PER2 mutant mouse⁶⁹. The blunted PER2 protein expressed in these mice is missing dimerization sites (PAS B and PAC domains)⁷⁰. These domains have been implicated in the ability of PER2 to

interact with other proteins and possibly act as a light sensor through FAD (Flavin Adenine Dinucleotide) binding^{71, 72}. Indeed, mice with mutant *Per2* (and also *Per1*) genes have altered responses to light⁷³. This group found that the mutant mice have higher heart rate during the light period than WT mice but have similar locomotor activity. In normal LD conditions, PER2 mutant mice had circadian periods of mean arterial pressure (MAP), heart rate (HR) and activity similar to WT mice. However, in the absence of light (DD), the mutant mice had shorter periods with a trend of decreased amplitude of MAP, HR and activity compared to WT controls. The mutant mice also were unable to maintain day-night differences in MAP, HR and activity when put into constant dark conditions for 8 days, but showed improved differences when returned to LD.

In addition to CRY and PER, DEC1 and DEC2 are also involved in the negative arm of the transcriptional circadian feedback loops⁷⁴. Gene expression of both *Dec1* and *Dec2* exhibits a circadian pattern of expression in the rat heart while only *Dec2* shows a comparable rhythm in the kidney⁷⁵. Since rats are nocturnal, they normally eat at night. When food intake was restricted to the light cycle/rest phase for seven days, *Dec1* and *Dec2* mRNA expression in the heart was shifted by about 8 hours. In the kidney, however, *Dec1* expression became rhythmic and the peak of *Dec2* expression was shifted 4 hours earlier than controls. The authors also reversed both feeding time and the LD schedule. After 7 days, the peak of *Dec1* expression was shifted only in the heart while the peak of *Dec2* expression was shifted in both the heart and kidney. This provides evidence that the timing of food cues can actually induce rhythmic expression of clock-controlled genes in the rat kidney.

Contrary to the rats from the previous experiments, *Dec1* exhibits a circadian rhythm of expression in the mouse kidney and liver, and this rhythm is disrupted in mice expressing a mutant CLOCK protein resulting from deletion of exon 19⁷⁶. In the kidney, mutant CLOCK expression was associated with decreased total *Dec1* expression. However, rhythmic expression of *Dec1* mRNA was not affected in LD or DD, suggesting that loss of WT CLOCK expression was not sufficient to alter circadian expression. Loss of DEC1 does not appear to negatively affect mice compared to WT controls⁷⁷. Since DEC1 and DEC2 have similar DNA binding domains, it seems that DEC1 may be redundant in the kidney with DEC2 able to compensate for loss of DEC1. This possible redundancy suggests the importance of maintaining the circadian clockwork in the kidney.

PER1 Action in the Kidney

Due to the important role that ENaC plays in maintaining sodium balance, it is tightly regulated at multiple levels. PER1 has been a focus of investigation in the kidney because it is a direct target gene of the sodium- and BP-regulating hormone aldosterone⁷⁸ which regulates ENaC via multiple mechanisms⁷⁹. Aldosterone-mediated transcriptional regulation of the *Scnn1a* gene, encoding α ENaC, occurs through the mineralocorticoid receptor (MR). PER1 appears to be involved in this regulation⁸⁰. In one of the first reports to link the molecular clock to renal sodium handling, Gumz and colleagues demonstrated that knockdown of PER1 in several renal CD cell lines resulted in decreased expression of α ENaC²⁰. Furthermore, *Per1* KO mice exhibited increased urinary sodium excretion, again supporting a role for the molecular clock in the regulation of renal sodium handling. In order for PER1 to get into the nucleus and affect its target genes, it must be phosphorylated by CK1 δ/ϵ . A CK1 δ/ϵ inhibitor

(PF670462) prevents PER1 nuclear entry in a murine cell line model of the CCD, mpkCCD_{c14} cells⁸¹. If PER1 is unable to enter the nucleus, it is therefore unable to associate with E-box elements of target genes such as α ENaC. Inhibition of PER1 nuclear entry decreases baseline α ENaC mRNA expression, suggesting that PER1 mediates basal expression of α ENaC as well as increasing transcription. α ENaC protein levels in the membrane are also decreased by about 60% after CK1 δ/ϵ blockade.

In addition to regulating α ENaC, PER1 regulates several other genes in the kidney¹⁹. *Per1* levels were decreased using siRNA in mpkCCD_{c14} cells which led to a decrease in mRNA expression of *Fxyd5*. Conversely, Ubiquitin-Conjugating Enzyme E2E3 (Ube2e3), Caveolin-1 (Cav-1) and Endothelin-1 (ET-1, encoded by *Edn1*) expression increased following PER1 knockdown. FXYD5 increases activity of the Na, K-ATPase which is responsible for pumping sodium reabsorbed from the filtrate back in to the blood⁸².

UBE2E3 is an E3 ubiquitin ligase, an enzyme which adds ubiquitin molecules to proteins, a common signal for proteasomal degradation. When UBE2E3 ubiquitinylates ENaC, it is removed from the membrane, thus decreasing sodium reabsorption⁸³. Reduction of PER1 levels lead to increased expression of this ligase, potentially leading to increased ENaC degradation. CAV-1 is a lipid raft protein associated with removal of ENaC from the membrane as well⁸⁴, leading to decreases in sodium reabsorption like UBE2E3. Finally, ET-1 leads to decreased ENaC open probability through a mechanism involving nitric oxide and the Endothelin type B (ET_B) receptor^{85, 86}. Following PER1 knockdown, ET-1 expression increased almost four-fold¹⁹. PER1 knockdown lead to

decreased membrane α ENaC protein levels, potentially due to contribution of the increased amount of ET-1, CAV-1 and UBE2E3⁸¹.

Per1 KO mice on a 129/sv background exhibit BPs about 18 mmHg lower than WT mice but maintain normal BP rhythms¹⁹. Although the extra-renal contributions to this phenotype have not been evaluated, many pieces of evidence support role of the renal clock. *Per1* KO mice also have decreased basal levels of α ENaC mRNA in the medulla compared to WT mice²⁰. These mice exhibit increased levels of ET-1 in the cortex and medulla of the kidney. While known as a potent vasoconstrictor in the majority of the body, ET-1 acts to decrease BP and increase natriuresis (sodium excretion) in the kidney through inhibition of ENaC^{85, 87}. Furthermore, ET-1 peptide levels are increased in *ex vivo* IMCD cells from *Per1* heterozygous mice (which have approximately 50% less PER1 expression than WT mice)⁸⁸. Increased ENaC inhibition by ET-1 coupled with decreased basal ENaC levels may contribute to the low BP phenotype observed in the global *Per1* KO mice. In healthy humans, plasma ET-1 levels decrease at night when BP dips, whereas patients with CKD have increased night time plasma ET-1 levels that correlate with loss of night time BP dipping⁸⁹. Normally, people experience a 10-20% decrease in BP at night and those who don't are called non-dippers. Non-dipping has been linked to increased risk of not only cardiovascular events⁹⁰ but also chronic kidney disease^{91, 92}. Together these results suggest that ET-1 may contribute to the circadian profile of BP.

Per1 heterozygous mice also have decreased levels of plasma aldosterone relative to WT 129/sv mice⁹³. Aldosterone regulation of ENaC expression is mediated at least in part by PER1. Treating mpkCCD_{c14} cells with aldosterone leads to increased

interaction of PER1 with E-box elements in the α ENaC promoter⁸⁰. Increased binding of RNA POLII to the ENaC promoter in the presence of aldosterone was observed as well, consistent with the known effect of aldosterone to increase α ENaC transcription. This is further validated by the observation that PER1 knockdown in the presence of aldosterone is associated with decreased α ENaC expression compared to aldosterone alone²⁰. Similar results were observed following nuclear blockade of PER1 using a CK1 δ/ϵ inhibitor⁸¹.

In addition to PER1 regulation by aldosterone, it seems that aldosterone may be regulated by PER1. In addition to lower plasma aldosterone levels, *Per1* heterozygous mice do not exhibit the normal increase in plasma aldosterone during their active phase that occurs in WT mice⁹⁴. One explanation for this phenotype may be that *Per1* heterozygous mice have a decrease in 3 β -HSD (3-(β)-hydroxysteroid dehydrogenase) expression. 3 β -HSD is an enzyme produced in zona glomerulosa cells of the adrenal glands, which are responsible for the production of aldosterone, among other hormones. This enzyme catalyzes the synthesis of progesterone, which is a precursor to aldosterone in the steroid hormone biosynthesis pathway⁹⁵. The time-dependent increase in 3 β -HSD during the active phase observed in WT mice is blunted in *Per1* heterozygous mice. PER1 knockdown in a human adrenal cell line (NCI-H295R) causes a 58% decrease in 3 β -HSD mRNA levels⁹⁶. A similar result was obtained *in vivo* in the adrenal gland in WT 129/sv mice treated with the CK1 δ/ϵ inhibitor.

Cry1/2 KO mice actually have increased levels of 3 β -HSD which correlates with the observed increase in their plasma aldosterone levels⁶⁵. These mice exhibit salt-sensitive hypertension. Interestingly, this phenotype is nearly opposite that of *Per1* KO

mice which exhibit reduced BP and that of *Per1* heterozygous mice which have decreased adrenal gland expression of 3 β -HSD^{19, 94}. Evidence suggests that PER1 and CRY1/2 have opposing actions on specific target genes encoding α ENaC and FXD5 in the kidney and PPAR α and DEC1 in the liver⁹⁴. Decreased expression of PER1 expression *in vitro* and *in vivo* in the liver and the kidney was associated with an increase in CRY2 protein levels. This is further supported by the observation that inhibition of PER1 nuclear entry with the CK1 δ/ϵ inhibitor in mouse liver cells (AML12) increases cytosolic and nuclear CRY2 expression⁹⁶. Further supporting opposing roles of CRY2 and PER1, mice lacking both *Per1* and *Cry2* have a normalization of their free running period compared to mice lacking *Per1* or *Cry2* alone⁹⁷.

The thiazide-sensitive NCC is also regulated by PER1¹⁶. Either PER1 knockdown or pharmacological blockade of nuclear entry in a model of the DCT (mDCT15 cells) resulted in decreased NCC expression. Furthermore, nuclear blockade of PER1 via CK1 δ/ϵ inhibition resulted in decreased NCC activity levels in these cells. NCC mRNA levels exhibited time-of-day-dependent changes with higher expression during the mouse active period. Consistent with the *in vitro* data from mDCT15 cells, NCC expression levels were also decreased in the cortex of *Per1* heterozygous mice compared to WTs. Similarly, CK1 δ/ϵ inhibitor treatment of WT mice decreased NCC expression compared to vehicle-treated controls. These data suggest that the circadian clock is critical for regulation of many important aspects of renal sodium reabsorption.

Development

The fetal SCN is synchronized by the mother and after birth, maternal care helps to keep the clock entrained⁹⁸. Eventually, the neonatal SCN matures enough to take over its job as the central clock⁹⁸. To investigate developmental changes in the

circadian clock in several tissues, transgenic rats expressing a PER2-LUCIFERASE fusion protein were utilized⁹⁹. These rats are transgenic for the mouse *Per2* promoter fused to a destabilized luciferase (luciferase fused with a modified PEST sequence¹⁰⁰) reporter gene (*Per2-dLuc*)¹⁰¹. This allows *Per2* promoter activity to be visualized by measuring luminescence. Significant differences were shown in *Per2-dLuc* activity between developmental stages E20 (embryonic day 20), P5 (postnatal day 5), P19 and adult rats. *Per2-dLuc* activity varied in total luminescence units as well as amplitude of change over time between the different developmental stages in the SCN, lung, kidney and liver in culture. In addition, other tissues were studied and similar tissue-to-tissue variability in *Per2-dLuc* expression was found. The peak phase of *Per2-dLuc* advanced after birth to adulthood by about 12 hours in the kidney and lung but not in the liver and only slightly in the SCN between E20 and adult. The SCN was one of the tissues in which *Per2-dLuc* activity did not vary or shift as much during development even though other tissues displayed significant changes, supporting the idea that peripheral clocks are not solely entrained by the SCN.

One reason for the differential entrainment of the SCN and peripheral clocks could be due to the fact that between 2 and 3 weeks after birth, pups switch from drinking milk to eating chow⁹⁹. The different nutrient composition may give different cues to the pups regarding entrainment. Another explanation is that pups usually feed while mothers rest, so they are eating during the day, or what should be their inactive period. The switch from milk to chow would be accompanied by a reversal of feeding time, resynchronizing the peripheral clocks. The different expression of PER2 may also be because organs are not fully mature at birth. The function changes as the pups age

and this may also account for the different expression of PER2 due to changing developmental needs¹⁰².

In order to investigate embryonic stages of development more thoroughly, gene expression from embryonic days E10-E19 was evaluated in PER2::LUC mice¹⁰³. Similar to the *Per2-dLuc* rats, these mice express a PER2-LUCIFERASE fusion protein. These mice are on a C57BL/6J background and have the luciferase gene added in frame to the end of the *Per2* gene¹⁰⁴. Real time PCR measurements of clock gene expression in the liver showed little to no circadian rhythms during this time even though maternal rhythms were robust. However, if these tissues were excised and cultured *in vitro*, large circadian rhythms in PER2::LUC protein were observed. Although this could be an artifact of cell culture, these results do not rule out the possibility that individual cells have rhythms and they are just not yet synchronized at embryonic stages.

Circadian variation during development of other clock controlled genes and kidney-specific clock controlled genes was studied in the kidneys of Sprague-Dawley rats. While small-amplitude rhythms of expression of *Bmal1*, *Rev-erba*, *Per2* and *Cry1* mRNA were seen at E20, the rhythms became more robust with increasing peak-to-trough differences from 1 week, 4 weeks and 12 weeks after birth¹⁰⁵. By 12 weeks after birth, peak expression levels of *Bmal1*, *Per2* and *Cry1* were observed during the dark period while peak expression of *Rev-erba* occurred during the day. Kidney-specific clock-controlled genes *Scnn1a* (α ENaC), *Sgk1* (serum and glucocorticoid-inducible kinase 1), *Slc9a3* (NHE3) and *Avpr2* (arginine vasopressin receptor 2) showed some rhythmic expression at E20 and these rhythms became more robust one week after delivery in addition to shifting the peak of expression for all four genes by about 12

hours. One week after birth, the peak expression levels of these genes occurred during the day but this again shifted three weeks later and peak expression times shifted closer to the night time, the rats' active period. Pups were then separated from their mothers between ZT3-7, the time of their highest feeding behavior, for one week after birth. This led to a 12 hour shift in *Bmal1* expression and caused most other genes that were studied to lose their rhythm of expression, possibly due to conflicting light and feeding cues.

Regulation of the Kidney Clock

After food deprivation for one day and in total darkness, male Wistar rats were allowed access to food for 30 minutes. This feeding stimulus led to a four hour shift in peak expression of a classic circadian gene, *Dbp* (D-site albumin binding protein), in the heart but had no effect on expression in the kidney¹⁰⁶). The same study also showed the 30 minute feeding stimulus decreased levels of expression of *Bmal1*, *Cry1*, *Dbp*, *Per1* and *Per2* in the heart while only *Per1* mRNA was significantly decreased in the kidney.

Feeding time also seems to be able to synchronize some peripheral clocks in the absence of a central clock. *Bmal1* forebrain KO mice crossed with PER2::LUC mice had no circadian rhythm of PER2::LUC in the kidney in constant darkness but time restricted feeding restored this rhythm in the kidney as well as in the liver⁶². This is further supported by the finding that feeding mice only during the day (when they would normally be inactive) shifted the peak of DBP protein levels by 12 hours in the kidney, liver and heart¹⁰⁷. Reversed feeding shifted PER1 and PER2 levels in the liver by 12 hours while there was no effect on either of these proteins in the SCN.

In addition to the time of feeding, specific composition of diets may affect entrainment as well. The type of diet can alter gene expression in order to adapt to changing requirements¹⁰⁸. Specifically, a high salt (HS) diet causes increased *Dbp* mRNA expression while decreasing *Bmal1* expression in the liver and kidney¹⁰⁹. HS diet also advanced the time of peak expression of enzymes involved in glycogen synthesis (*Gys2*), gluconeogenesis (*G6pc*) and the synthesis of fatty acids, cholesterol and bile acid (*Fasn*, *Hmgcr* and *Cyp7a1*, respectively). Even though HS diet affected many genes important to metabolism, activity, feeding and drinking behavior were not affected. Timing of food intake and food content both appear to be important cues with respect to the entrainment of peripheral clocks.

Although it is clear that food is a dominant Zeitgeber for peripheral clock entrainment, the dominant cue for the central clock is light. The effects of reversing the LD cycle, restricting time of feeding or changing both the light cycle and feeding times were investigated specifically in the kidney¹¹⁰. LD reversal did not alter the circadian pattern of expression of *Bmal1*, *Cry1*, *Clock* or *Per2* mRNA but did delay the peak expression of *Per1* by 4 hours. In addition, the amplitude of expression of *Per1*, *Cry1*, *Clock* and *Bmal1* were altered only with the reversal of the LD cycle. Shifting feeding time by 12 hours caused 8-12 hour shifts in the peak expression of *Clock*, *Cry1* and *Bmal1* after 7 days while causing 4 hour shifts in *Per1* and *Per2*. After 7 days of LD and feeding time reversal, a total inversion of expression of all 5 genes occurred. Thus, separately, light and food cues partially entrain the circadian clock while both synchronize the clock throughout the whole body. Together these results suggest that food cues are a critical synchronizing signal for the kidney clock.

Transgenic PER2::LUC mice were further used to study the effects of temperature on time-dependent entrainment¹¹¹. These mice were placed in water baths at 35°C, 37°C and 41°C. The treatment at 41°C led to phase advancement by about four hours in the kidney and liver after 1-2 days but peak PER2::LUC expression started to return to normal after repeated treatments for more than two days. After a 2 hour treatments at 41°C, PER2::LUC expression levels were also increased in the kidney and liver. Thus, temperature appears to be another important cue for peripheral clock entrainment.

The circadian clock is classically considered to be temperature compensated, meaning that the free-running period, associated with translation and degradation of clock proteins, does not change much with changes in temperature. This is an intriguing piece of the circadian clock puzzle as the rate of enzymatic reactions should increase with temperature¹¹². However, other experiments in PER2::LUC mice suggest that the SCN is not affected by temperature while peripheral clocks are more susceptible to temperature-induced changes in PER2::LUC expression and this may be mediated by the heat shock pathway¹¹³.

In a more recent study, this same mouse model was used to investigate the effect of sleep deprivation on PER2. Sleep deprivation for 6 hours led to increased PER2::LUC levels in the brain, liver and kidney¹¹⁴. Thus, even relatively short disruptions in the sleep pattern can lead to disrupted circadian rhythms of PER2::LUC protein levels in peripheral tissues including the kidney.

Potassium

Potassium is another urinary electrolyte that has been shown to be regulated by the circadian clock and potassium excretion oscillates with sodium in multiple species

(reviewed in^{115, 116}). In the first study of its kind, Firsov and colleagues performed microarray analysis to characterize circadian gene expression in the kidney, revealing that the expression of many renal genes exhibited time-dependent changes in expression^{18, 64}, including many genes involved in potassium handling (reviewed in¹¹⁵). Also like sodium, potassium plays an important role in BP. In addition to disrupted patterns of potassium excretion in diseases such as CKD, potassium supplementation is beneficial in the treatment of hypertension^{117, 118}. It has been shown that potassium supplementation leads to increased sodium excretion and therefore decreased BP. In addition to decreasing BP, potassium may also be beneficial in restoring a night time dip in BP, thus reducing the risk of cardiovascular events.

Crambert and colleagues investigated the mechanism of regulation of potassium reabsorption using WT mice, *Clock* KO mice or mice null for the α subunit of the non-gastric H,K-ATPase (HK α 2, encoded by *Atp12a*)¹¹⁹. In WT mice, HK α 2 was present in significantly higher amounts in the luminal membrane at the end of the rest period while there were no sufficient differences in *Clock* KO mice. HK α 2 deficient mice excreted more potassium compared to WT mice during the rest period but they had no differences in sodium excretion. On a low potassium diet, HK α 2 mRNA expression peaked about 9 hours later than controls and amplitude of expression increased by 45%. This diet also affected expression of *Nrf2*, a transcription factor which participates in HK α 2 mRNA expression. In controls, expression preceded peak HK α 2 mRNA expression by about 4 hours. On a low potassium diet, peak *Nrf2* expression was delayed by about 10 hours, again peaking before HK α 2 mRNA.

ERR β is a nuclear receptor specific to the TAL which regulates NKCC2¹⁵. ERR β mRNA expression is circadian in male CD1 mice with expression peaking at ZT4. Furthermore, this rhythm is lost in mice without *Clock*. Blockade of ERR β increased the Na/K urinary excretion ratio and decreased NKCC2 mRNA expression and activity, suggesting a positive regulation of NKCC2. NKCC2 expression is also circadian in mouse kidney, with a peak early in the light period (CircaDB¹⁴), suggesting that it is under transcriptional regulation by the molecular clock. In addition to complex regulation of sodium balance in the kidney, the circadian clock is also integral to maintenance of potassium levels.

Clinical Implications

Many widely used treatments for hypertension such as ACE inhibitors and Ang II receptor blockers (ARBs) aim to inhibit the RAAS. Due to their demonstrated regulation of renal sodium transporters and BP, clock genes may represent novel targets for hypertension treatment. Another potential hypertension treatment target is the elevated 3 β -HSD observed in the *Cry1/2* KO mice that exhibit salt-sensitive hypertension.

In addition to targeting clock genes to treat disease, another strategy is to work with the clock. The aim of chronotherapy is to administer medicines at certain times of the day to maximize efficacy and reduce side effects. Many hypertension medications are usually taken in the morning, however convincing evidence concerning the effects of chronotherapy shows the benefit of taking at least one hypertension medication at night (for an excellent review, see¹²⁰). The MAPEC study followed over 2000 hypertensive patients over the course of a median of 5.6 years⁹¹. The study divided the volunteers into two groups, one taking all BP medications upon waking and the other group taking at least one medication at night. The night time dosing group showed significant

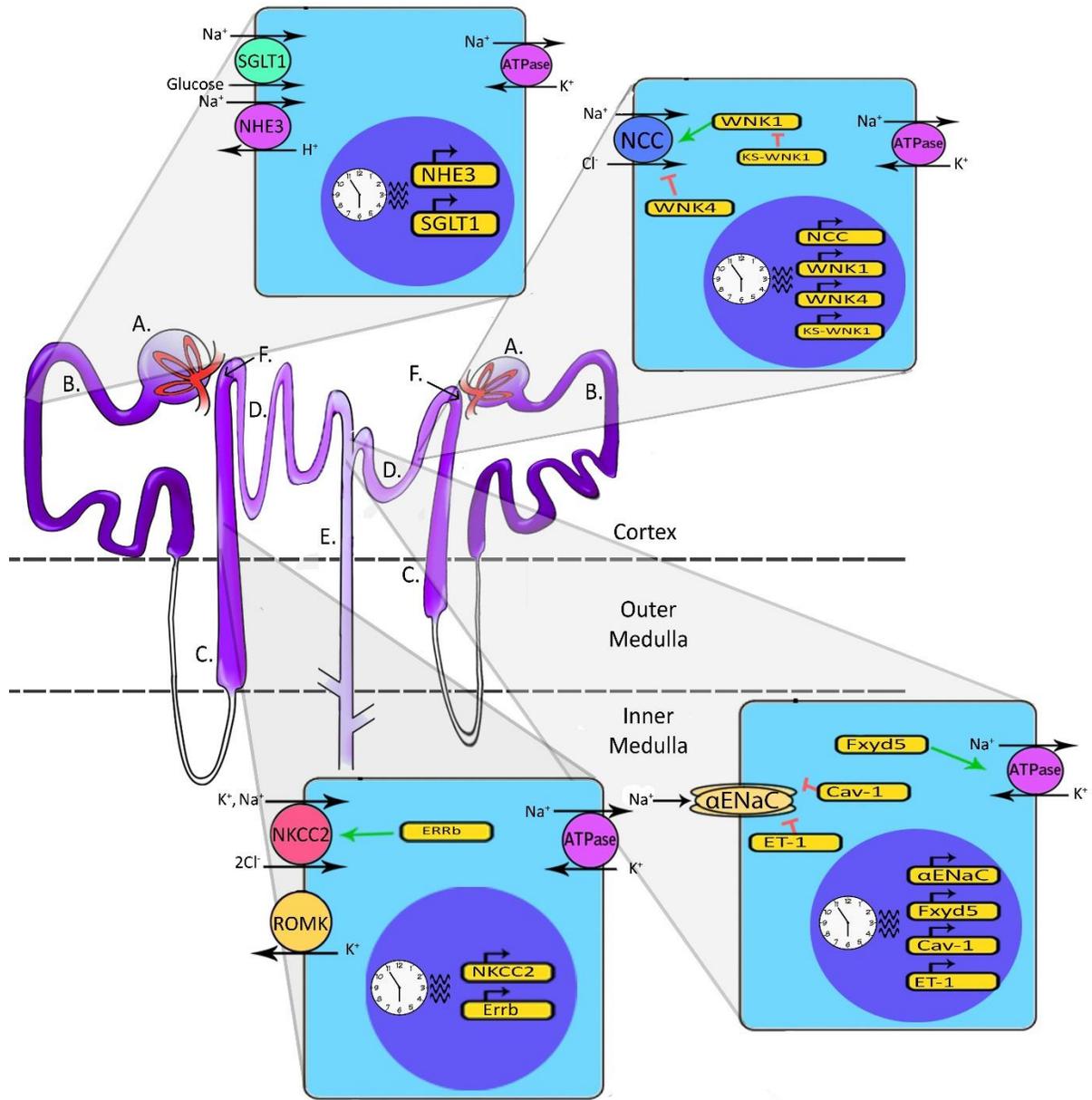
decreases in nocturnal BP and an increase in dipping prevalence of almost 30% compared to the morning dosing group. At the follow up of the study, the night time dosing group had a lower risk of cardiovascular events. They also had a significantly lowered morbidity and mortality due to cardiovascular disease.

Similar to timing the treatment of hypertension, timing of dialysis treatment was also shown to be important with respect to the overall well-being of patients. People undergoing dialysis have been observed to lose a normal night time peak of melatonin production¹²¹. This hormone is important to the maintenance of the circadian cycle of sleep¹²². Normally, melatonin shows a circadian rhythm of expression with high production at night that is suppressed by light. This helps to keep the body in sync with day and night. The loss of this rhythm would imply a dysregulation of the sleep cycle. It has been shown that patients undergoing dialysis during the day experience sleep disturbances and daytime tiredness¹²³, while night time dialysis helps to reduce these effects. The night time dialysis patients also have a restored melatonin peak at night¹²⁴.

It has also been shown that lack of sleep can cause disturbances in kidney function and BP patterns. Compared to people who slept 8-10 hours at night, those who were sleep deprived (no sleep for the night of the experiment) had a greater than 50% increase in nocturnal urine output¹²⁵. While potassium and sodium excretion were increased in both genders in the sleep deprived group, the effect was greater in men. The sleep deprived group exhibited an attenuation of the normal dipping that was observed in the control group. These observations indicate that disruption of circadian rhythms via sleep deprivation can affect kidney function which has important implications for maintaining overall health.

The circadian clock appears to be an important player in the regulation of the cell cycle and disruption of the molecular clock has been associated with cancer¹²⁶. Renal cell carcinoma is known to be associated with hypoxic signaling pathways and these pathways have recently been linked with the circadian clock¹²⁷. In an important human study linking dysregulation of circadian clock gene expression to renal cell carcinoma, Mazzoccoli et al. demonstrated that several core clock genes were differentially expressed in tumor samples compared to matched normal tissue¹²⁸. These findings likely have important implications for the treatment of kidney cancer. Further studies exploring the role of the molecular clock in mechanisms underlying renal cell carcinoma are needed.

Figure 1-1. Diagram of Two Nephrons and Representative Cell Types. A. Blood enters the nephron and is filtered at the glomerulus. B. Filtrate then moves to the PT (PT). This segment of the nephron contains the sodium-glucose linked transporter isoform 1 (SGLT1) and the sodium-hydrogen exchanger isoform 3 (NHE3), both of which are located on the apical membrane (facing the filtrate) and are regulated by the circadian clock. Sodium reabsorbed from the filtrate is pumped back into the blood by the basolateral Na/K-ATPase, found on each of the cell types illustrated here. C. Filtrate then moves to the Loop of Henle. Cells in the Thick Ascending Loop (TAL) contain the sodium-potassium-chloride cotransporter isoform 2 (NKCC2) which is regulated by the nuclear receptor $ERR\beta$. These cells also contain renal outer medullary potassium channel (ROMK), responsible for pumping K^+ back into the filtrate. Again, these cells have a basolateral Na, K-ATPase. D. The DCT (DCT) cells contain apically located sodium chloride cotransporters (NCC) which reabsorb Na^+ and Cl^- from the filtrate. This co-transporter is regulated by the circadian clock and well as with-no-lysine (WNK) kinases. E. The last part of the nephron filtrate enters is the CD (CD). The ENaC (ENaC) reabsorbs Na^+ from the filtrate and is regulated by the clock. FXYD5, CAV-1 and ET-1 are regulated by the circadian clock as well and in turn FXYD5 positively regulates the Na/K-ATPase, while CAV-1 negatively regulates ENaC. F. Juxtaglomerular apparatus: Cells in the macula densa communicate with the glomerulus to modulate GFR in a process known as tubule-glomerular feedback (TGF). Glomeruli, PTs and DCTs are located in the cortex of the kidney while the Loop of Henle and part of the CD span into the medulla. Multiple CDs converge to ultimately transport urine out of the kidney.



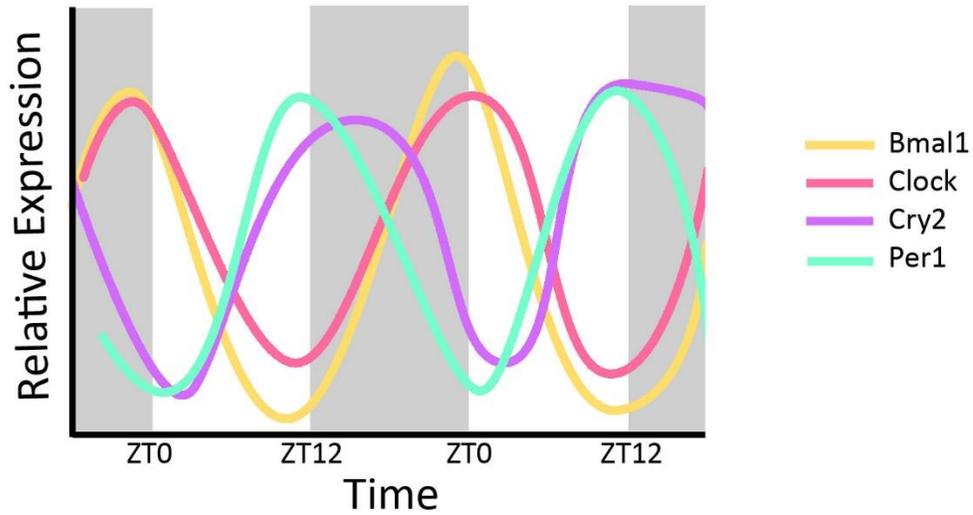


Figure 1-2. Circadian Expression of Core Clock Genes in the Kidney. Relative expression levels are plotted on the y-axis with Zeitgeber time on the x-axis. Shaded areas represent time when lights are off. Peak Expression of *Bmal1* and *Clock* are in phase and approximately antiphase to *Cry2* and *Per1* mRNA expression. Data derived from CircaDB ¹⁴.

CHAPTER 2 TRANSCRIPTIONAL REGULATION OF NHE3 AND SGLT1 BY THE CIRCADIAN CLOCK PROTEIN PER1 IN PT CELLS

Background

The circadian clock is an important regulator of multiple physiological functions including BP, the immune response, sleep-wake cycle, metabolism, vascular function, and renal function (reviewed in^{129, 130}). Urinary sodium excretion, renal blood flow, glomerular filtration rate, and plasma aldosterone levels have been shown to undergo rhythmic fluctuations (reviewed in²⁶). The circadian clock on the molecular level consists of four core proteins, which interact with one another to regulate expression of circadian target genes³. These four core circadian proteins are: CLOCK, Bmal1, Per, and Cry. CLOCK and Bmal1 heterodimerize and interact with E-box response elements to transcriptionally up-regulate circadian target genes, including Per and Cry. Per and Cry interact and then repress the transcriptional activity of CLOCK and Bmal1¹³¹. The circadian clock also undergoes post-translational modifications through the phosphorylation of the Per proteins by the circadian kinases, Casein Kinase 1 isoforms δ/ϵ (CK1 δ/ϵ). Phosphorylation by CK1 δ/ϵ allows Per1 nuclear entry¹³²⁻¹³⁴.

We have previously demonstrated that Per1 positively regulates the basal and aldosterone-mediated expression of α ENaC, a subunit of the renal ENaC^{19, 20, 133-135}. We also recently demonstrated that Per1 coordinately regulates the expression of multiple genes involved in the regulation of renal sodium reabsorption in CD cells¹⁹. It was initially assumed that Per1 behaves primarily as a repressor of CLOCK and Bmal1

*Reprinted with Permission from: Solocinski K, Richards J, All SC, Cheng KY, Khundmiri SJ, Gumz ML. Transcriptional regulation of nhe3 and sgl1 by the circadian clock protein per1 in PT cells. *Am J Physiol Renal Physiol*. 2015:ajprenal 00197 02014

activity. However, we and others have shown that Per1 appears to activate gene expression in a manner that appears to be gene and tissue-specific^{19, 97, 134-137}. Concordantly, we proposed a putative mechanism for Per1 action involving repression of the circadian repressor Cry2¹³⁴. These data predict that loss of Per1 should result in decreased renal sodium reabsorption, with subsequent decreased plasma volume and decreased BP. Consequently, we have demonstrated that Per1 KO mice have significantly lower BP compared to WT controls¹⁹ and increased urinary sodium levels²⁰. Consistent with these results, we have recently shown that mice with reduced Per1 expression exhibit reduced plasma aldosterone levels and renal sodium wasting⁹⁶.

Per1 appears to play an integral role in the regulation of α ENaC and other genes that modulate renal sodium reabsorption. We have also recently demonstrated that Per1 regulates NCC and the WNK kinases in a model of the murine distal convolute tubule¹³⁸. However the role of Per1 in the PT has not been investigated. The PT is the major site of sodium reabsorption in the nephron. A majority of sodium is reabsorbed in this segment through the actions of the sodium hydrogen exchanger NHE3 (reviewed in¹³⁹). The sodium glucose co-transporters (SGLT1 and SGLT2) also play an important role in the reabsorption of sodium and glucose (reviewed in¹⁴⁰). Previous work has demonstrated that NHE3 mRNA oscillates with a circadian pattern in mouse renal medulla^{13, 141}. Likewise, SGLT1 is regulated in a similar manner in the colon^{142, 143}. However, it is unknown if expression of NHE3, SGLT1, or SGLT2 are regulated by Per1.

The goal of this study was to test whether Per1 contributes to the regulation of sodium transporters in the PT via transcriptional modulation of NHE3 and SGLT1.

Pharmacological blockade of nuclear Per1 entry *in vivo* led to decreased mRNA expression of NHE3 and SGLT1 in the renal cortex of mice. Using several additional, independent methods in the human PT cell line HK-2, we demonstrated a role for Per1 in the transcriptional regulation of NHE3 and SGLT1 but not SGLT2. Importantly, pharmacological blockade of Per1 nuclear entry resulted in decreased intracellular and membrane protein levels of NHE3 and SGLT1. Taken together, these data demonstrate a potential role for the circadian protein Per1 in the regulation of NHE3 and SGLT1 in human PT cells.

Methods

Animals

All animal use protocols were approved by the University of Florida and North Florida/South Georgia Veterans Administration Institutional Animal Care and Use Committees in accordance with the NIH Guide for the Care and Use of Laboratory Animals. WT and Per1 KO mice (129/sv) were originally provided by Dr. David Weaver (University of Massachusetts¹⁴⁴). WT and Per1 heterozygote (het) mice were bred in house by UF Animal Care Services Staff and maintained on a normal 12 hours light:dark cycle. For *in vivo* PF670462 (CK1 δ/ϵ inhibitor) experiments, weight matched male WT 129/sv mice were either given vehicle (20% hydroxypropyl β -cyclodextrin) or 30 mg/kg PF670462 subcutaneously every 12 hours for 2.5 days starting at noon and sacrificed at midnight 12 hours after last injection as previously described^{96, 138}. Mice were anesthetized by inhalant isoflourane and tissues were collected and snap frozen in liquid nitrogen. Kidneys were later dissected and cortex removed for RNA isolation or protein collection.

Cell Culture

HK-2 cells were kindly provided by Kirstan Meldrum (University of Florida, Gainesville, FL)^{145, 146}. HK-2 cells were maintained in DMEM-F-12, 10% FBS, and 1% penicillin-streptomycin at 37°C. For RNA silencing experiments, Non-Target and Per1 siRNA (Dharmacon) were used according to the manufacturer's instructions. HK-2 cells were transfected using Dharmafect 1 (Dharmacon). Casein Kinase 1 δ/ϵ inhibitor (PF670462) experiments were performed as described previously^{133, 134}. For the dose and time-course, HK-2 cells were treated with either 0.1, 1, 10, or 100 μ M PF670462 or vehicle (water) for 24, 48, or 72 hours as previous described^{133, 138}. For nuclear entry experiments, HK-2 cells were treated with 10 μ M PF670462 or vehicle for 24 hours.

RNA Isolation and quantitative real-time PCR

Total RNA was isolated and DNaseI-treated using Direct-Zol™ RNA Miniprep (Zymo Research) as per the manufacturer's instructions. DNaseI-treated RNA (2 μ g) samples were used as template for reverse transcription with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNAs (20 ng) were then used as template in quantitative real-time PCR (qPCR) reactions (Applied Biosystems) to evaluate changes in Per1, NHE3, SGLT1, SGLT2, and Actin mRNA levels. Cycle threshold (Ct) values were normalized against β -actin and relative quantification was performed using the $\Delta\Delta$ Ct method¹⁴⁷. Fold change values were calculated as the change in mRNA expression levels relative to the control. Mouse and human TaqMan primer/probe sets were purchased from Applied Biosystems.

Membrane Protein Analysis, Nuclear Protein Isolation, and Western Blot Analysis.

Membrane protein was isolated using the Cell Surface Protein Isolation Kit (Pierce) according to the manufacturer's instructions. HK-2 cells were grown to confluence and

then treated with vehicle (water) or 10 μ M PF670462 for 24 hrs. Nuclear extracts were isolated using the NE-PER kit (Pierce) according to the manufacturer's instructions. For the Per1 nuclear entry experiments, protein concentrations were then quantified by BCA assay (Pierce). Western blots were performed as previously described^{96, 133, 134}. Proteins were separated on a 4-20% Tris-HCl Ready Gel (BioRad), and transferred to a PVDF membrane. The membrane was blocked with 2% non-fat dry milk in TBS-S (TBS plus 0.05% Rodeo™ Saddle Soap) (USB) and incubated overnight at 4°C with anti-Per1 (1:500) (Pierce), anti-NHE3 (Millipore), anti-SGLT1 (Millipore), or anti- β -actin (1:500) (Santa Cruz) antibodies. β -Actin was used as a loading control. The membrane was washed with 2% non-fat dry milk in TBS-S for 15 minutes and then incubated with horseradish peroxidase conjugate anti-rabbit secondary antibody and incubated in 2% non-fat dry milk in TBS-S for 1 hour at 4 °C. After incubation, the blot was washed with TBS-S for 15 minutes. Detection was performed using Novex® ECL Chemiluminescent Substrate reagents (Invitrogen). Densitometry was performed using ImageJ (rsbweb.nih.gov/ij).

hnRNA Analysis.

hnRNA analysis was performed as previously described^{20, 135, 138}. Primers were designed to amplify regions spanning the intron/exon boundary of the NHE3, SGLT1, and GAPDH genes. GAPDH was used as a cDNA loading control. Sequences and exon numbers are shown in Table 2. Total RNA was isolated from vehicle- and CKinh-treated cells, DNase I treated, and converted to cDNA as described above. PCR reactions were performed using 40 ng of cDNA as template. Reactions were heated to 95°C for 15 minutes to activate the Taq polymerase. 35 amplification cycles were performed using

the following parameters: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, followed by a final 10-minute extension at 72°C.

Chromatin Immunoprecipitation (ChIP).

HK-2 cells were grown to ~80% confluency and then treated with vehicle (water) or 10µM PF670462 for 24 hrs. ChIP was performed using the ChIP-IT™ Express Enzymatic Kit (Active Motif) according to the manufacturer's instructions and as previously described^{80, 138}. Chromatin concentrations were calculated and equal amounts of vehicle-treated and 10µM PF670462 treated chromatin were used per pull down. Pull downs were performed using 3µg of either anti-Per1 (Pierce), anti-CLOCK (Pierce), anti-Pol II (Santa Cruz), or rabbit IgG (Bethyl). Complexes were incubated overnight at 4°C with end-over-end rotation. Immunoprecipitated DNA was amplified by End Point PCR. Band intensities were quantitated using densitometry, which was performed using ImageJ (rsbweb.nih.gov/ij/). Bands were relativized to the relevant vehicle or PF670462-treated 10% input. Putative E-boxes in the NHE3 and SGLT1 promoters were analyzed using ALGGEN-PROMO (http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)^{148, 149}. For each gene, three primer sets were designed as shown in Table-1.

Ouabain-sensitive ⁸⁶Rb uptake

Human kidney PT cells, HK-2 (ATCC) or HKC11 (provided by Dr. Racusen, Johns Hopkins University) were treated with 10 µM PF670462 for 24 h in serum free DMEM:F12 containing 5 mM glucose. Ouabain-sensitive ⁸⁶Rb uptake was measured as an index of Na⁺/K⁺ ATPase-mediated ion transport as described previously¹⁵⁰. Briefly, cells were treated with 5 µM monensin for 30 min to short-circuit Na channels so as to measure Na⁺/K⁺ ATPase-mediated ion transport at V_{max}. A trace amount of ⁸⁶Rb (~1

$\mu\text{Ci/ml } ^{86}\text{RbCl}$) was added to the cells. Uptake was carried out for 10 minutes, after which the cells were washed five to six times with ice-cold PBS. One half of the cells were treated with 1 mM ouabain along with monensin for 30 min. The cells were lysed overnight in 0.5 N NaOH containing 0.1% Triton X-100 at 37°C. An aliquot (100 μl) of the lysate was used to measure radioactivity or to measure protein. The difference between ^{86}Rb uptake measured in the presence of 1 mM ouabain and the absence of ouabain was used as a measure of Na^+/K^+ ATPase-mediated transport activity. Uptake data are calculated as nanomoles of ^{86}Rb accumulated per milligram of protein per 10 min and expressed as percent difference from the vehicle treated cells.

Statistical Analysis.

All data are represented as mean \pm standard error of the mean (SEM) with $n=3$ or more. Statistical analyses were performed using Graphpad Prism v6. All graphs/plots were made with Graphpad Prism v6. An unpaired Student's *t*-test was used to compare differences between control and treated groups. All P values less than 0.05 were considered significant.

Results

Per1 must be phosphorylated by CK1 δ/ϵ in order to enter the nucleus¹³². Our lab has previously shown that pharmacological inhibition of CK1 δ/ϵ recapitulates the effects of Per1 knockdown, including decreased αENaC mRNA levels, protein levels, and ENaC activity^{133, 134}. To determine if Per1 regulates NHE3, SGLT1, and SGLT2 *in vivo*, WT mice were treated with vehicle or the CK1 δ/ϵ inhibitor PF670462 as previously described⁹⁶. Kidneys were harvested and cortex dissected. mRNA levels of NHE3, SGLT1, and SGLT2 were measured by qPCR. PF670462 treatment resulted in significantly decreased levels of NHE3 (Figure 2-1A) and SGLT1 (Figure 2-1B), when

compared to vehicle treated mice. SGLT2 mRNA levels did not appear to be affected by PF670462 treatment (Figure 2-1C). We have previously demonstrated that PF670462 treated mice had significantly reduced levels of nuclear Per1 in kidney cortex¹³⁸.

To further investigate our *in vivo* results, the human PT cell line HK-2 was employed for subsequent studies^{151, 152}. Per1 was knocked down using siRNA in HK-2 cells and mRNA levels of NHE3, SGLT1, and SGLT2 were measured by qPCR. As expected, Per1 knockdown resulted in significantly decreased mRNA expression of Per1 (Figure 2-2A) as we have previously demonstrated^{19, 134, 135}. Consistent with our *in vivo* results, Per1 knockdown resulted in significantly decreased expression of NHE3 (Figure 2-2B) and SGLT1 (Figure 2-2C), but not SGLT2 (Figure 2-2D).

To further explore the potential role of Per1 in the regulation of NHE3 and SGLT1, HK-2 cells were treated with PF670462 and NHE3, SGLT1, and SGLT2 mRNA expression was determined by qPCR. After 24 hours, treatment with 10 μ M PF670462 resulted in significant reduction of NHE3 and SGLT1 mRNA (Figure 2-3A and B). SGLT2 expression was not affected at any time point or dose tested (Figure 2-3C). To insure that CK1 δ/ϵ inhibition decreased Per1 nuclear entry, HK-2 cells were treated with vehicle or 10 μ M PF670462 for 24 hours and nuclear extracts were collected. Western blotting was performed to assess nuclear Per1 levels. As expected, and as we have shown previously in other cell types^{133, 134}, nuclear Per1 levels were significantly decreased with CK1 δ/ϵ inhibition (Figure 2-4A; quantified in 2-4B).

Measurement of short-lived heterogeneous nuclear RNA (hnRNA) is a measure of transcriptional activity^{153, 154}. To assess if the effect of CK1 δ/ϵ inhibition or Per1 knockdown on NHE3 and SGLT1 was transcriptional, hnRNA levels were assessed by

PCR amplification of intron-exon junctions using cDNA templates from HK-2 cells treated with either PF670462 for 24 hours or Per1 siRNA for 48 hours. Per1 siRNA-mediated knockdown or blockade of Per1 nuclear entry led to significantly decreased hnRNA expression of both NHE3 (Figure 2-5A) and SGLT1 (Figure 2-5B).

As mentioned above, regulation of target genes by circadian clock proteins is mediated through interaction of these proteins with E-box response elements in the promoters of target genes. Therefore, to assess if Per1 and CLOCK interact with the endogenous promoters of NHE3 and SGLT1, ChIP was performed in HK-2 cells treated with either vehicle or 10 μ M PF670462 for 24 hours. Three primer sets per gene were designed to amplify short regions containing putative E-box sites upstream of the transcription start site (Panel A in Figures 2-6 and 2-7). Loading was demonstrated by performing the PCR on 10% input (Panel B in Figures 2-6 and 2-7). IgG was used as a negative control (Panel F Figures 2-6 and 2-7). CK1 δ/ϵ inhibition led to significantly decreased binding of RNA Pol II at all three sites in the NHE3 promoter, indicative of decreased transcription, further corroborating our mRNA and hnRNA data (Figure 2-6C). CK1 δ/ϵ inhibition led to significant decrease of CLOCK and Per1 with the second and third promoter regions, but neither CLOCK nor Per1 appeared to interact with the promoter region amplified by the first primer set (Figure 2-6D and E). On the SGLT1 promoter, CK1 δ/ϵ inhibition led to significantly decreased RNA Pol II binding at all three promoter regions (Figure 2-7C). As with NHE3, CK1 δ/ϵ inhibition led to a significant decrease of CLOCK and Per1 interaction with the second and third promoter regions, but there was no apparent interaction with the promoter region amplified by the first primer set (Figure 2-7D and E).

To test whether the effects of CK1 δ/ϵ inhibition on NHE3 and SGLT1 expression extended to the level of protein, plasma membrane and intracellular fractions were isolated from HK-2 cells treated with vehicle or 10 μ M PF670462 for 24 hours. Western blot analysis was performed to determine changes in NHE3 and SGLT1 protein levels from intracellular and membrane fractions. CK1 δ/ϵ inhibition led to significant decrease of both NHE3 and SGLT1 in both the membrane and intracellular fraction (Figure 2-8A and B), consistent with the hypothesis that inhibition of Per1 nuclear entry leads to decreased transcription of NHE3 and SGLT1^{134 20, 96 96, 138}.

In order to test the effect of CK1 δ/ϵ inhibition on ion transport, Na⁺/K⁺ ATPase activity was measured as ouabain-sensitive ⁸⁶Rb uptake in two cell lines representative of human PT cells (HK-2 and HKC11¹⁵⁵). Ouabain-sensitive Na⁺/K⁺ ATPase activity was significantly decreased in both cell lines following treatment with 10 μ M PF670462 (Figure 2-9). Although a direct effect of CK1 δ/ϵ on the Na pump cannot be ruled out, decreased Na⁺/K⁺ ATPase activity indirectly provides support for the hypothesis that pharmacological blockade of CK1 δ/ϵ results in decreased NHE3 and SGLT1 activity.

Discussion

The present study demonstrates a role for Per1 in the transcriptional regulation of NHE3 and SGLT1 in PT cells. Pharmacological blockade of Per1 nuclear entry *in vivo* and *in vitro* resulted in decreased mRNA expression of NHE3 and SGLT1. CHIP and hnRNA assays performed in HK-2 cells consistently demonstrated that the effect of Per1 on NHE3 and SGLT1 appears to involve a transcriptional mechanism. Importantly, we demonstrated that the regulation of NHE3 and SGLT1 by Per1 extends to the protein level. Pharmacological blockade of nuclear Per1 entry resulted in decreased protein expression of NHE3 and SGLT1 at the membrane and in the cytosol.

Consistent with decreased expression of NHE3 and SGLT1 at the apical membrane, which would predict decreased apical sodium reabsorption, pharmacological inhibition of CK1 δ/ϵ was associated with decreased Na⁺/K⁺ ATPase activity in two different PT cell lines, HK-2 and HKC11. Together, these data suggest a role for Per1 in the positive regulation of genes that function in sodium reabsorption in PT cells; this model is entirely consistent with our previously proposed model for Per1 action on ENaC in cortical CD cells¹⁹ and on NCC in distal convoluted cells¹³⁸.

Previous work has demonstrated that NHE3 is a potential circadian target in mouse renal medulla^{13, 141}, while others studies have demonstrated that SGLT1 is similarly regulated in the colon^{142, 143}. However, it was unknown if NHE3 and SGLT1 were regulated by Per1 in the kidney. For the first time, we have demonstrated that both NHE3 and SGLT1 are Per1 targets in a cell model of the human PT. Along with our previous data on Per1 regulation of transporters in the cortical CD^{19, 20, 80, 133-135}, and in the DCT¹³⁸. These data predict that loss of Per1 should result in decreased renal sodium reabsorption, leading to decreased plasma volume and decreased BP. Consistent with these predictions, we have recently shown that mice with reduced levels of Per1 expression exhibit renal sodium wasting⁹⁶ and that Per1 KO mice display lower BP compared to WT controls¹⁹.

Increasing evidence supports an important role for the circadian clock in the regulation of BP control and renal sodium handling (Reviewed in^{26, 129, 130, 156}). Per2 KO mice display decreased 24hr diastolic BP, increased heart rate, and reduced diurnal dipping⁶⁹. Bmal1 KO mice have reduced BP in the active phase, leading to a blunting of circadian BP variation⁶¹. CLOCK KO mice are hypotensive and display dysregulated

urinary sodium excretion with mild diabetes insipidus^{18, 64}. Cry1/2 KO mice display salt sensitive hypertension partially due to increased aldosterone production and serum aldosterone levels⁶⁵. This was potentially due to increased expression of 3-beta dehydrogenase-isomerase, an enzyme in the aldosterone synthesis pathway. We have shown that Per1 KO mice have significantly lower BP than WT controls¹⁹ and excrete more sodium in their urine^{20, 96}. Mice with reduced levels of Per1 also have decreased plasma levels of aldosterone and decreased renal sodium reabsorption⁹⁶. While a multitude of factors contribute to the BP phenotypes of these animals (including, but not limited to, the heart (reviewed in¹⁵⁷), sympathetic activity^{158, 159}, the vasculature¹⁶⁰, and/or nitric oxide^{161, 162}), accumulating data from multiple labs suggests that salt handling by the kidney is likely to play an important role.

Consistent with our findings, a recent human study demonstrated that Per1 was overexpressed in the renal medulla of human hypertensive patients compared to normotensive controls¹⁶³. Given the magnitude of the BP and renal sodium phenotypes in Per1 KO or Per1 heterozygous mice, it is perhaps not surprising that multiple sodium transporter genes along the nephron are regulated by Per1. Taken together, the data presented here demonstrates a novel role for Per1 in the transcriptional regulation of NHE3 and SGLT1 in the kidney *in vivo* and in PT cells *in vitro*. Determining the functional effects of this regulation will require further investigation. Reduced NHE3 expression may lead to reduced GFR¹⁶⁴, which was not tested in the present study. On the other hand, knockout of NHE3 in a rodent model was associated with reduced BP without alteration in the autoregulation of GFR¹⁶⁵. Per1 appears to regulate gene expression in a manner consistent with driving sodium reabsorption in cortical CD

cells^{19, 20, 80, 133-135} and DCT cells¹³⁸. Now for the first time, we have demonstrated a role for Per1 in the regulation of sodium transporter gene expression in PT cells.

Table 2-1. Sequences and exon numbers.

Gene	Spanning Exon	Forward Primer	Reverse Primer
NHE3	7	5'CTGTGACTCTGGGCCT AC-3'	5'ACTGCTCCGAGATGTTGG- 3'
SGLT1	3	5'GCTCCTTCTCCAATCCT C-3'	5'AGCAAAGAGGGAGGCTCC- 3'
GAPDH	4	5'AAGAAATGTGCTTTGGG G-3'	5'GACTCCACGACGTACTIONCA- 3'

NHE3, Na⁺/H⁺ exchanger 3; SGLT1, Na⁺-glucose transporter 1.

Table 2-2. Primer sets for each gene.

	Forward Primer	Reverse Primer
NHE3		
Primer set 1	5'GTCAGAAGACACATCCAT-3'	5'CTCATGCATACATCCC-3'
Primer set 2	5'TGGAAACCTTGTGAGCGA-3'	5'ACCCCAGGCTCTGAGATG-3'
Primer set 3	5'CAGGTTCTGCTGAAGAC-3'	5'CGCAGCTCCTGGGATG-3'
SGLT1		
Primer set 1	5'GACTGCAACCTCCACCT-3'	5'TTCTTAGTTTAGGCCGGG-3'
Primer set 2	5'CGGCAAGAGAGAGTCCAA-3'	5'TTAGGGAGGGACTAGCTG-3'
Primer set 3	5'CAAGTCCTTAGAAGGCAT-3'	5'AGGTAAGAGCTGTCCTGC-3'

NHE3, Na⁺/H⁺ exchanger 3; SGLT1, Na⁺-glucose transporter 1.

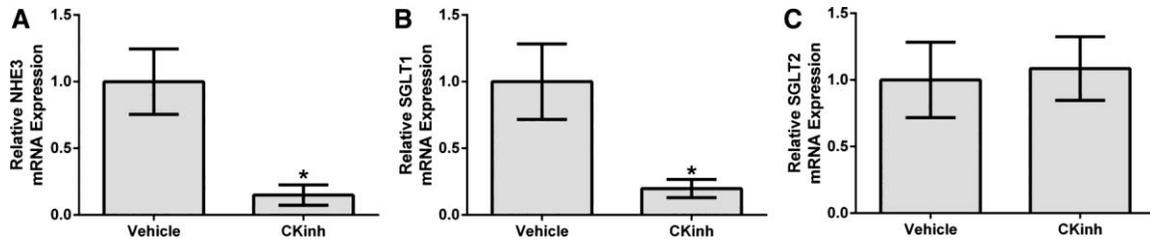


Figure 2-1. Pharmacological blockade of Per1 nuclear entry *in vivo* results in decreased NHE3 and SGLT1 expression, but not SGLT2 expression in the renal cortex. Weight matched male WT 129/sv mice were injected subcutaneously with vehicle (20% hydroxypropyl b-cyclodextrin) or 30 mg/kg PF670462 every 12 hrs for 2.5 days starting at noon and euthanized at midnight 12 hours after last injection as previously described⁹⁶. Kidneys were harvested, cortex dissected, and NHE3 (Panel A), SGLT1 (Panel B), or SGLT2 (Panel C) mRNA expression was measured by qPCR, n=4 *p<0.05 compared to WT. Values are represented as the mean ± SEM.

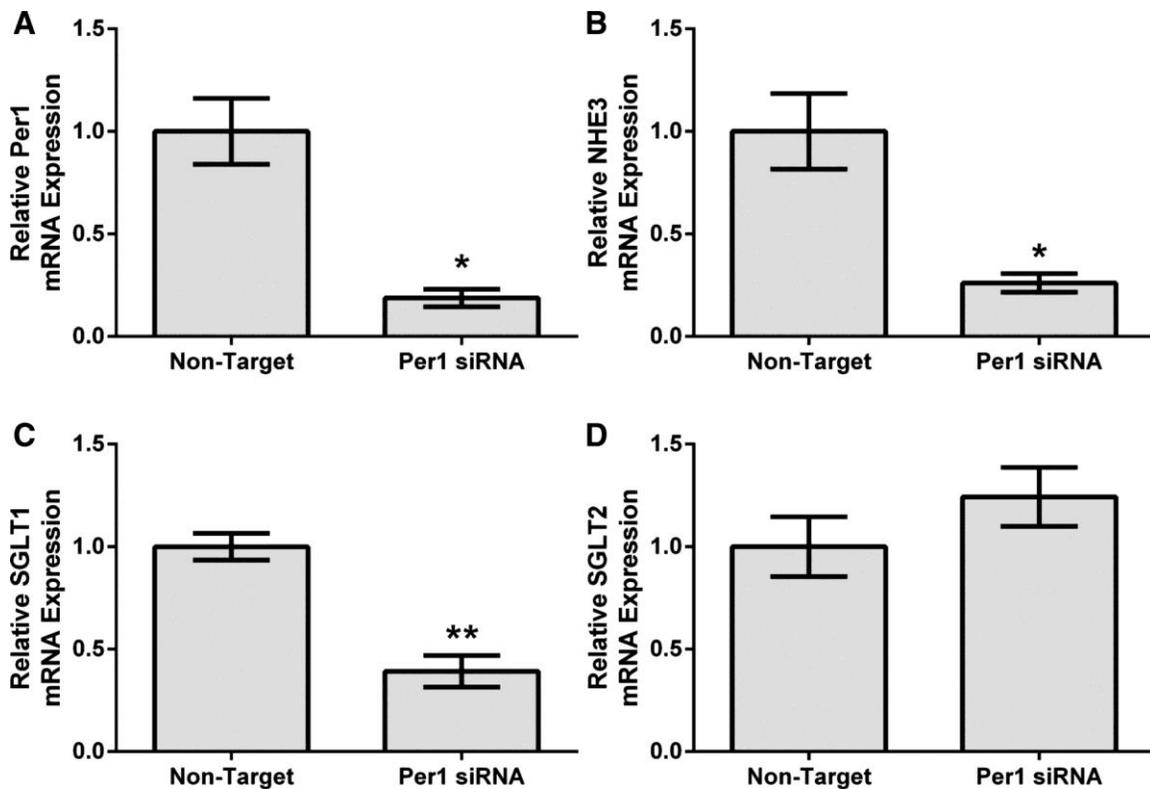


Figure 2-2. Per1 knockdown results in decreased NHE3 and SGLT1 expression, but not SGLT2 expression in HK-2 cells *in vitro*. HK-2 cells were treated with Non-Target or Per1 siRNA for 48 hours. qPCR was used to evaluate changes in Per1 (Panel A), NHE3 (Panel B), SGLT1 (Panel C), or SGLT2 (Panel D) mRNA expression in Per1 siRNA versus non-target siRNA control, n=3. *p<0.05, **p<0.01, Values are represented as the mean ± SEM.

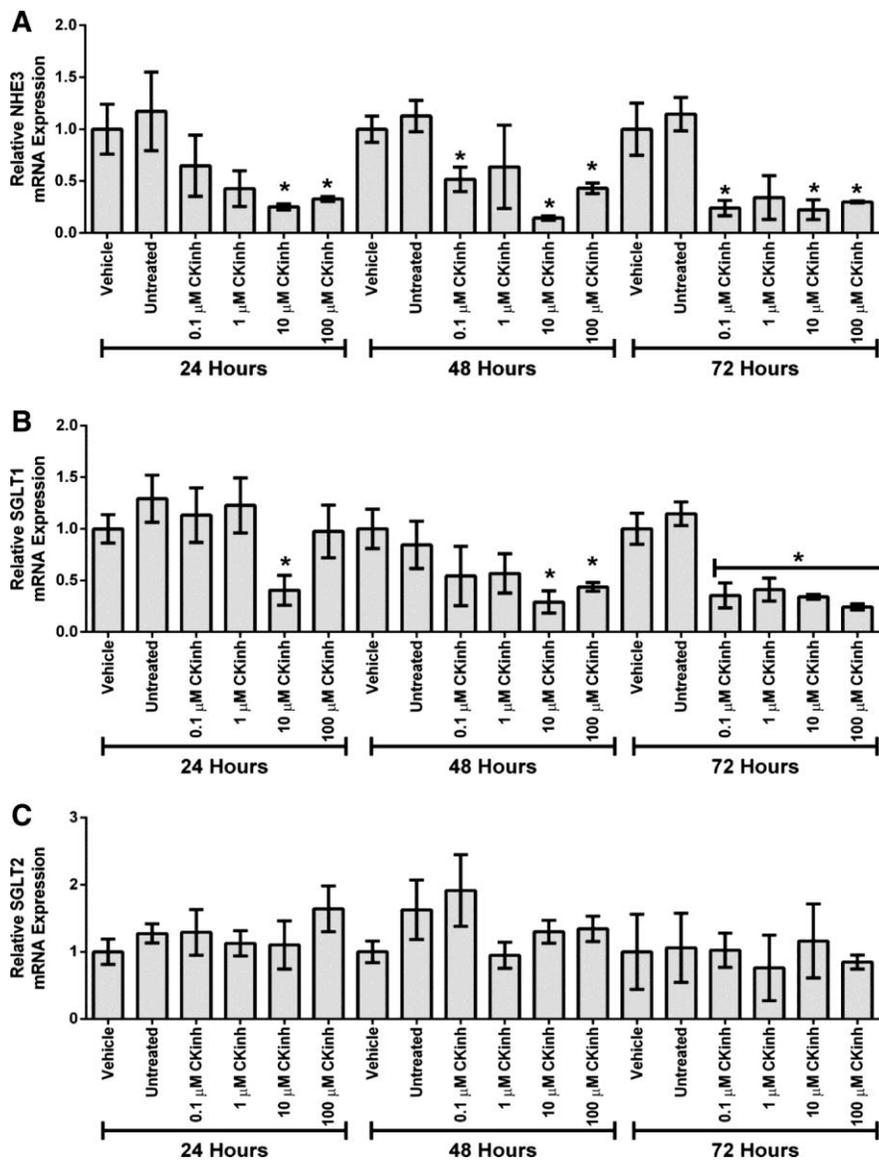


Figure 2-3. Pharmacological blockade of Per1 nuclear entry results in decreased NHE3 and SGLT1 expression, but not SGLT2 expression *in vitro*. A-B. HK-2 cells were treated with the Casein Kinase 1 δ/ϵ inhibitor PF670462 with either 0.1, 1, 10, or 100 μ M for 24, 48, or 72 hours. qPCR was used to evaluate changes in NHE3 (Panel A), SGLT1 (Panel B), or SGLT2 (Panel C) expression in PF670462-treated cells versus vehicle (water) treated cells, n=3. *p<0.05. Values are represented as the mean \pm SEM. CKinh - Casein Kinase 1 δ/ϵ inhibitor.

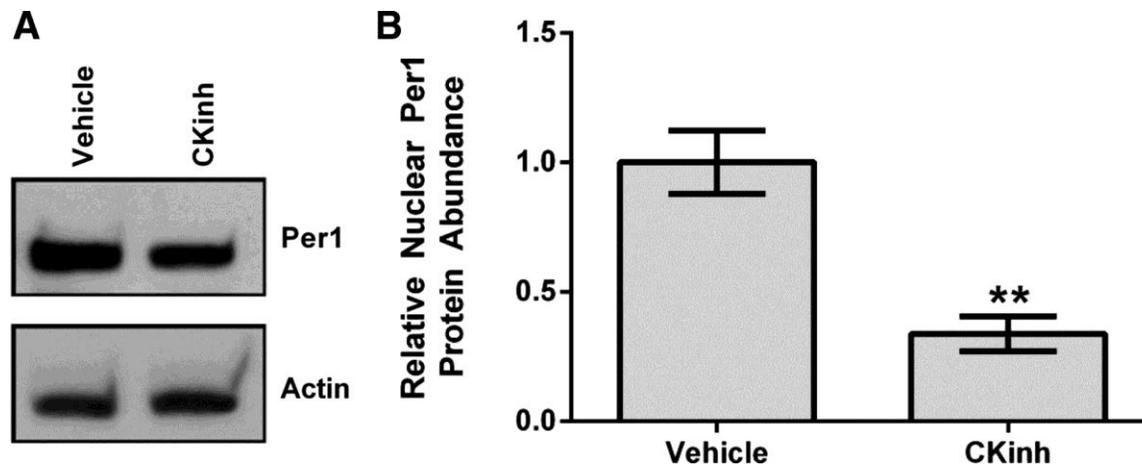


Figure 2-4. Pharmacological blockade of Per1 nuclear entry results in decreased nuclear Per1 *in vitro*. A. Nuclear extracts were collected from HK-2 cells treated with 10 μ M Casein Kinase 1 δ/ϵ inhibitor PF670462 or water for 24 hours. Western blot analysis was performed using anti-Per1 (nuclear Per1 ~50 kDa) or anti- β -actin (~42 kDa) antibodies as a loading control. Data are representative of three independent experiments. B. Densitometry analysis was used to quantitate the level of Per1 in panel A, n=3 **p<0.01. Values are represented as the mean \pm SEM. CKinh - Casein Kinase 1 δ/ϵ inhibitor.

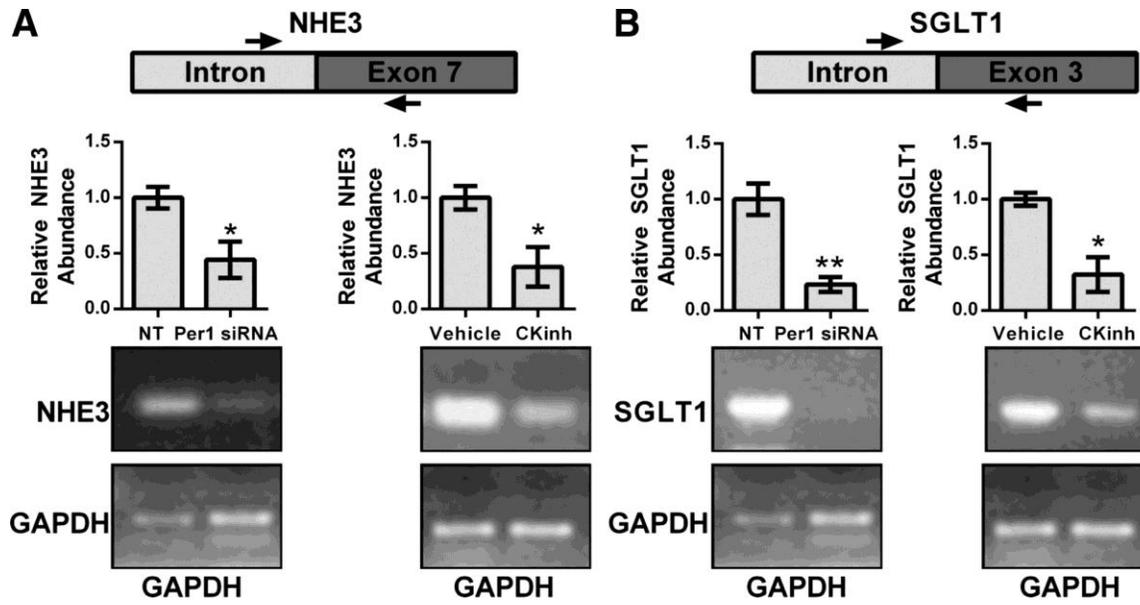


Figure 2-5. Pharmacological blockade of Per1 nuclear entry results in decreased transcription of NHE3 and SGLT1. Primers were designed to amplify regions of hnRNA spanning the intron/exon boundary of the NHE3 (Panel A) and SGLT1 (Panel B). GAPDH was used as a cDNA loading control. Arrows are representative of primer location. Bands were quantitated using ImageJ densitometry (rsbweb.nih.gov/ij). Signal strength was normalized to the relevant vehicle or CKinh treated GAPDH control, n=3 *p<0.05, values are represented as the mean ± SEM. CKinh - Casein Kinase 1 δ/ϵ inhibitor.

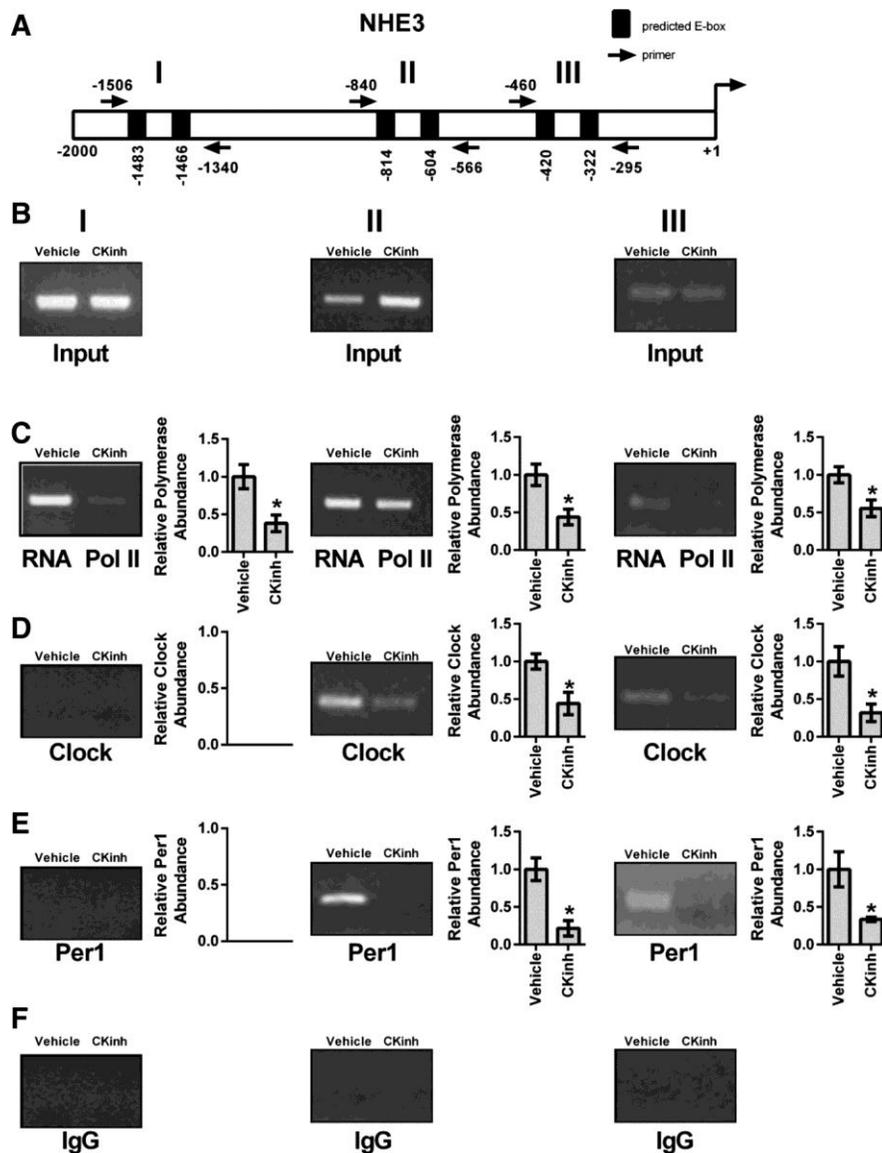


Figure 2-6. Pharmacological blockade of Per1 nuclear entry leads to decreased occupancy of CLOCK and Per1 on the NHE3 promoter in HK-2 cells. Chromatin immunoprecipitation experiments were performed using HK-2 cells treated with either vehicle (water) or 10 μ M PF670462 for 24 hrs. Endpoint PCR was performed using primer sets flanking three putative E-boxes in the NHE3 promoter (Panel A). Chromatin immunoprecipitations were performed using anti-Pol II (Santa Cruz) (Panel C), anti-CLOCK (Pierce) (Panel D), anti-Per1 (Pierce) (Panel E), or rabbit IgG (Bethyl) (negative control) (Panel F) antibodies. Bands were quantitated using densitometry, which was performed using ImageJ (rsbweb.nih.gov/ij/). Signal strength was normalized to the relevant vehicle or CKinh treated input control, n=3. *p<0.05, values are represented as the mean \pm SEM

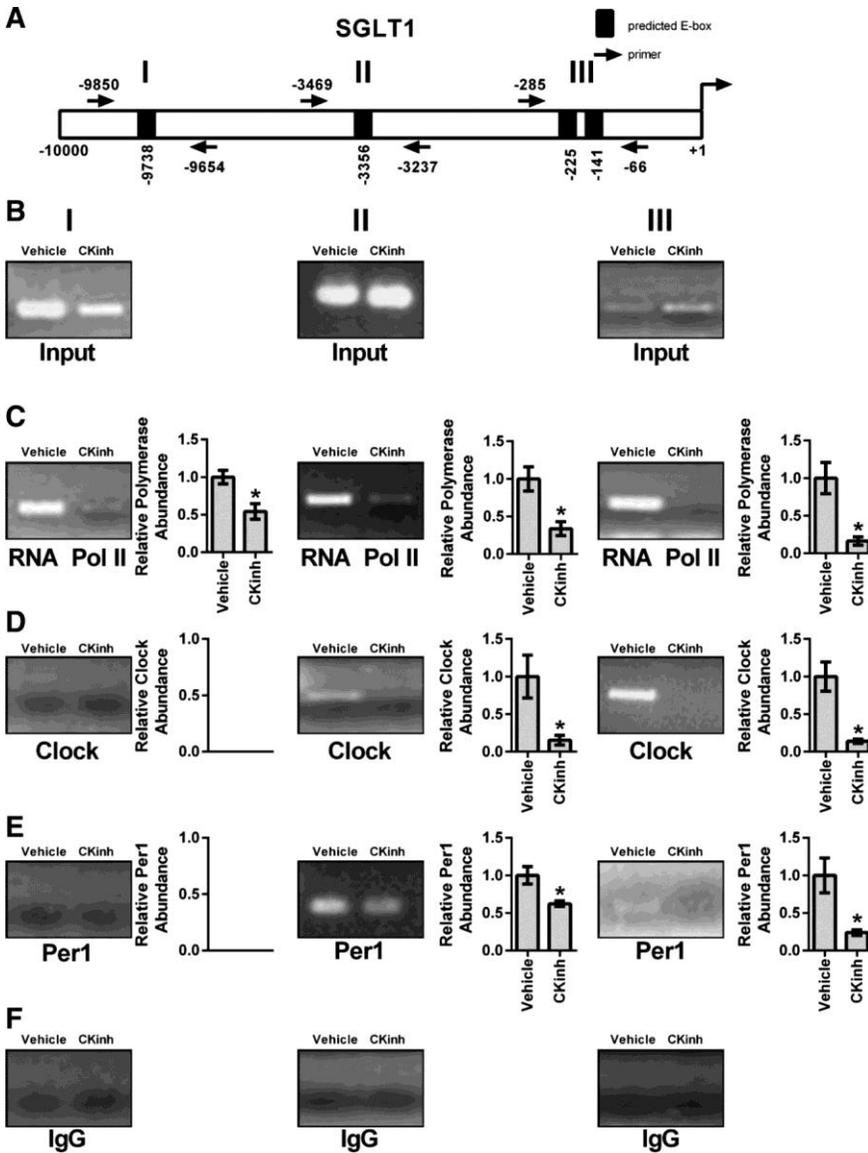


Figure 2-7. Pharmacological blockade of Per1 nuclear entry leads to decreased occupancy of CLOCK and Per1 on the SGLT1 promoter in HK-2 cells. Chromatin immunoprecipitation experiments were performed using HK-2 cells treated with either vehicle (water) or 10 μ M PF670462 for 24 hrs. Endpoint PCR was performed using primer sets flanking three putative E-boxes in the SGLT1 promoter (Panel A). Chromatin immunoprecipitations were performed using anti-Pol II (Santa Cruz) (Panel C), anti-CLOCK (Pierce) (Panel D), anti-Per1 (Pierce) (Panel E), or rabbit IgG (Bethyl) (negative control) (Panel F) antibodies. Bands were quantitated using densitometry, which was performed using ImageJ (rsbweb.nih.gov/ij). Signal strength was normalized to the relevant vehicle or CKinh treated input control, $n=3$. * $p<0.05$, values are represented as the mean \pm SEM.

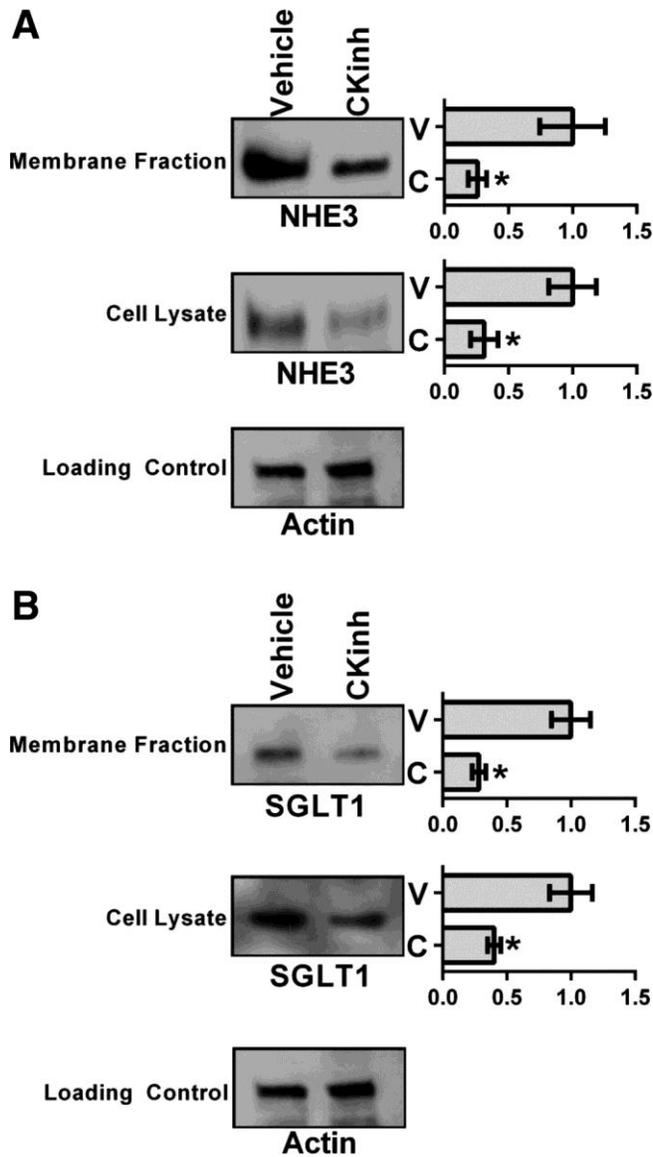


Figure 2-8. Pharmacological blockade of Per1 nuclear entry results in decreased membrane and intracellular protein expression of NHE3 and SGLT1 in HK-2 cells. Membrane and intracellular extracts were collected from HK-2 cells treated with 10 μ M Casein Kinase 1 δ/ϵ inhibitor PF670462 or water for 24 hours. Western blot analysis was performed using anti-NHE3 (~85 kDa) (Panel A), anti-SGLT1 (~75 kDa) (Panel B) or anti- β -actin (~42 kDa) antibodies as a loading control. Data are representative of three independent experiments. Densitometry analysis was used to quantitate the level of NHE3 and SGLT1 in panel A-B, n=3 *p<0.05. Values are represented as the mean \pm SEM. CKinh - Casein Kinase 1 δ/ϵ inhibitor.

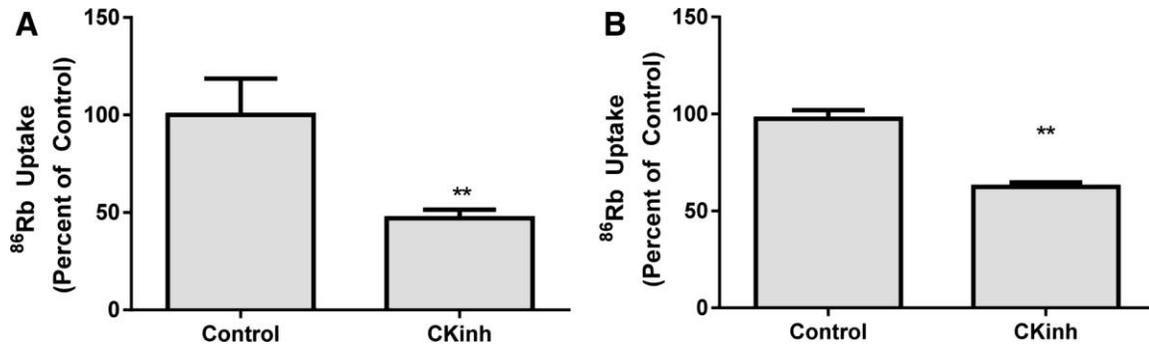


Figure 2-9. Na^+/K^+ ATPase activity is reduced in HK-2 and HKC11 cells following pharmacological blockade of CK1 δ/ϵ . HK-2 (Panel A) and HKC11 (Panel B) cells both show significantly reduced Na^+/K^+ ATPase activity following treatment with the Casein Kinase 1 δ/ϵ inhibitor. Cells were treated with 10 μM PF670462 for 24 h in serum free DMEM:F12 containing 5 mM glucose. They were then treated with 5 μM monensin to short circuit Na channels and thus measure Na^+/K^+ ATPase transport at V_{max} . Half of the cells were treated with 1 μM ouabain for 30 min followed by addition of $^{86}\text{RbCl}$ for 10 min to determine Na^+/K^+ ATPase specific activity. Cells were then lysed and protein was measured to normalize uptake to total protein. Values are calculated as nmoles of ^{86}Rb accumulated per milligram of protein per 10 min and expressed as percent activity relative to the vehicle treated control cells. $n=6$ for Panel A, $n=12$ for Panel B, $**p<0.01$. Values are represented as the mean \pm SEM.

CHAPTER 3 DOCP-SALT TREATMENT LEADS TO NONDIPPING HYPERTENSION IN PER1 KNOCKOUT MICE

Background

The circadian (Latin for “around a day”) clock functions to synchronize our daily activities, on a physiological and molecular level, with the time of day and certain behaviors that usually occur around the same time each day^{2*}. Many physiological functions exhibit circadian rhythms, including BP. Healthy individuals experience a 10-20% decrease in BP at night. Individuals who do not exhibit this “dip” in resting BP are termed “non-dippers.” Non-dipping is associated with an increased risk of chronic kidney disease^{91, 92}, adverse cardiovascular events^{90,166} and can also be used to predict future cardiovascular events¹⁶⁷. These risks are further increased by chronic hypertension, or high BP, which is also a risk factor for CVD. CVD is the leading cause of death of Americans¹², accounting for nearly 1 million deaths/year¹⁶⁸. A subset of hypertensive patients exhibit salt-sensitive hypertension in which BP is abnormally elevated after high salt intake, implicating aberrant sodium handling in the pathogenesis of the disease. The underlying mechanisms of these pathologies remain incompletely understood.

A central clock located in the suprachiasmatic nucleus of the brain is entrained by light signals from the retina²¹. These signals are then relayed through neuronal and humoral pathways to peripheral clocks located in other tissues and organs of the body. The importance of circadian proteins for BP control is illustrated by clock KO mice.

*Reprinted with Permission from: Solocinski K, Holzworth M, Wen X, Cheng KY, Lynch IJ, Cain BD, Wingo CS, Gumz ML. Desoxycorticosterone pivalate-salt treatment leads to non-dipping hypertension in per1 knockout mice. *Acta Physiol (Oxf)*. 2017;220:72-82

Every clock gene KO mouse that has been tested exhibits a BP phenotype¹⁶⁹. Global *Bmal1* KO mice lose circadian rhythmicity of BP and are hypotensive⁶¹. Loss of *Bmal1* in renin-producing cells in the kidney also leads to a significant decrease in BP compared to controls⁶³. Loss of *Clock* causes mice to become hypotensive yet retain the circadian rhythm of BP¹⁸. *Cry1/Cry2* global KO mice exhibit salt-sensitive hypertension^{13,65}. Mice lacking all three *Period* isoforms lose their BP dip on a low salt diet¹⁷⁰. Thus, the clock is a major regulator of baseline BP and rhythmic BP.

The kidney is a critical regulator of BP and many aspects of renal function oscillate in a circadian manner including sodium excretion, renal blood flow²⁴ and glomerular filtration rate²³. Aldosterone, a critical regulator of renal sodium handling, is also released in a circadian manner⁵³. Gumz et al. identified *Per1* as a novel aldosterone target gene in renal CD cells⁷⁸. Circadian clock-mediated regulation of a renal sodium transporter was first reported by Okamura and colleagues who demonstrated that the Na⁺,H⁺ exchanger NHE3 exhibited time-dependent changes in expression¹³. Gumz et al. provided the link between the clock and the ENaC ENaC with the finding that PER1 transcriptionally regulates α ENaC²⁰. Subsequent studies provided further evidence that PER1 coordinately regulates several genes encoding proteins that function in renal sodium reabsorption^{16, 88, 171}. Global loss of *Per1* on the hypertensive, salt-sensitive 129/sv mouse strain resulted in significantly reduced BP compared to WT (WT) controls on a normal diet¹⁹.

The goal of the present study was to characterize the role of PER1 in the normotensive C57BL/6J background strain. Small but significant differences in mean arterial pressure (MAP) were observed between WT and PER1 KO mice at baseline.

Since *Per1* is an aldosterone target gene and coordinately regulates many renal sodium transport genes, we subjected WT and *Per1* KO mice to a high salt (HS) diet in combination with desoxycorticosterone pivalate (DOCP) injection as a model for evaluating salt-sensitive changes in BP. DOCP is a long-acting aldosterone analog that bypasses the sodium-excreting effects of Renin-Angiotensin-Aldosterone System (RAAS) suppression normally seen with high salt intake (reviewed in¹⁷²) and leads to increased renal sodium reabsorption. Interestingly, loss of PER1 in C57BL/6 mice subjected to HS/DOCP treatment resulted in significantly elevated BP accompanied by disruption of the normal BP rhythm. Thus, PER1 appears to be an important regulator of baseline and rhythmic BP in C57BL/6J mice.

Methods

Mice

Male WT C57BL/6J mice and C57BL/6J *Per1* KO between the ages of 14-18 weeks were used for these experiments (original heterozygous mice were the kind gift of Dr. Shin Yamazaki, Vanderbilt¹⁷³). WT and *Per1* KO mice were litter mates derived from mating mice heterozygous for *Per1*. Mice were maintained on a normal 12:12 L:D cycle at the NF/SG VA Healthcare animal facility. Experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of UF and VA Medical Center IACUCs. Mice were implanted with telemetry devices from Data Sciences International through the carotid artery and extended into the aortic arch following established protocol¹⁷⁴ and as described previously¹⁹. Mice were allowed at least seven days to recover. Mice were then placed on control diet (0.2% NaCl, Harlan) for at least 3 days, during which time baseline BP recordings were made. Mice were then placed on a high salt (4% NaCl, Harlan) diet for 3 more days, followed by injection

with 75 µg/g bw DOCP. Three days after the injection and after six total days on a high salt diet, telemetry recordings for the HS/DOCP period were made.

Tissue dissection and quantitative real time RT-PCR (qPCR)

Tissue dissection and QPCR were performed as described previously in¹⁷¹.

Flame Photometry

Flame photometry was used to determine urine and plasma Na and K concentrations.

Aldosterone ELISA

Plasma aldosterone was measured by ELISA as described in⁹⁶.

Statistics

Day values are the 3-day averages of MAP between 6am (ZT0) through 5pm (ZT11). Night values are the 3-day averages of MAP recorded between 6pm (ZT12) and 5am (ZT23). Paired t-tests and standard and repeated measures 2-way ANOVAs were performed as appropriate using GraphPad Prism software (version 6.07 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Cosinor analysis was done using ClockLab software (Actimetrics) and the generous help of Dr. Joshua Speed (University of Alabama Birmingham). $p < 0.05$ was considered significant. JTK Cycle was used as described¹⁷⁵. The program cosinor was downloaded from www.Circadian.org (last accessed 09/01/2016). Values are shown as mean \pm SEM.

Results

Given our previous findings that *Per1* regulates a number of genes related to renal sodium reabsorption in vitro and in vivo in hypertensive 129/sv mice, we investigated the expression of candidate genes in WT normotensive C57BL/6 mice compared to *Per1* KO mice. Real time quantitative PCR (QPCR) was used to assess

relative differences in gene expression in the renal cortex between WT and *Per1* KO mice at two distinct time points in the circadian cycle. Kidneys were collected at noon and midnight in order to capture time points at the midpoint of the rest phase and the active phase, respectively. We investigated time and PER1-dependent changes in steady state mRNA expression for genes known to contribute to renal sodium reabsorption or the regulation of this process. The Angiotensin II receptor (AT₁R) leads to increased sodium reabsorption and BP through the RAAS and has positive effects on ENaC¹⁷⁶ and expression displays a significant change over time due to the interaction of time and genotype (p=0.0075) (Figure 3-1A). UBE2E3, a ubiquitin E3 ligase, has been previously shown to regulate the alpha subunit of the ENaC (α ENaC)⁸³ and is regulated by PER1¹⁶. As shown in Figure 3-1B, there was a significant effect of time on UBE2E3 expression (p=0.015) with a trend towards a genotype effect as well (p=0.065). Nitric oxide synthase 1 (NOS1) also regulates α ENaC¹⁷⁷ (Figure 3-1C) and trended towards an interaction between genotype and time. With-no-lysine kinase 1 (WNK1) is a regulator of the sodium chloride cotransporter¹⁷⁸ and trended towards a genotype-time interaction as well (Figure 3-1D). Figure 3-1E shows that α ENaC expression changed significantly with time (p=0.024) and a possible trend for an interaction between time and genotype was observed (p=0.12) (Figure 3-1E). In contrast to these changes, PCSK9, an ENaC-regulating protease, did not undergo any changes in expression relative to time or *Per1* KO (Figure 3-2A). Likewise, mRNA levels for the Endothelin A Receptor, a regulator of renal sodium balance and BP, did not change with time or *Per1* KO (Figure 3-2B). Therefore, *Per1* appears to selectively regulate only certain genes which govern sodium transport and reabsorption.

Given our previous findings that PER1 acts as a coordinate regulator of several genes in the kidney that participate in the regulation of renal sodium reabsorption, we hypothesized that *Per1* KO mice would exhibit altered BP. Due to the altered gene expression patterns observed in Figure 3-1, we compared MAP between WT and KO mice under baseline conditions. The mice were implanted with telemetry devices and after recovery MAP was monitored over a 3 day period. During the day when mice are inactive, WTs have a MAP of 102.1 ± 1.7 mmHg which increases to 112.5 ± 1.08 mmHg during the night when they are active, an increase of 10.4 ± 1.5 mmHg (Figure 3-3A). *Per1* KO mice exhibit an inactive phase MAP of 103 ± 1.4 mmHg which rises to $119.8 \pm .9$ during the active phase. This is a change of 16.8 ± 1.7 mmHg which is significantly higher than the inactive/active phase difference observed in WT mice ($p < 0.05$). In Figure 3-3B, hourly MAP data are shown. The 12 hour period of the inactive phase is indicated by the white areas between Zeitgeber Time 0 (ZT0, "lights on") and ZT12. The active phase is indicated by the shaded areas between ZT12 and ZT 0. The dip in MAP during the inactive phase is easily visualized, as well as the slightly higher MAP in the *Per1* KO mice seen during the dark/active period. Under these baseline conditions, significant differences between WT and *Per1* KO mice in terms of plasma aldosterone levels, plasma Na or K, or body weight were not observed (Table 3-1).

Given the subtle but significant differences in MAP between WT and *Per1* KO mice under control conditions, we examined the response of both groups to an established model of salt-sensitivity: high salt diet plus mineralocorticoid treatment. Following the baseline recordings presented in Figure 3-3, the same mice were given a high salt diet (4% NaCl) in addition to injection of DOCP. Figure 3-4A shows day and

night averages taken the same way as described in Figure 3-3. WT mice exhibit a significant inactive/active phase difference in BP with an increase from 101.5 ± 1.92 mmHg during the inactive phase to 114.5 ± 1.1 mmHg during the active phase. This is a 13 ± 2.2 mmHg increase which is not different from the inactive/active difference in BP of WT mice observed under control conditions. In contrast, *Per1* KO mice exhibited an inactive phase average MAP of 118.1 ± 1 mmHg that increased to 125.3 ± 1.5 mmHg during the active phase. This is a difference of only 7.2 ± 1.8 mmHg, which is significantly different from the WT and, importantly, classifies these mice as non-dippers. Panel B shows the hourly BP measurements, clearly illustrating the attenuated dip of the KO mice during the inactive phase.

In order to analyze the dipping pattern of the mice, we compared inactive/active phase differences in MAP of WT and *Per1* KO mice under control and HS/DOCP conditions (Figure 3-5). It is clear that *Per1* KO mice have a greater decrease in MAP from the active to inactive phase compared to WT on control diet (Figure 3-5A). The *Per1* KO dip of $14 \pm 1.4\%$ is significantly larger than the WT $9.2 \pm 1.3\%$ dip ($p < 0.05$) under baseline conditions. On HS/DOCP, WT mice maintain their dip in BP whereas *Per1* KO mice exhibit an attenuated inactive/active phase difference in BP (panel B). WT MAP decreases by $11.4 \pm 1.9\%$ from night to day while *Per1* KO mice decrease only $5.7 \pm 1.4\%$ ($P < 0.05$). Diastolic and systolic BP changed in parallel to MAP (Figure 3-6). In contrast to these differences in BP, inactive/active phase differences in activity patterns were higher in WT mice compared to *Per1* KO mice under control and HS/DOCP conditions (Figure 3-7). Thus, activity levels do not account for the higher MAP observed in *Per1* KO mice.

Finally, in order to determine if there was a change in the 24hr rhythm of BP, we subjected the hourly telemetry data to cosinor analysis. This allowed us to examine changes in circadian parameters that may have occurred in response to HS/DOCP and loss of PER1. Table 3-2 lists the values for amplitude (difference between the highest and lowest BP values), mesor (mean value for MAP across all time points), and phase (Zeitgeber time at which MAP peaks)¹⁷⁹. Consistent with our findings in Figure 3-4, there was a significant change in mesor between WT (107.6 ± 5) and *Per1* KO (121.2 ± 5.3) on HS/DOCP. *Per1* KO mice on HS/DOCP also had a significant phase shift (22.3 ± 1.3) compared to WT mice on HS/DOCP (19 ± 0.3 , $p=0.018$) as well as KO mice on control diet (18.2 ± 0.1 , $p=0.013$). We also used JTK cycle (Table 3-3) and cosinor analysis (Table 3-4) to evaluate circadian rhythms demonstrated significant rhythms in MAP ($p<0.0001$ for each mouse in every group).

Discussion

The most striking finding of this study was that loss of a single gene, *Per1*, in combination with high salt and mineralocorticoid treatment resulted in a non-dipping phenotype. Importantly, these significant results were observed in a relatively mild HS/DOCP model without surgical nephrectomy. This is also accompanied by a significant phase shift in the peak of MAP in *Per1* KO compared to WT mice. To the best of our knowledge, this is the first demonstration of a non-dipping, phase-shifted BP phenotype in a single clock gene KO mouse in response to a model of salt-sensitive hypertension.

We have previously demonstrated a role for PER1 in the regulation of renal sodium handling with accompanying effects on BP^{16, 80, 96, 171, 180}. The results of the present study further implicate PER1 as an important regulator of BP. When challenged

with HS/DOCP, *Per1* KO mice undergo increased BP as well as a disruption of the normal 24 hr rhythm of BP. Our previous work in the hypertensive 129/sv mouse strain demonstrated a protective effect of *Per1* KO on BP. This is not surprising given that strain-dependent differences in BP regulation are an established phenomenon, with wide-ranging baseline BP¹⁸¹, sodium sensitivity^{182,183} and susceptibility to renal damage¹⁸⁴ and diabetic nephropathy¹⁸⁵. Thus, the differences in baseline BP between *Per1* KO mice on 129/sv and C57BL/6J backgrounds are likely due to strain-dependent differences in background genetics.

Even though non-dipping is a recognized problem with estimates of non-dipping prevalence as high as 45%¹⁸⁶, the need for better treatments and thus better animal models remains. Other animal models of non-dipping hypertension have been identified using chronic models¹⁸⁷ or ablation of multiple genes^{170, 188}. In depth testing of Period isoform KOs in 129/sv mice by Rudic and colleagues further supports the importance of the PER proteins in BP regulation (Pati et al., 2016). In this study, Angiotensin II infusion into *Per2* KO mice in total darkness for 3 weeks elicited a non-dipping phenotype. Interestingly, a low salt diet resulted in non-dipping in *Per1/2/3* KO mice in normal light conditions as well. It is difficult to directly compare these results due to the differences in background strain and treatment conditions. However, it is clear that the PER proteins are important regulators of BP. Thus the novel model presented in this study is the first single gene knockout model to produce a hypertensive non-dipping phenotype which develops quickly under normal light/dark conditions. In addition, WT C57Bl/6J mice are quite resistant to the current study regimen and usually require some additional insult to see a phenotype^{183, 189}, making the non-dipping exhibited by *Per1* KO

mice even more remarkable. Moreover, C57BL/6J *Per1* KO mice are slightly hypertensive at baseline, which is a common characteristic of many non-dippers^{190, 191}.

Non-dipping in humans can likely be attributed to many factors including kidney damage and salt-sensitive hypertension (reviewed in¹⁹²). The model presented here is pertinent to human disease as it has been demonstrated that high salt intake can blunt the nocturnal dip in humans¹⁹³ and conversely, restricted salt intake can help increase this dip¹⁹⁴. The changes observed in these mice likely involve the contribution of multiple organ systems since the current model is a global KO. It is evident that the vasculature and heart contribute to rhythms in cardiovascular function (reviewed in¹⁹⁵) and the contribution of these systems to the regulation of BP rhythms is an active area of investigation. For example, db/db mice displayed a nondipping phenotype along with altered expression of clock gene mRNA levels in vascular smooth muscle tissue, which likely contributed to the nondipping phenotype observed in this mouse model of diabetes¹⁹⁶. While the actions of PER1 in the vasculature remain largely unknown, the vasculature is an important mediator of BP regulation through changes in vessel compliance. Decreased compliance is accompanied by an increase in SBP and no change or a decrease in DBP¹⁹⁷. The parallel changes in DBP and SBP observed in these mice suggest a largely volume-dependent mechanism of hypertension which likely involves the action of the kidneys. Diuretics have been shown to reverse non-dipping in humans¹⁹⁸, suggesting a volume-dependent mechanism as well. The kidney is further implicated in the etiology of non-dipping through the association of renal transplantation with an improvement in dipping status in humans¹⁹⁹. While certain SNPs in *Bmal1* have been associated with hypertension²⁰⁰, to the best of our knowledge

there have been no studies large enough to identify SNPs in *Per1* which could be attributed to salt-sensitivity or non-dipping in humans. Taken together with the results of the present study, these findings support a role for the circadian clock and the kidney in the maintenance of baseline BP and rhythmic BP. In conclusion, *Per1* KO C57BL/6J mice on HS/DOCP regimen may represent an important new model for understanding mechanisms underlying non-dipping hypertension.

Limitations of this study include the fact that the mice used in this study are global KO. Thus, tissue-specific contributions to the non-dipping phenotype cannot be assessed. These studies were also limited to male mice. The experiments presented here were performed in a normal 12:12 light/dark cycle. Since a truly circadian process is defined as one that persists in the absence of light cues, determination of a true circadian defect would require the use of total darkness, an experimental maneuver that is beyond the scope of the present report.

To place these findings in a clinical perspective, accumulating evidence suggests that chronotherapy, or dosing of medication at a specific time of day, can be helpful in treating non-dipping in certain patients^{201,202}. While chronotherapy or lifestyle changes can decrease non-dipping prevalence in some patients, not all are helped by these interventions. Since non-dipping increases the risk of cardiovascular events, there is a need for better models and treatments. Our novel model of *Per1* KO mice treated with a HS/DOCP regimen models a diminished dipping phenotype similar to that seen in humans and may provide a new way to test interventions. PER1 is again implicated in BP control and thus presents as a putative target for modulation of dipping status.

The correlation between non-dipping and cardiovascular risk highlights not only the importance of normal circadian rhythms to overall health but also the way in which BP measurements are taken. 24 hour ambulatory BP monitoring (ABPM) is a much more accurate predictor of dipping status and therefore future risk as it markedly reduces white-coat hypertension and placebo effects^{203,204,205}. Somewhat similar to white-coat hypertension experienced by humans, mice can become stressed by tail-cuff BP measurements^{206,207}. ABPM is a powerful method for BP studies since recordings are taken continuously as opposed to the snapshots generated from tail-cuff or office measurements.

In summary, here we present a new model of non-dipping hypertension: *Per1* KO mice on a HS/DOCP regimen. This is the first report of a single clock gene KO resulting in a non-dipping phenotype in response to high salt plus mineralocorticoid. The data implicate PER1 in the control of a normal dip in BP during the inactive period. This is especially significant given that C57BL/6J mice are often resistant to perturbations in BP. The new model of non-dipping proposed here (*Per1* KO mice on HS/DOCP) can also serve as a means of discovering new ways to treat non-dipping. In contrast to WT mice, global C57BL/6J *Per1* KO mice are relatively hypertensive at baseline and are sensitive to a high salt/mineralocorticoid model of hypertension. These *Per1* KO mice display attenuated dipping of BP which is similar to non-dipping hypertension in humans that is associated with increased risk of adverse cardiovascular events.

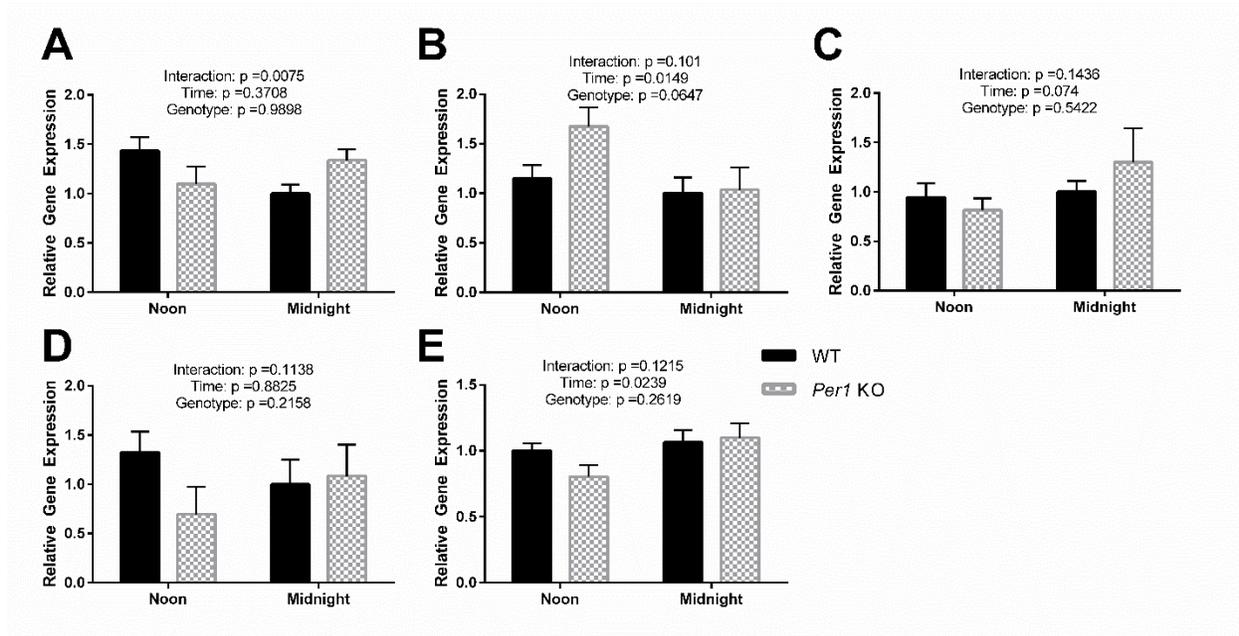


Figure 3-1. Differences in renal gene expression between WT and *Per1* KO mice at noon and midnight on control diet. Kidney cortex from WT (black bars) and *Per1* KO (checkered bars) mice collected at noon (inactive phase) and midnight (active phase) time points was analyzed by qPCR for differences in genes related to renal sodium transport. (A) AT₁R, (B) UBE2E3, (C) NOS1, (D) WNK1 and (E) α ENaC. Significance was calculated by two-way ANOVA. n=3 for KO at noon, n=4 for all others. p<0.05 was considered significant.

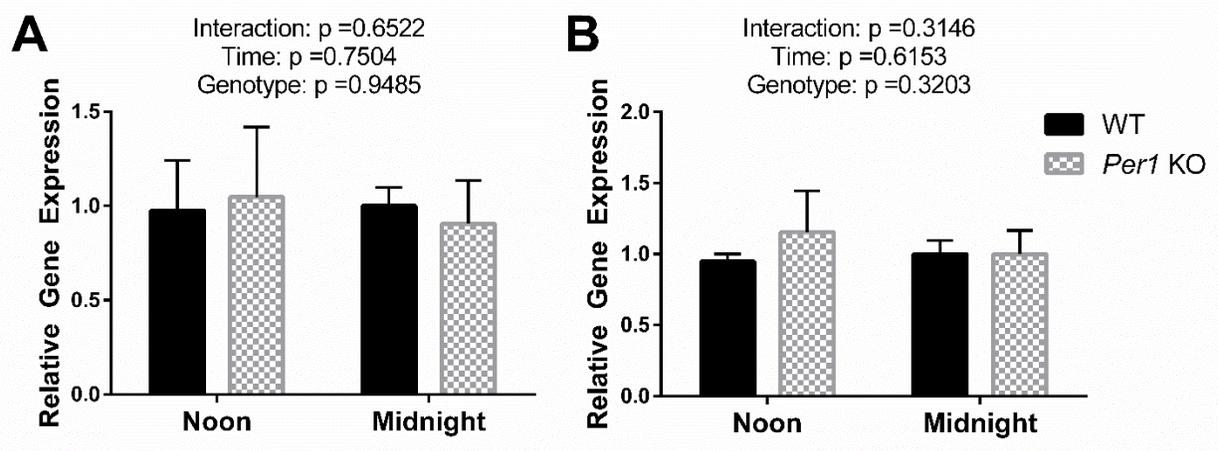


Figure 3-2. There are no significant differences in expression of Pcsk9 or ETA in WT or *Per1* KO mice at noon and midnight. Kidney cortex was collected at noon and midnight time points and made into cDNA. Gene expression measured by qPCR is not significantly different between WT and *Per1* KO mice at either noon or midnight for Pcsk9 (A) or ETA (B). Significance was calculated by two-way ANOVA. n=4 animals per group.

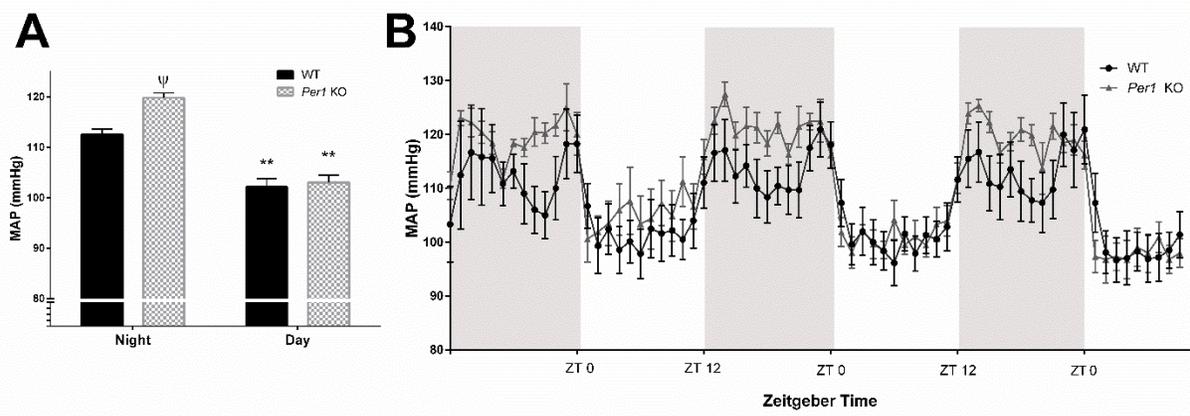


Figure 3-3. Inactive/active averages and 72 hour telemetry recordings of MAP in WT and *Per1* KO mice on control diet. A. Day and night MAP averages. Day values are the 3-day averages of MAP between 6am ZT0-ZT11. Night values are the 3-day averages of MAP recorded between ZT12-ZT23. B. Telemetry recording of MAP with lights on from ZT0-ZT12 depicted with light areas and ZT12-ZT0, the time when lights are off, shaded gray. n=8 for WT, n=6 for *Per1* KO. Significance was calculated by two-way ANOVA. * p< 0.01, ** p< 0.001 compared to same genotype, ψ p< 0.05 compared to same time.

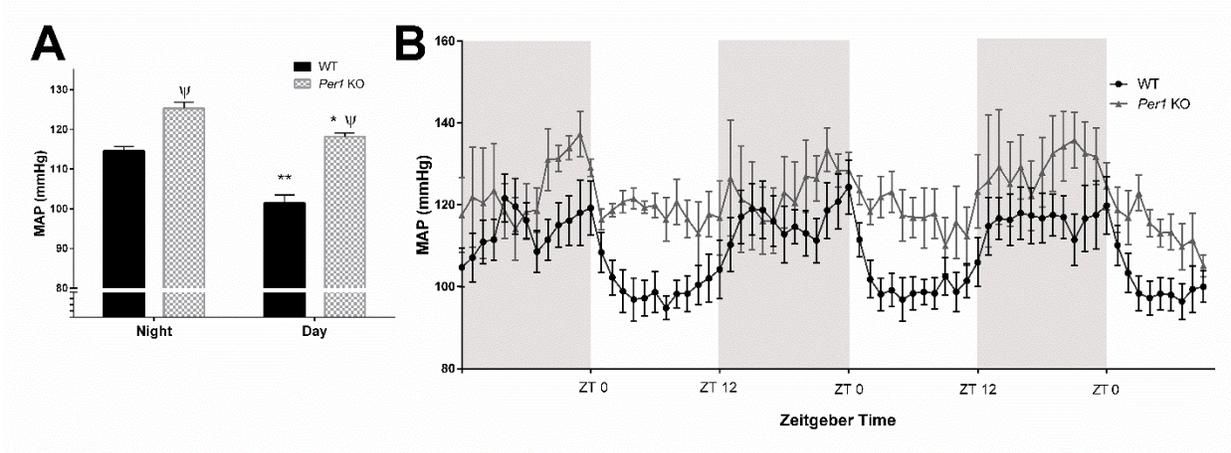


Figure 3-4. Inactive/active averages and 72 hour telemetry recordings of MAP in WT and *Per1* KO mice on HS/DOCP diet. MAP recordings from day 3 to day 6 on HS/DOCP. A. Day and night MAP averages. Values were calculated as in Figure 2. B. Telemetry recording of MAP depicted the same as in Figure 2. n=8 for WT, n=6 for *Per1* KO. Significance was calculated by two-way ANOVA. * p< 0.01, ** p< 0.001 compared to same genotype, ψ p< 0.05 compared to same time.

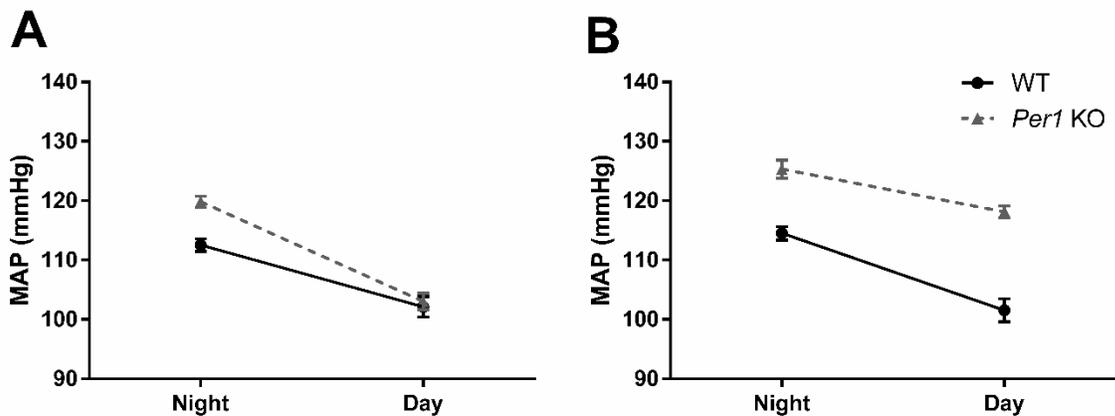


Figure 3-5. *Per1* KO mice lose the inactive/active difference in MAP on HS/DOCP diet. A. The change in MAP from night to day is shown for WT mice and *Per1* KO mice on control diet. B. The change in MAP from night to day is shown for WT and *Per1* KO mice on HS/DOCP diet for 6 days. Percent dip is given for each group. Significance was calculated using a t-test. *p<0.05. n=8 for WT, n=6 for *Per1* KO.

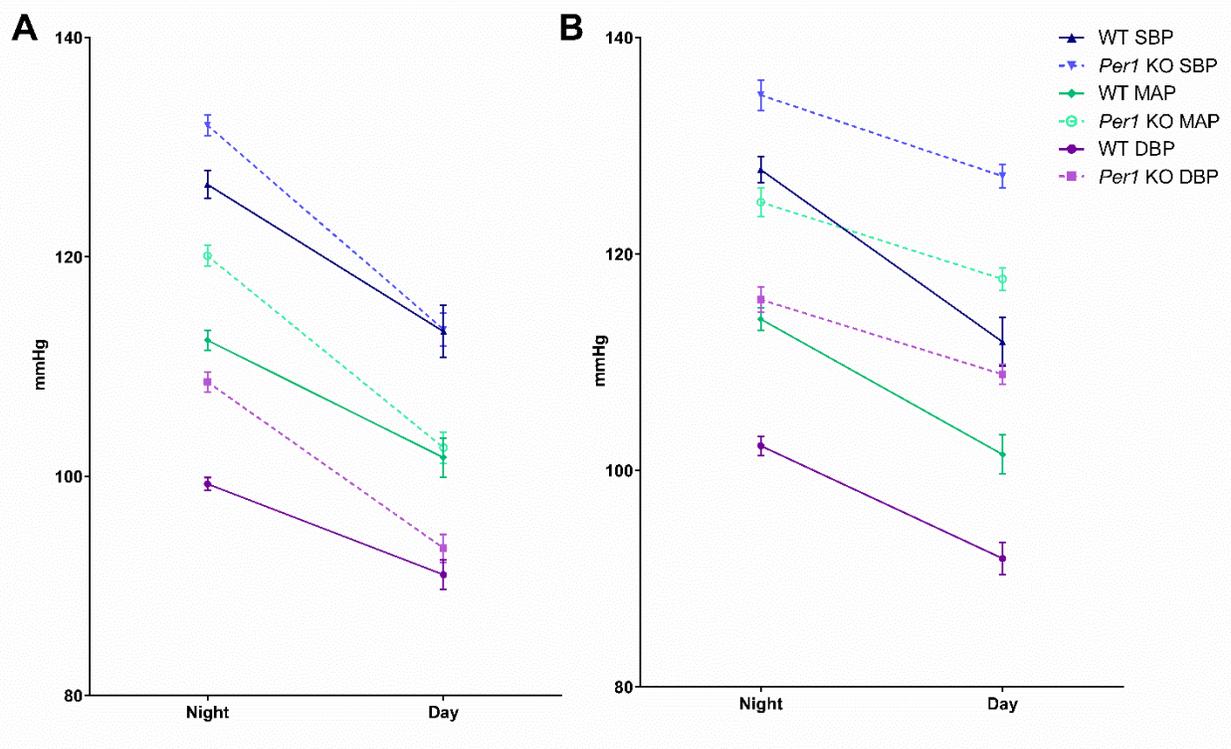


Figure 3-6. Parallel changes in DBP, SBP and MAP. DBP, SBP and MAP as measured by telemetry change in parallel in WT mice as well as *Per1* KO mice on both diets. n=8 for WT, n=6 for *Per1* KO.

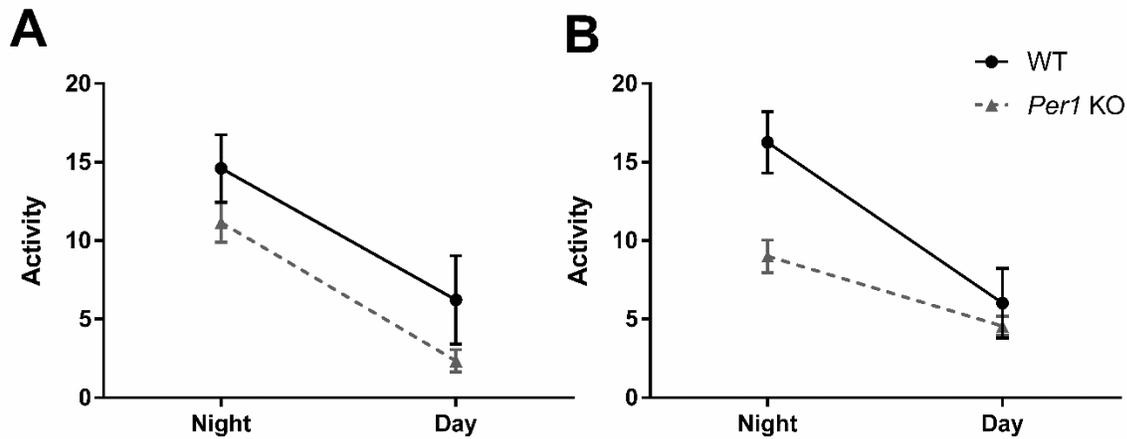


Figure 3-7. WT and *Per1* KO mice maintain inactive/active activity patterns on control and HS/DOCP diet. Activity patterns measured by telemetry were higher during the night than day in both genotypes on both control (A) and HS/DOCP (B) diets. Activity was significantly lower for *Per1* KO on HS/DOCP compared to WT and thus does not account for the higher MAP observed. Statistics were calculated by two-way ANOVA. n=8 for WT, n=6 for *Per1* KO.

Table 3-1. Baseline characteristics of WT and *Per1* KO mice.

	WT mice		<i>Per1</i> KO mice		Significance	
	Noon	Midnight	Noon	Midnight	Time	Genotype
Plasma Aldosterone (pg/mL)	551.6±193	292.1±47.9	384.2±29.2	260.894±76.5	*	NS
Plasma Na ⁺ (mM)	150.7±1.1	152.5±0.62	149.9±1.38	153±0.72	*	NS
Plasma K ⁺ (mM)	4.54±0.14	4.71±0.07	4.98±0.21	4.69±0.07	NS	NS
Body weight (g)	27.6±0.56		27.3±0.35		NS	

Plasma concentrations of aldosterone, sodium (Na) and potassium (K) were determined from samples taken at noon and midnight from WT and *Per1* KO mice. Significance was determined by two-way ANOVA (excluding body weight). Data are presented as mean ± SEM. *p<0.05. n=4 mice per group.

Table 3-2. Cosinor analysis of HS/DOCP effects on MAP in WT and *Per1* KO mice.

	WT Control	<i>Per1</i> KO Control	WT HS/DOCP	<i>Per1</i> KO HS/DOCP
Amplitude	15.8 ± 1.8	22.4 ± 3.4	21.1 ± 2.7	23.3 ± 4.5
Mesor	106.9 ± 4	111.4 ± 1.3	107.6 ± 5	121.2 ± 5.3¥
Phase	18.9 ± 0.56	18.2 ± 0.1	19 ± 0.3¥	22.3 ± 1.3*

Using ClockLab software, we determined amplitude, mesor and phase data from MAP measurements. All values were calculated separately for each animal and the mean is given ± SEM. Significance was calculated by t-test. *p<0.05 compared to same genotype, ¥ p<0.05 compared to same treatment. n=6 for KO, n=8 for WT.

Table 3-3. Analysis of MAP by JTK Cycle.

	WT Control	KO Control	WT HS/DOCP	KO HS/DOCP
Phase	24.63 ± 0.76	23.33 ± 0.49	24.81 ± 0.45	27.08 ± 1.66*
Amplitude	7.26 ± 0.93	10.58 ± 1.6†	10.47 ± 1.5†	9.53 ± 1.95
Period	24	24	24	24

Amplitude and phase data from MAP measurements were calculated using JTK Cycle. All values were calculated separately for each animal and the mean is given ± SEM. Significance was calculated by t-test. *p<0.05 for treatment effect (p<0.1 for †genotype effect, †treatment effect). n=6 for KO, n=8 for WT.

Table 3-4. Analysis of MAP by Cosinor Analysis.

	WT Control	KO Control	WT HS/DOCP	KO HS/DOCP
Period	24.82 ± 0.44	24.02 ± 0.29	24.05 ± 0.25	23.55 ± 0.63
Mesor	108 ± 4.04	111.72 ± 1.78	109.31 ± 4.89	122.06 ± 5.82†
Amplitude	8.03 ± 0.82	12.11 ± 0.96¥	10.91 ± 1.25*	11.51 ± 1.9

Amplitude and phase data from MAP measurements were calculated using Cosinor. All values were calculated separately for each animal and the mean is given ± SEM. Significance was calculated by t-test. *p<0.05 effect of treatment, ¥ p<0.05 effect of genotype. (†p=0.09 and 0.14 for effect of genotype and treatment, respectively). n=6 for KO, n=8 for WT.

CHAPTER 4 LOSS OF THE CIRCADIAN CLOCK PROTEIN PER1 DOES NOT LEAD TO NONDIPPING IN FEMALE C57BL/6 MICE

Background

Hypertension, is the greatest risk factor for death around the world^{208, 209}. It is also the number one risk factor for stroke and CVD which is the leading cause of death of Americans¹², claiming 1/3 of the total deaths in the U.S. annually¹⁶⁸. While about 30% of U.S. adults have hypertension, nearly half of them do not have adequate BP control (less than 140/90 mmHg)²¹⁰. Clearly, better awareness and treatments are needed, not only for the sake of patients but because of the direct and indirect costs of CVD and stroke. The American Heart Association estimates this to be \$320 billion annually and predicts an increase to \$918 billion by 2030¹⁶⁸.

While the primary cause of the majority of hypertension is not known, table salt (NaCl) is known to increase sensitivity to BP dysregulation. Salt-sensitivity affects people across a spectrum but it is known to be more prevalent in African Americans, affecting almost $\frac{3}{4}$ of hypertensive patients, compared to Caucasians, in which about half of hypertensive patients are also salt-sensitive²¹¹. Salt-sensitivity has also been associated with increased risk of cardiovascular events²¹², which is possibly independent of high BP²¹³.

The kidney is the organ responsible for water and ion (including Na⁺Cl⁻) homeostasis via filtration by the functional unit of the kidney: the nephron. The DCT and the CD reabsorb the final 10-15% of filtered sodium and are the most highly regulated segments. The kidney has been implicated in the pathogenesis of hypertension in cross-transplantation studies in mice²¹⁴ and human kidney transplant recipients²¹⁵. Similar experiments in rats²¹⁶ showed that salt-sensitivity tracks with the kidney as well.

Many of the sodium transporters located along the nephron are regulated by the circadian clock. This ancient mechanism for energy conservation and physiological synchronicity can be traced all the way back to archaebacterial (reviewed in ²). Very briefly, on a molecular level the clock is comprised of four core proteins (CLOCK, CRY, PER and BMAL1) whose expression patterns oscillate over a ~24 hour period (for a review on this mechanism, see⁴). Through their actions as transcription factors, these core clock components regulate over 40% of all expressed genes⁸ including NCC and the WNK pathway in the DCT^{16, 17}, α ENaC as well as regulators of α ENaC in the CD¹⁸⁻²⁰. While α ENaC is often thought of as the regulated subunit of ENaC, there is also evidence that the gamma subunit of ENaC is regulated as well (CircaDB)^{14, 217}.

Numerous circadian gene knockout mice have been created and all of them develop a BP phenotype, highlighting the close relationship between the clock and BP regulation^{18, 19, 61, 63, 65, 68, 69}. However, all of these studies have focused only on male mice. Previously, we investigated the BP response of C57BL/6 male mice with global loss of PER1 on a HS diet in combination with DOCP, a long-acting aldosterone analog²¹⁸. Normally on high salt diet, aldosterone production is suppressed (reviewed in¹⁷²) so through the addition of DOCP, we are able to increase sodium reabsorption and thus create a model of hypertension similar to that seen in salt-sensitive African Americans²¹⁹ with low renin yet high aldosterone^{220, 221}. In order to investigate sex differences in this model, we used the same experimental technique with female mice. In contrast to male mice on HS/DOCP, female *Per1* KO mice maintain a normal circadian rhythm of BP and appear to be protected from the harmful non-dipping

phenotype seen in males. To our knowledge, this is the first investigation of this treatment in a clock gene KO model in female mice.

Methods

Mice

Female WT and *Per1* KO C57BL/6J mice between the ages of 14-24 weeks were used for these experiments (original heterozygous mice were obtained from Dr. Shin Yamazaki, Vanderbilt¹⁷³). Mice were housed as described in²¹⁸ and telemetry surgery was performed as described in^{19, 174}. After at least a week of recovery after surgery, mice were given control diet for 3 days and baseline recordings were done. They were then given 0% NaCl diet for 3 days, 4% NaCl (Harlan) diet for 3 more days, followed by injection with 75 µg/g bw DOCP. HS/DOCP recordings were taken 3 days after injection. All animal use was in compliance with the American Physiological Society's Guiding Principles in the Care and Use of Laboratory Animals, and animal use protocols were approved by the Institutional Animal Care and Use Committees of the University of Florida and the North Florida/South Georgia Veterans Administration.

Tissue dissection and Real-Time PCR

Dissection and PCR were performed as previously described in¹⁷¹.

Flame Photometry

Flame photometry was used to determine urine and plasma Na and K concentrations.

Aldosterone ELISA

Plasma aldosterone was measured by ELISA as described in⁹⁶.

Statistics

Graphpad was used to perform t-tests and 2-way ANOVAs. All values are presented as mean \pm SEM.

Results

Figure 4-1 shows a representative tracing of mean arterial pressure recordings of the mice over 4 days of control diet. Time is given as Zeitgeber time (ZT), indicating lights on between ZT0 and ZT12 and lights off between ZT12 and ZT0 (grey bars). Both WT and KO mice display a normal circadian rhythm of BP with an increase in the night when they are active and a decrease during the day when mice are inactive. On control diet, *Per1* KO mice have a MAP about 4-5 mmHg higher than WT mice. This is in contrast to male mice who exhibit little to no difference in MAP between WT and *Per1* KOs at baseline²¹⁸.

The mice were challenged with a series of different diets to test their ability to regulate BP with different salt loads. Mice on each diet maintained circadian rhythms similar to that shown in Figure 4-1 but for simplicity, the results in Figure 4-2 are given as an average of 3 days of diet shown as one cycle. KO mice maintain consistently higher MAP than WTs on all diets, but the difference between the two groups is amplified by HS/DOCP. This figure also highlights the progressive increase in BP in both groups over the course of the experiment. In addition, it is important to note that both groups of female mice maintain a day time dip in BP, unlike the male mice²¹⁸.

In order to quantify the results in Figure 2, MAP was calculated as day (ZT0-ZT11, 6am-5pm) and night (ZT12-ZT23, 6pm-5am) values for each diet. A similar trend of increasing MAP with increasing salt is seen in WT and KO mice. KO mice also have a consistently higher MAP than WT, averaging about 5 mmHg higher at all times. While

on control diet, the difference in MAP between WT and KO was 4 mmHg at night and on HS/DOCP this difference rose to about 7.5 mmHg at night. This again is in contrast to male mice who only show differences in BP on HS/DOCP diet²¹⁸.

Cosinor analysis was done on MAP to investigate differences in the diurnal pattern of MAP on control, 0% NaCl, 4% NaCl and 4% NaCl + HS/DOCP. There were no significant differences in mesor (the MAP halfway between the highest and lowest MAP recorded) or the amplitude (the difference between the highest and lowest MAP recorded) except for a small difference in amplitude on control diet between WT and *Per1* KO mice (Table 1). This may be due in part to the fact that the animals were on a normal light/dark cycle and not in total darkness.

Since there seemed to be little difference between how WT and PER1 KO mice react to the different diets, we wanted to compare baseline characteristics of these mice to attempt to explain the higher MAP observed in KOs. We compared many effectors of BP between WT and KO mice (summarized in Table 4-1), focusing on parameters likely to be affected by changes in renal regulation. We chose to focus on the kidney because changes in BP that we do observe occur in parallel between diastolic and systolic measurements (Figure 4-4), suggesting a volume-dependent mechanism which points to the kidney. Isolated increases in SBP suggest increased arterial stiffness²²² which leads to decreased DBP²²³. Isolated changes in diastolic BP occur due to increased peripheral resistance²²⁴. Thus, the parallel changes in SBD and DBP suggest increased blood volume, which is regulated by the kidney. No significant differences in baseline plasma Na, K or aldosterone were observed at either noon or midnight time points. However, there was a trend of increased plasma aldosterone at noon for each

group though significance was not reached due to high animal-to-animal variability, following the trend of varying levels of aldosterone over the course of a day^{96 64}. There was also no difference in urine Na or K as well as mouse age or hematocrit. There was a small but significant difference in mouse weight.

To consider a mechanism, expression levels of genes involved in renal sodium handling were assessed. Kidneys were obtained from WT and *Per1* KO mice at noon, the midpoint of their inactive period to match the experiments done in males. Kidneys were roughly dissected into cortex and medulla and isolated RNA was used to make cDNA for Real-Time PCR analysis. Selected results are shown in Figure 4-5. In general, female *Per1* KO mice show higher expression of renal genes associated with sodium reabsorption relative to WT females. Expression is higher in the cortex and medulla of the gamma subunit of the ENaC (γ ENaC) (Figure 4-5, A and D). The sodium potassium chloride cotransporter is more highly expressed in the cortex (Figure 4-5B), but not medulla (Figure 4-5E) of KOs. The Endothelin A (ETA) receptor and is also significantly upregulated in both cortex and medulla in KOs (Figure 4-5, C and F). There were no significant changes in α ENaC, Endothelin-1 (in the medulla) or the ETB receptor, however (Figure 4-6).

Discussion

It is known that sex differences in BP and cardiovascular parameters exist but the cause for these differences is not well understood. Even less explored is the role of the circadian clock in cardiovascular sex differences. The aim of this study was to explore the role of a core circadian clock protein, PER1, in BP regulation disparities between male and female mice and compare the results to what we have previously observed in male mice. We found that while male mice on a HS/DOCP diet develop

harmful non-dipping hypertension²¹⁸, females are protected from this phenotype and retain normal circadian BP rhythms. In contrast to the male phenotype, female *Per1* KO mice maintain slightly higher MAP over a range of sodium intake compared to WTs.

It has been well-established that there are sex (used here to refer to the genetic/phenotypic contribution of sex chromosomes and hormones) differences in the prevalence of hypertension in humans with women having lower rates until the age of menopause (reviewed in²²⁵) (with exceptions to this general rule between different races). Females of other species also have slightly lower BP than males including dogs²²⁶, birds²²⁷ and rats^{228, 229} at baseline as well as experimental rodent models of hypertension²³⁰⁻²³⁴, though some studies do show similar baseline BP^{230 235 236}.

In general, females are protected compared to males with respect to cardiovascular morbidities. In animal models these include but are not limited to the response to Angiotensin II^{230, 232, 237-239}, DOCA-salt (similar to DOCP)^{236, 240-242} and excretion of a salt load²³⁵. In humans, male sex generally increases the risk for cardiovascular disease, chronic kidney disease and hypertension¹⁶⁸. It has also been suggested that postmenopausal women have a higher incidence of non-dipping²⁴³ and this may be lessened by the use of hormone replacement therapy²⁴⁴.

The role of hormones, specifically estradiol and progesterone, has been investigated as a source of reported sex differences. In addition to different expression patterns between sexes of ETAR and ETBR in the nephron, it has been shown that ENaC is regulated by estrogen and progesterone^{217, 245}. Ovariectomy reduced ETAR and ETBR expression in kidney cortex and this result was abolished by supplementation with estrogen²⁴⁶. In addition, salt sensitivity was shown to be increased

by ovariectomy in spontaneously hypertensive and dahl salt sensitive rats^{247, 248}. Many more instances have been demonstrated and are reviewed in great detail in²²⁵.

Although it is tempting to speculate that ovarian hormones are the underlying mechanisms of protection, a limitation of our study is that we did not perform ovariectomy. This is a likely future direction of this line of research

Our lab is particularly interested in the first homolog of Period, PER1, in the kidney. This was initiated by the discovery that the sodium- and BP-regulating hormone aldosterone regulates PER1 expression⁷⁸. Subsequent work in our lab and that of collaborators solidified the role of PER1 in the kidney by showing that it regulates expression of α ENaC²⁰, Endothelin-1¹⁹, which acts to promote sodium excretion⁸⁷, NCC¹⁶, and NHE3¹⁷¹. PER1 global KO mice on a 129/sv background have a mean arterial BP 18mmHg lower than WT controls, at least in part likely due to reduced plasma aldosterone⁹⁶ as well as reduced expression of the sodium channels and transporters mentioned above. Conversely PER1 global KO mice on a C57BL/6J background have slightly increased BP compared to WTs. Although our understanding of the role of background strain genetics is limited in rodent cardiovascular studies, there are reports of differences in diabetic nephropathy¹⁸⁵, baseline BP¹⁸¹, susceptibility to renal damage¹⁸⁴ and salt sensitivity^{182,183}. If we think in terms of each mouse strain being representative of a different human being and realize that SNPs, environment, genetics and many other factors influence the different responses to stimuli and disease seen in humans, it follows that two mouse strains would react differently to the same to the same treatment.

One of the predominant regulators of BP is Endothelin-1 which signals through A and B receptors. In the kidney, ETA receptors promote sodium reabsorption while ETB receptors lead to sodium excretion through the blockade of ENaC^{85, 249}. Since the ratio of ETAR/ETBR is higher in males²⁵⁰ suggesting sex-specific regulation and the Endothelin axis is regulated by PER1⁸⁸, we hypothesized that this could be a contributing factor to the higher MAP in KOs. Indeed, the female KO mice do have higher expression of ETA in the cortex and medulla of the kidney as measured by real-time PCR. Higher expression of ENaC and NKCC2 can also help explain the increased BP through increased sodium reabsorption. Further experiments are needed to delineate a functional role for these transporters and signaling pathways.

The fact that there is a much less dramatic phenotype seen in females compared to male *Per1* KO mice indicates that between sexes, PER1 may have different roles or be regulated differently. Our lab has previously demonstrated the important role of PER1 in the regulation of BP and renal sodium handling^{16, 19, 80, 96, 171, 180}. However, these studies were conducted in male mice exclusively. This is the first demonstration of a BP phenotype in female mice lacking a core circadian clock gene. That they are protected from non-dipping hypertension relative to male *Per1* KO mice is consistent with human literature that pre-menopausal women are less likely to exhibit non-dipping hypertension. This is an important first step in understanding sex differences in the regulation of cardiovascular function by the circadian clock. Future studies are clearly needed to ascertain the precise role of sex hormones and the kidney-specific role of PER1 in BP regulation.

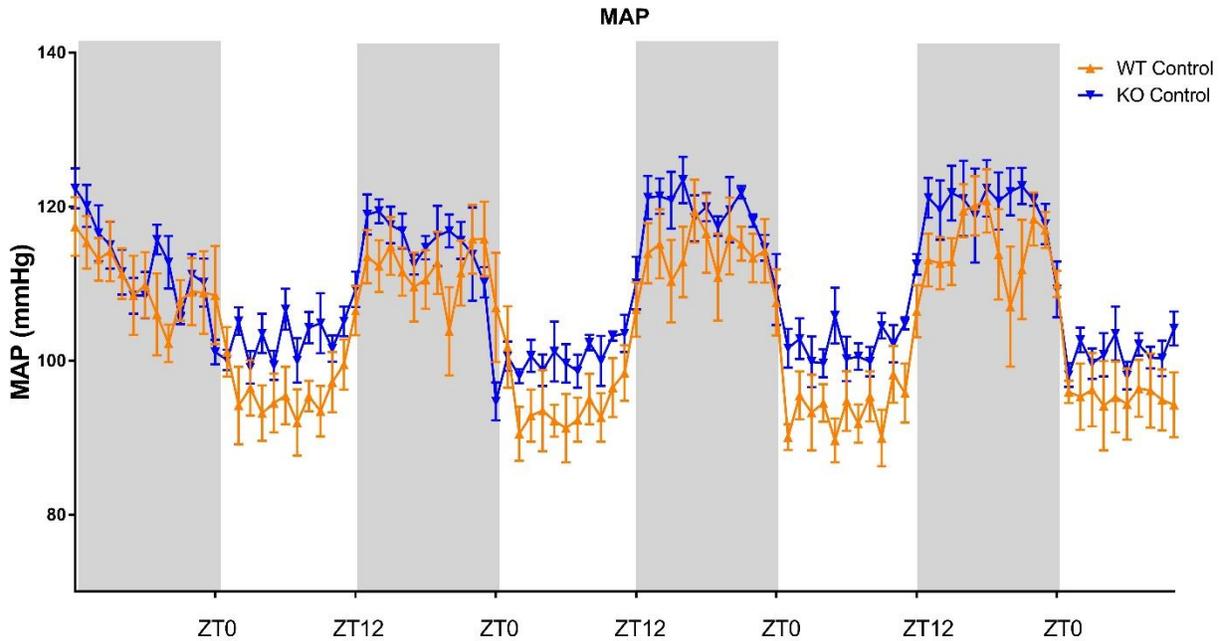


Figure 4-1. Circadian Rhythm of BP on Control Diet. Recordings of mean arterial pressure from implanted telemetry devices in WT and *Per1* KO female mice are shown. Time on the x axis is presented as Zeitgeber time, denoting the time of lights on (ZT0, white bars) and lights off (ZT12, grey bars). Both WT and *Per1* KO mice maintain a normal circadian rhythm of BP on control diet, with MAP peaking at night when mice are active. n=6WT, 4KO.

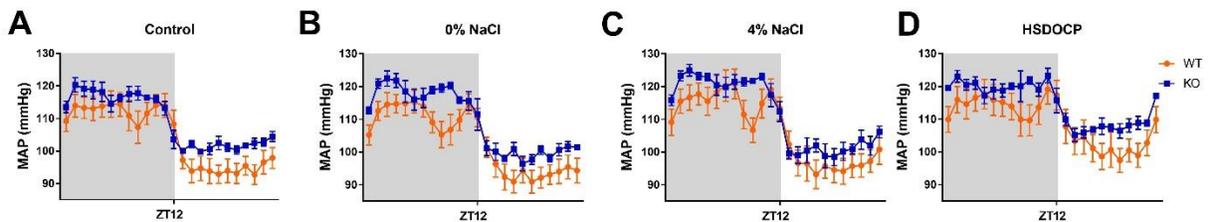


Figure 4-2. Mean Arterial Pressure Averaged Over Multiple Days of Diets. Three days of diet are shown as an average of one cycle starting at midnight (ZT18). MAP is consistently higher in the KOs (red lines) compared to WT (blue lines) but each group shows an increase in MAP with increasing salt and the addition of DOCP. n=6WT, 4KO.

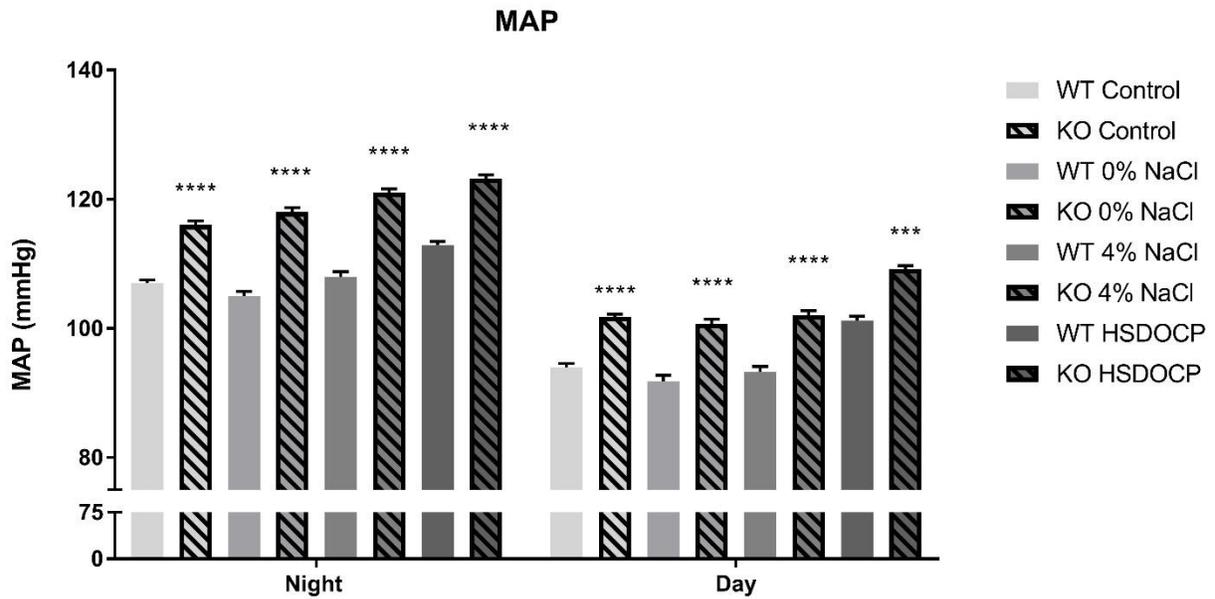


Figure 4-3. Average MAP for All Diets. MAP was calculated for the day (ZT0-ZT11, 6am-5pm) and night (ZT12-ZT23, 6pm-5am) periods on the different diets. In all groups and diets, MAP is lower during the inactive period when lights are on. There is also a trend of increasing MAP from control to 0% NaCl to 4% NaCl to HS/DOCP diet in both WT and KO animals. $n=6WT, 4KO$. *** $p \leq 0.001$, **** $p \leq 0.0001$ compared to WT. Values shown are mean \pm SEM.

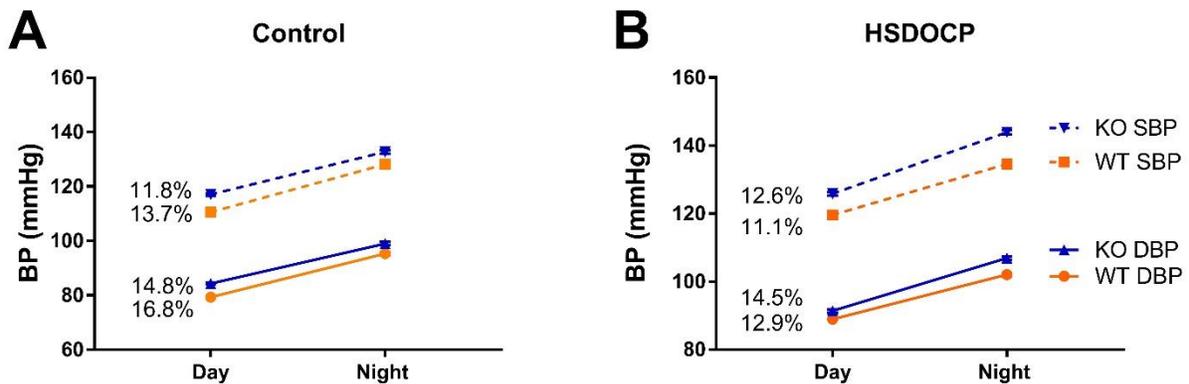


Figure 4-4. Changes in DBP and SBP on Control and HS/DOCP Diets. Parallel changes can be seen in DBP and SBP in both genotypes and on control and HS/DOCP diets, suggesting a volume-dependent mechanism of BP changes. Percentage dip is given next to each corresponding line.

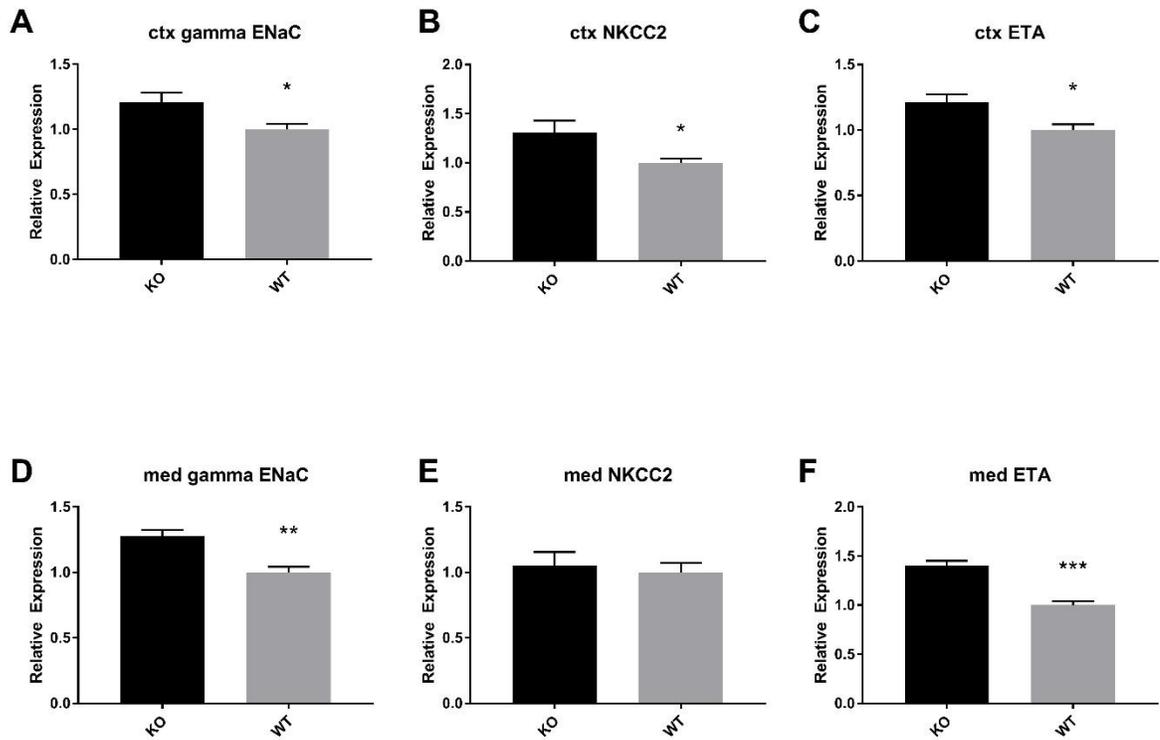


Figure 4-5. Real Time PCR Expression of Selected Genes in Kidney Cortex and Medulla. Real time PCR expression of selected renal genes involved in sodium transport is shown. KO mice have higher expression of genes responsible for sodium reabsorption including the gamma subunit of the ENaC in the cortex and medulla (A and D), the sodium potassium chloride cotransporter in the cortex (B) but not the medulla (E) and the Endothelin A receptor in the cortex and medulla (C and F). n=6WT, 6KO. *p ≤0.05, **p ≤0.01, ***p ≤0.001 compared to WT.

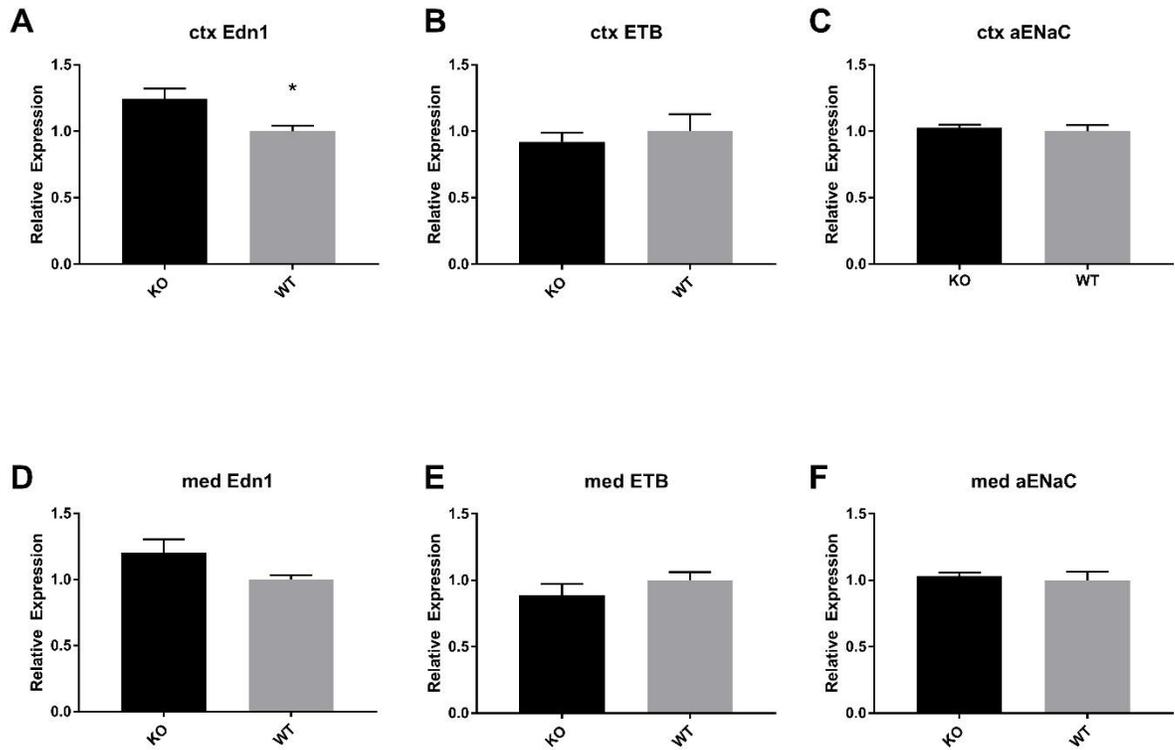


Figure 4-6. Additional Real Time PCR Expression of Selected Genes in Kidney Cortex and Medulla. Real time PCR expression of selected renal genes involved in sodium transport is shown. There were no differences observed in EDN1 expression in medulla (D), the Endothelin B receptor in cortex and medulla (B and E) or α ENaC in cortex or medulla (C and F). n=6WT, 6KO. *p \leq 0.05 compared to WT.

Table 4-1. Cosinor analysis of mean arterial BP on different diets.

Genotype	Diet	Mesor	Amplitude
WT	Control	103.78 ± 3.17	11.4 ± 0.4
KO	Control	108.93 ± 0.86	9.67 ± 0.58 *
WT	0% NaCl	103.63 ± 3.05	11.61 ± 0.51
KO	0% NaCl	109.63 ± 0.95	12.5 ± 0.95
WT	4% NaCl	106.67 ± 3.88	13.03 ± 0.71
KO	4% NaCl	111.56 ± 1.61	13.48 ± 1.05
WT	4% NaCl & DOCP	109.13 ± 4.2	8.64 ± 0.59
KO	4% NaCl & DOCP	114.82 ± 0.78	8.3 ± 0.64

Cosinor analysis was done to determine changes in circadian parameters of MAP. Mesor is the MAP halfway between the highest and lowest MAP recorded. Amplitude is the difference between the highest and lowest MAP recorded. n=6 WT, 4 PER1 KO. *p≤0.05 compared to WT by T-test.

Table 4-2. Baseline Characteristics of WT and KO Mice.

	WT	KO
Plasma Na (noon)(mM)	157.33 ± 2.38 (6)	152.23 ± 2.09 (6)
Plasma Na (midnight) (mM)	163.54 ± 3.78 (6)	155.88 ± 2.77 (6)
Plasma K (noon) (mM)	4.49 ± 0.25 (6)	4.30 ± 0.13 (6)
Plasma K (midnight) (mM)	4.18 ± 0.19 (6)	4.56 ± 0.24 (6)
Urine Na, 48h average (mEq/g BW)	0.01233 ± 0.00122 (12)	0.01282 ± 0.00049 (12)
Urine K 48h average (mEq/g BW)	0.01834 ± .000292 (12)	0.01934 ± 0.000412 (12)
Weight (g)	21.72 ± 0.38 (12)	20.31 ± 0.43* (12)
Age (weeks)	16.5 ± 0.90 (12)	17.24 ± 1.02 (12)
Hematocrit	41.5 ± 0.59 (12)	42.46 ± 0.90 (12)
Plasma Aldosterone (pg/ul) (noon)	504.05 ± 46.78 (5)	530.88 ± 40.61 (6)
Plasma Aldosterone (pg/ul) (midnight)	417.76 ± 79.02 (6)	390.00 ± 109.46 (6)

All values are mean ± SEM (n). * p≤0.05 compared to WT by T-test.

CHAPTER 5
IDENTIFICATION AND INITIAL CHARACTERIZATION OF A lncRNA ANTISENSE TO
THE EDN1 GENE: EDN1-AS

Background

Endothelin-1 (ET-1, encoded by the *Edn1* gene) is one of the most potent vasoconstrictors known²⁵¹. When first translated, prepro Endothelin-1 is a 212 amino acid protein that gets progressively cleaved to a 21 amino acid, mature ET-1 peptide (reviewed in²⁵²). Though each of the cleavage steps are likely regulated, the major regulation of Endothelin-1 occurs at a transcriptional level (reviewed in²⁵³). ET-1 binds to two different receptors (ETAR and ETBR) which are expressed by almost every cell in the body and generally produce opposite effects (reviewed in²⁵²). For example, stimulation of ETAR produces vasoconstriction, kidney damage and increased oxidative stress^{254, 255} (reviewed in²⁵⁶) while ETBR stimulation generally promotes BP lowering and antifibrotic effects (reviewed in²⁵²). These two receptors also bind the two other isoforms of Endothelin, Endothelin-2 and -3, but these isoforms are not as abundant or widespread as ET-1²⁵⁷.

The inner medulla of the kidney is the site of the highest levels of ET-1 production in the body²⁵⁸ and almost every cell in the kidney synthesizes ET-1 and contains ET receptors (reviewed in²⁵²). The renal vasculature is also highly sensitive to the actions of ET-1²⁵⁹. While ET-1 is generally viewed as a vasoconstrictor, in the kidney tubule ET-1 has natriuretic effects, promoting the excretion of sodium and subsequent fall in blood volume and BP. The natriuretic effects of ET-1 appear to be perpetuated via ETBR and possibly ETAR and subsequent blockade of ENaC open probability^{85, 87, 260} through a mechanism likely involving nitric oxide²⁶¹. Localization of

ETBR to the basolateral membrane of tubular cells in the kidney suggest an autocrine or paracrine mechanism of regulation by ET-1 in the CD²⁵².

Due to the almost ubiquitous expression of ET-1 throughout the body, dysregulation of ET-1 has been implicated in the etiology of numerous diseases including cancer (reviewed in²⁶²), hypertension^{263, 264} and chronic kidney disease (CKD)²⁶⁵. ET receptor antagonists have been used in the treatment of pulmonary arterial hypertension²⁶⁶, a life-threatening disease that involves aberrant ET actions. However, there are not many successful treatments targeted at the kidney. A small clinical trial of CKD patients showed benefits of ETAR blockade²⁶⁷. However in diabetic CKD patients, this was harmful²⁶⁸. The ASCEND trial was prematurely terminated due to increased congestive heart failure outcomes in patients with type II diabetes taking an ETAR blocker (Avosentan) compared to placebo groups²⁶⁸. With estimates of over 10% of the populations of the United States and Europe diagnosed with CKD²⁶⁹, better therapies are needed. Since the renal ET system is activated in almost all CKD cases²⁵⁶, it represents a promising target. Indeed, a number of human clinical studies suggest the possibility that ETAR antagonists in combination with other therapies may be beneficial in the treatment of CKD, with careful dosing and mindfulness of patient populations with increased likelihood of fluid retention²⁵⁶. Our lab has recently discovered a long, non-coding RNA (lncRNA) antisense to the EDN1 gene, providing a potential new target in the endothelin axis for therapeutic treatment.

lncRNAs are a relatively new class of RNAs that have been implicated in gene regulation. These >200 bp species arise from parts of the genome that does not code for proteins, hence their name, and come from what was long thought to be “junk

DNA²⁷⁰. However, largely due to the ENCODE Project²⁷¹ it is now clear that this DNA is anything but junk. lncRNAs have been shown to regulate many aspects of the genome with perhaps one of the most well-known being Xist. Xist is the lncRNA responsible for female X chromosome inactivation and is itself regulated by a ncRNA transcribed antisense to itself, Tsix²⁷². Many examples of lncRNAs are known and have been shown to possess a vast assortment of regulatory methods including epigenetic regulation²⁷³, trafficking and transcription (reviewed in²⁷⁴). While most lncRNAs regulate their targets in a negative manner, there are instances of positive regulation as well (reviewed in²⁷⁵). It has also been suggested that RNAs could provide a bridge for the interaction of histone-modifying enzymes and DNA²⁷⁵ since histone-modifying enzymes lack DNA-binding domains²⁷⁶.

A specific class of lncRNAs, antisense lncRNAs, are transcribed from the opposite strand of DNA as an mRNA and thus are capable of regulating their complementary strand of DNA or mRNA (reviewed in²⁷⁷). Antisense lncRNAs can also regulate other genes and transcripts as well depending on post-translational processing. When acting in a *cis* manner, antisense lncRNAs regulate genes close to their site of synthesis, as in the case of Xist which is transcribed from the soon to be inactivated X chromosome²⁷⁸. COOLAIR is an antisense lncRNA which silences its sense gene (FLC) through polycomb complex recruitment in response to cold stimulus in *Arabidopsis*²⁷⁹. When acting in *trans*, antisense RNAs act on distal genes and can regulate many targets due to imperfect complementarity²⁸⁰. HOTAIR is the first lncRNA discovered to regulate gene expression in this manner. HOTAIR downregulates HOXD locus genes while it is actually transcribed in the HOXC gene locus. It has also been implicated in

regulation of a number of genes related to cell cycle progression and metastases and therefore has also been implicated in cancer etiology (reviewed in²⁸¹).

Experiments to verify the existence of EDN1-AS and start initial characterization are detailed in this chapter. EDN1-AS was shown to be expressed in multiple cell lines of mouse and human origin as well as authentic human kidney RNA. Preliminary experiments suggest that EDN1-AS is expressed in a time-dependent manner which may be due in part to Per1 residence in the promoter region of EDN1-AS as shown by chromatin immunoprecipitation (ChIP). Further studies using CRISPR/Cas9 to delete regions of the promoter region of EDN1-AS have resulted in heterozygous knockout (KO) mIMCD3 and HK-2 cell lines and 2 homozygous KO HK-2 lines. One ET-1 ELISA of media from these cells did not show significant differences in ET-1 levels from WT cells. Experiments using a different method of CRISPR/Cas9 KO will be undertaken in the near future.

Methods

Exploration of EDN1 Chromatin State and Regulation with the UCSC Genome Browser

Human EDN1 chromatin state was assessed using the UCSC Genome Browser²⁸² at <http://genome.ucsc.edu/>. Using primarily the Genome Segments, Transcription Factor ChIP, Layered H3K2Ac, DNase Clusters, Broad Chrom HMM and Broad Histone Modification tracks, it was observed that there was open chromatin with multiple important transcription factors bound at the 3' end of the EDN1 gene. This was indicated to be a predicted promoter region with high confidence by the genome browser (Figure 5-1). This ~800 bp region was termed the “promoter region” of EDN1-AS. Further investigation into the promoter region showed binding of multiple

transcription factors that suggest active transcription and regulation including TFIIID, USF1 and GR (Figure 5-2).

Strand-Specific Primer Design

Human Strand-Specific primers (SS-primers) were designed by two previous lab members (Lauren Jeffers and Dr. Sarah Barilovits). SS-Primers (LJ3, LJ4, LJ5) were chosen to lay down at locations progressively closer to the 5' end of the EDN1 gene (illustrated in Figure 3) for use in reverse transcriptase reactions. Oligo dT primers were used as a positive control for any polyA tailed mRNA. Primers have a complimentary sequence to the EDN1 sense strand so they will only anneal to antisense RNA. PCR primers (LJ7 and LJ8) were designed to amplify the same region of cDNA regardless of the SS-Primer used. Mouse SS-Primers were designed by the same method and in roughly homologous locations (e.g. mEDN1-AS3 lays down in a homologous location to LJ3).

RNA Isolation and DNase Treatment

RNA was isolated from cells and tissue using Trizol (Ambion) per manufacturer instructions. In general, 1ml Trizol was used per well in a 6 well plate. 0.5 ml Trizol was used for inner medulla tissue, 1ml for outer medulla and 2-3 ml for cortex tissue. Tissue was homogenized by hand in Trizol and processed to total RNA (tRNA). Tissue and cells were treated with DNase (Ambion) per manufacturer instructions.

cDNA Conversion and PCR

Reverse Transcriptase (RT) reactions were carried out using a kit from Thermo Fisher. Random hexamers were used as primers to make cDNA (final concentration 4ng/ μ l) to assess EDN1 mRNA levels. SS-Primers were used +RT and -RT to assess EDN1-AS levels (+RT) and DNA contamination (-RT). Any sample which had a band in

–RT lanes was considered contaminated and not used. Preliminary experiments used all SS-Primers but subsequent experiments to compare multiple samples used LJ4 or mEDN1AS4 as SS-Primers. Most RT reactions were brought down to 5 μ l total volume due to the precious nature of the RNA. LJ7 and LJ8 (or mouse equivalent) were used in a PCR reaction for all samples including random hexamer RT samples. PCR was done at 35 cycles for all reactions to better visualize bands, however PCR using 30 cycles still showed the presence of EDN1-AS. Real time PCR for EDN1 was done using TaqMan probes and mastermix.

Cell Culture

mIMCD3 cells were cultured in DMEM/Hamm's F12 media supplemented with 10% FBS and 0.5% Gentamycin. Cells were passaged when they reached 80-100% confluence. HK-2 cells were cultured in DMEM/Hamm's F12 media supplemented with 10% FBS and 1% Penicillin/Streptomycin (and after 7/2017 with 0.5% Gentamycin). Cells were passaged when they reached about 80% confluence.

Aldosterone Treatment

mIMCD3 cells were treated with 100 nM aldosterone for 4 hours in 6 well dishes in 3 separate experiments. RNA was then isolated and SS-RT PCR was done to check antisense expression.

Dexamethasone Treatment

HK-2 cells were grown to ~80% confluence in 6 well cell culture plates. 100 nM Dexamethasone was added for 30 minutes and then media was changed. This allows the circadian clock to be synchronized in all cells (Figure 5-4). After 24 hours, cells were trypsinized and RNA was isolated every 2 hours for 24 hours.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was done using the Active Motif ChIP Kit following manufacturer instructions.

Mice

Male and Female 129sv/s1s4 and Male 129sv/s2 mice were housed in normal 12:12 L:D conditions and fed a normal diet with *ad libitum* access. Animals were euthanized with 5% isoflurane and cardiac puncture. Kidneys were collected and flash-frozen in liquid nitrogen. All animal use was in compliance with the American Physiological Society's Guiding Principles in the Care and Use of Laboratory Animals, and animal use protocols were approved by the Institutional Animal Care and Use Committees of the University of Florida and the North Florida/South Georgia Veterans Administration.

CRISPR/Cas9

gRNA Design

Guide RNAs (gRNAs) were designed using the online tool CC Top²⁸³ from <https://crispr.cos.uni-heidelberg.de/>. A sequence of ~150 bp of the promoter region of (mouse or human) EDN1-AS was used as input and the program produced multiple possible gRNAs. The region was selected based on identified (in human, Figure 5-5A) or predicted (in mouse, Figure 5-5B) transcription factor binding sites hypothesized to be important to initiation of transcription. gRNAs were selected based on location and predicted off-target sites. If possible, only gRNAs with no off-target sites or those with sites only in regions between genes were chosen. None were used if the gRNA had predicted off-target sites in exonic or intronic regions of any gene to minimize possible off-target effects. Originally gRNAs were designed to remove ~100 bp of sequence but all combinations were used in the end.

Creation of the gRNA Plasmid

gRNA design from CC Top included an overhang for insertion into the plasmid. Cloning was carried out according to the protocol provided by the Zhang Lab^{284, 285}. Briefly, the LentiCRISPR v2.0 plasmid (a gift from Dr. Kevin Brown (Addgene plasmid # 52961)) was digested with *Bsmbl* to remove a 2 kb fragment with overhangs complimentary to the sites added to the gRNAs. The larger fragment was gel-purified using the Qiagen kit and was ligated to phosphorylated and annealed gRNA oligos with Quick Ligase (NEB). AT NO POINT IS CIP (Calf Intestinal Phosphatase) TO BE USED. The ligated plasmid and gRNA was transformed into Stbl3 bacteria following manufacturer instructions.

Puromycin Kill Curve

Kill curves were obtained for HK-2 and mIMCD3 cells with puromycin. Initially, 80-100% confluent cells in a 24 well plate were subjected to puromycin concentrations of 0 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 3 µg/ml, 5 µg/ml, 8 µg/ml and 10 µg/ml in triplicate. This was subsequently narrowed down to a range of 0-3 µg/ml to confirm that 2 µg/ml puromycin was an optimal concentration, killing all cells after 72 hours but not before 48h.

Creation of Virus and Infection of Cells

With the gracious help of Dr. Kevin Brown, HEK293 cells were transfected with the LentiCRISPR + gRNA 1, 2, 3, and 4 plasmids in addition to pCMV-VSV-G (Addgene plasmid # 8454) for viral envelope proteins and psPAX2 (Addgene plasmid # 12260) for lentiviral packaging. After sufficient time, media containing virus particles with the LentiCRISPR plasmids encapsulated was collected and spun down to remove cell debris. The respective virus containing human or mouse gRNA/LentiCRISPR plasmid

was then added onto HK-2 and mIMCD3 cells in every combination (e.g. gRNA 1+2, 1+3, 1+4 etc.). 2 µg/ml puromycin was used for both lines to select for cells successfully infected with virus.

96 well cloning

First attempts to clone CRISPR cells were done in 96 well plates. Cells were plated into the first of 4 columns of a 96 well plate in 200 µl of media. 20 µl of cells were then diluted using a multichannel pipette into the 3 subsequent columns of wells containing 180 µl of media in order to have single cells in the third and fourth columns. After one to two weeks, when media started to indicate acid build up in columns 1 and 2, wells were inspected for the presence of one to three colonies of cells (illustrated in Figure 5-6). These wells were then trypsinized with 1 drop of 0.25% Trypsin per well and plated into a 6 well plate. Then gDNA was isolated using the Zymo gDNA isolation spin kit and cells were genotyped. Depending on the genotyping results, cells were replated into 96 well plates and the process was repeated to attempt to obtain homozygous cell lines.

10 cm dish cloning

When 96 well cloning did not result in homozygous cell lines (either due to mixed cell populations or heterozygous KO of single cells), cloning was attempted in 10 cm dishes. Cells were plated in order to obtain a plate with ~100 cells total. These were allowed to grow for over a week undisturbed. Colonies were observed as small cloudy points on the bottom of the plate. They were marked with a dot and then examined under the microscope. If it was clear to be one colony sufficiently separated from others, colonies were removed from the plate. Cloning rings were dipped in vacuum grease to create a seal and pressed down over the colony. Media was aspirated and 1 drop of Trypsin was added. After ~5 minutes, trypsinized cells were removed and plated in 6 well plated for

genotyping. Depending on genotyping results, cells were replated and the process repeated to obtain homogenous cell lines.

Nomenclature

Cell line names were derived based on the well they were removed from in a 96 well plate and the gRNAs used. For example, if a cell line is named 23A8A11, these cells were subjected to gRNAs 2 and 3 and clones were isolated first from well A8 and a subsequent round of cloning produced a cell colony from well A11. Note: after sequencing, many of the gRNA combinations noted in the cell line name appear to be in error. However, to avoid even more confusion, names remain unchanged.

CRISPR Genotyping Primer Design

Genotyping primers were designed using Primer3 software²⁸⁶. Initially, two sets of primers were designed to amplify the region containing hEDN1AS CRISPR 1 and 2 (hCRISPRpcr1F/R) and hEDN1AS CRISPR 3 and 4 (hCRISPRpcr2F/R). Subsequently, hCRISPRpcr1F and hCRISPRpcr2R were used for genotyping because they worked better. WT/ Het/ KO cells were determined by subtracting the calculated Cas9 cut size from the WT amplicon size (Table 5-1).

Sequencing

Gel-purified PCR products from hCRISPRpcr1F and hCRISPRpcr2R amplification were sent to GENEWIZ for sequencing and analyzed with the help of Dr. Kevin Brown.

Mycoplasma Decontamination and Detection

Mycoplasma was detected with the Venor®GeM OneStep PCR Kit (Minerva Biolabs) following manufacturer instructions. gDNA as well as cell culture media was used in reactions. Mycoplasma was successfully eliminated from infected cell lines (HK-2 23G7H3 and 23A8A11) using Mynox® Gold (Minerva Biolabs).

ET-1 ELISA

Media from HK-2 and mIMCD3 cell lines was taken, spun down to remove cell debris and then frozen at -80 °C until all samples were ready to use. When ready, media was thawed and concentrated using a speed-vac on medium heat setting (no heat re-froze the samples). ELISA for ET-1 was done with the Human Endothelin-1 QuantiGlo ELISA Kit (R&D Systems) according to manufacturer instructions. Luminescence was assessed using the Synergy HT Microplate reader with the following settings: emission through hole in filter wheel (no set wavelength), 1 min lag time (time after plate is inserted into reader), 0.5 sec integration time (time each well is measured) and automatic gain. Summation mode is recommended in the kit instructions but not available on the Synergy. R&D customer service said it is not critical.

Results

In order to confirm the presence of EDN1-AS predicted from the genome browser, strand-specific reverse transcriptase followed by PCR (SS-RT-PCR) was done on multiple cell lines (Figure 5-7). DNase treated RNA was used as a template in reverse transcriptase (RT) reactions using strand-specific (SS) primers (LJ3, LJ4, LJ5, SB1, SB2 and SB3). As a control for DNA contamination, each sample was also ran without RT. This cDNA was then amplified with PCR primers (LJ7 and LJ8). Absence of bands in –RT lanes confirms that bands in +RT lanes are due to EDN1-AS and not DNA contamination. EDN1-AS expression was shown in s9 (human bronchoendothelial) cells (Figure 5-7A), HMEC (human primary mammary endothelial cells) (Figure 5-7B), HK-2 (human kidney PT epithelial cells) (Figure 5-7C) and total human kidney RNA (Figure 5-7D).

Since all of the samples shown to express EDN1-AS were of human origin, we investigated the expression of EDN1-AS in mice. Kidneys from 129sv/s2 mice sacrificed at midnight were dissected into cortex and medulla and processed to cDNA with mouse SS-primers in the same way as human samples. As shown in Figure 5-8, EDN1-AS is expressed in mouse cortex (Figure 5-8A) and outer medulla (Figure 5-8B). Even though the gel cannot be quantified, it does appear that expression of EDN1-AS is more highly expressed in outer medulla compared to cortex. We also checked the aorta of C57BL/6J mice on control and HS/DOCP diet and found that while there were multiple bands in –RT lanes, two mice did not show bands in –RT yet there were bands in the +RT lanes, confirming expression of EDN1-AS in aorta as well (Figure 5-9).

Next, we wanted to investigate sex differences in expression. Following the same protocol for the 129sv/s2 mice, male and female kidneys from 129sv/s1s4 mice were examined for EDN1-AS expression (Figure 5-10). EDN1-AS expression was detected but was much more variable than in the 129sv/s2 strain. Female estrus cycle was not synchronized, possibly accounting for variation as well.

Since *Edn1* is regulated by aldosterone, we wanted to see if the same was true of EDN1-AS. We used a model of mouse inner medullary CD cells (mmIMCD3) treated with 100 nM aldosterone in three separate experiments (Figure 5-11). It appears that expression varies from passage to passage but within passages, EDN1-AS expression is higher in aldosterone treated samples compared to vehicle (100% ethanol).

Edn1 is also regulated by the circadian clock. In order to see if EDN1-AS is similarly regulated, we treated human kidney PT cells (HK-2) with 100 nM dexamethasone for 30 minutes to synchronize their circadian clocks (Figure 5-12). 24

hours later, RNA was isolated every 2 hours for 24 hours and SS-RT-PCR was done. In addition to using SS-primers, random hexamers were used in RT reactions to assess EDN1 expression. Figure 5-11A shows that EDN1-AS expression varies over 24 hours and is in phase with EDN1 expression as measured by real time PCR (curve overlay on panel A). GAPDH expression as measured by semi-quantitative PCR did not change. This is a preliminary experiment with n=1.

To determine if the mineralocorticoid and circadian regulation was occurring at the expected promoter region of EDN1-AS, chromatin immunoprecipitation was done on an area of the promoter shown to have glucocorticoid and clock binding sites (Figure 5-13, experiment done by Dr. J. Richards of the Cain lab). Indeed, in a preliminary experiment, Per1 and the glucocorticoid receptor were shown to bind at the promoter region.

Since EDN1-AS expression is conserved and appears to be regulated in multiple ways, we wanted to investigate the regulatory effect EDN1-AS has on EDN1. In order to accomplish this, CRISPR/Cas9 was used to delete parts of the EDN1-AS promoter region selected based on shown (in human) or predicted (in mouse) binding of important transcription factors. With the aid of Dr. Kevin Brown, HEK293 cells were transfected with plasmids containing guide RNAs for Cas9 as well as viral assembly proteins. The viruses were then used to infect HK-2 and mIMCD3 cells as human and mouse models, respectively. After multiple rounds of cloning, genotyping and sequencing, it was determined that all mIMCD3 clones were either heterozygous cell populations or had heterozygous deletion in individual cells. HK-2 cells comprised a mix of homozygous KO, heterozygous KO and heterozygous cell populations. A

representative genotyping gel is shown in Figure 5-14 with examples of A) heterozygous KO, B) WT and C) homozygous KO HK-2 cells as well as D) mIMCD3 cells, displaying bands of multiple sizes most likely due to heterozygous cell populations of heterozygous KOs.

After almost a year of cloning and genotyping, two HK-2 cell lines were genotyped as homozygous KOs and this was confirmed by sequencing. These cells along with six different mIMCD3 lines including WT, CRISPR WT and heterozygous lines were grown and media was taken to use in an ET-1 ELISA (Figure 5-15). The preliminary data appears to show increased ET-1 levels in CRISPR cells, however, all of the high data points are from media collected after 3 days compared to the other points which were taken after 1 day on the cells. Thus, more experiments are required to determine if changes are due to promoter disruption or simply the time that media was on the cells.

Discussion

The work summarized here has shed light on a possible new mechanism of EDN1 regulation – EDN1-AS. We have shown that this antisense transcript is expressed in multiple human cell lines with apparent differences in expression level (measured by semi-quantitative PCR) depending on the origin of the cells. In addition, EDN1-AS is expressed in mouse kidney and aorta, again with seemingly different levels of expression based on location. Preliminary experiments indicate that expression of EDN1-AS is regulated by the circadian clock and aldosterone and this is further supported by preliminary ChIP data showing the presence of Per1 protein and the glucocorticoid receptor at the EDN1-AS promoter region. Studies of EDN1-AS promoter activity KO in cell lines have resulted in ambiguous data so far. However, the presence

of EDN1-AS in human and mouse as well as its apparent regulation by multiple means are highly suggestive of the importance of this transcript.

Endothelin-1 is an integral part of normal development throughout the entire body (reviewed in ²⁵³). Dysregulation of Endothelin-1 and its receptors has been implicated in many disease states including diabetic nephropathy²⁸⁷, pulmonary arterial hypertension (PAH)²⁸⁸, preeclampsia^{289, 290}, atherosclerosis ^{291, 292}, cancer²⁹³ and coronary artery disease²⁹⁴. Endothelin receptor antagonists have not had great success at treating various diseases in which ET-1 is implicated. ETA receptor antagonists have shown some efficacy in treating PAH, however it seems that often the side effects are worse than the treatment (reviewed in²⁹⁵).

ET-1 plays a role in the regulation of almost every part of the kidney including the renal vasculature, glomerular mesangial cell proliferation, matrix production and contraction, nerve activity, acid/base handling and sodium and water transport in the nephron (reviewed in²⁹⁶). Increased levels of ET-1 have been correlated with chronic kidney disease, diabetic nephropathy as well as increased renal cell proliferation and inflammation (reviewed in²⁹⁷). While ETA receptor antagonists appear to be useful in opposing the unwelcome actions of ET-1^{298, 299}, none are yet approved for use in humans to treat any of the conditions in the kidney.

Our recent discovery of a lncRNA antisense to the Endothelin-1 gene (EDN1-AS) may provide another route of treatment and regulation of ET-1 levels. Antisense lncRNAs can regulate genes in a discordant manner, negatively regulating expression of their sense RNA, or in a concordant manner, leading to increased levels of corresponding sense RNA³⁰⁰. It appears that most antisense regulation is of the

discordant category³⁰¹. Many natural antisense transcripts (NATs) have been implicated in diseases including breast cancer³⁰², renal cancer³⁰³, schizophrenia³⁰⁴, Alzheimer's³⁰⁵, leukemia³⁰⁶ and Huntington's disease³⁰⁷, to name a few.

It has also been demonstrated that targeting antisense transcripts with strand-specific oligonucleotides can be therapeutically beneficial. Wahlestedt et al. were the first group to demonstrate this *in vivo*³⁰⁸. This group investigated Brain-Derived Neurotrophic Factor (BDNF) which is responsible for neuronal growth, differentiation and memory and is regulated by a NAT (BDNF-AS). They infused AntagoNATs (small oligonucleotides which antagonize sense-antisense interaction) by minipump into mice and found that after 28 days BDNF mRNA and protein levels were increased 50-100%. This lays the foundation for the proposal that EDN1-AS may also be a practical target for disease therapy in the future. Since lncRNAs also seem to be expressed and regulated in a tissue-specific manner³⁰¹, tissue-specific delivery could decrease the risk of side effects compared to chemical compounds. Preliminary data suggests that EDN1-AS expression does vary between tissue (outer medulla and cortex of kidney) as well as between mouse strain (129sv/s2 and 129sv/s1s4). Additionally, antisense transcripts tend to be low abundance and are not as extensively spliced as sense transcripts³⁰⁹. EDN1-AS again seems to fall into this category based on the fact that EDN1-AS is difficult to amplify, especially with RNA that is over 2 weeks old, but when it is, it is amplified regardless of which SS-primer is used. Thus, it appears that EDN1-AS is likely at least as long as EDN1 (due to amplification with SB3 which extends just into the 5' UTR of EDN1) and is not extensively spliced or processed, but further experiments are needed to confirm this.

Further experiments are also needed to investigate sex differences in EDN1-AS expression and regulation. We know that EDN1-AS and EDN1 are both regulated by the clock and expression is in phase with each other and that both are positively regulated by aldosterone. Thus, it follows that since EDN1 expression is different between the sexes, the expression of EDN1-AS would differ as well. Preliminary experiments concur with this prediction, showing that in one experiment female mice seem to have higher expression levels of EDN1-AS in the kidney compared to males. Tantalizingly, the estrous cycle of the females was not synchronized, which could explain the high variability of expression between female mice. ET-1 protein as well as EDN1, EDNRA and EDNRB mRNA levels vary in corpus luteum over the course of the bovine estrous cycle³¹⁰. In two a small studies of 10 and 16 participants, ET-1 plasma levels were shown to vary over the course of the menstrual cycle in humans^{311, 312}.

Another possibility is that EDN1-AS is not a lncRNA but an enhancer RNA (eRNA). eRNAs are transcripts produced from enhancers but their role is not yet clear (reviewed in³¹³). The first evidence of eRNAs was produced about 25 years ago while investigating the β -globin locus control region^{314, 315}. However, eRNAs were not named until 2010 with two papers confirming the production of transcripts from enhancers^{316, 317}. Thus, since the idea of an eRNA is relatively young, it is not clear what their function is. Li et al. have proposed three possible classes of eRNAs: those which are transcriptional noise, having no function, those which function to recruit transcription machinery to aid in transcription of other genes or those which actually act in *cis* or *trans* as an RNA to regulate other genes³¹³. The authors have also listed a few ways to differentiate lncRNAs from eRNAs including the presence of H3K36me3 (not present at

eRNA but present at lncRNA) and whether splicing has occurred (it is uncommon with eRNAs however lncRNAs have a bias for containing 2 exons). However, unpublished data which I was able to see recently suggests that EDN1-AS is actually a long, intergenic non-coding RNA (lincRNA) which starts thousands of bases further downstream than we thought and thus the area we have been targeting with CRISPR was very incorrect.

No one can deny the importance of Endothelin-1 as evidenced by its ubiquitous expression and conservation across species. What is also undeniable is the implication of ET-1 in many diseases, most of which cannot be adequately treated by targeting Endothelin-1 binding to its receptors. EDN1-AS provides a new possibility for treatment and although it is not well-characterized yet, the data we have gathered shows promise for EDN1-AS an important regulator of EDN1. Expression in multiple cell lines, authentic human kidney RNA as well as mice in addition to multiple means of regulation and possible sex-dependent changes in expression all suggest that this transcript is important and worth the trouble of conserving over the course of evolution. Only future studies will be able to address this prediction.

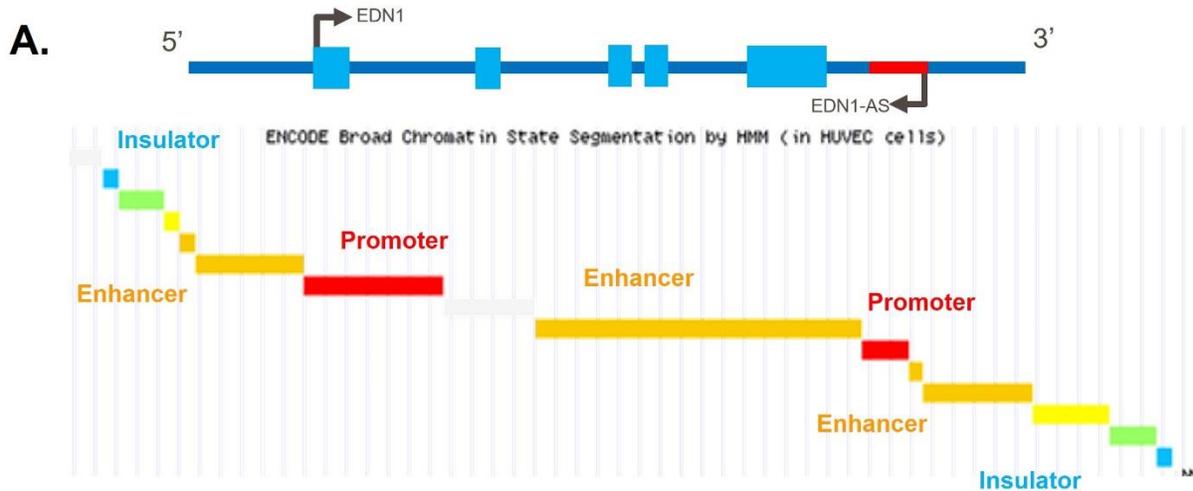


Figure 5-1. The UCSC genome browser predicted chromatin state of EDN1 in HUVEC (human umbilical vein endothelial cells). There is a predicted promoter at the end of the EDN1 sense transcript.

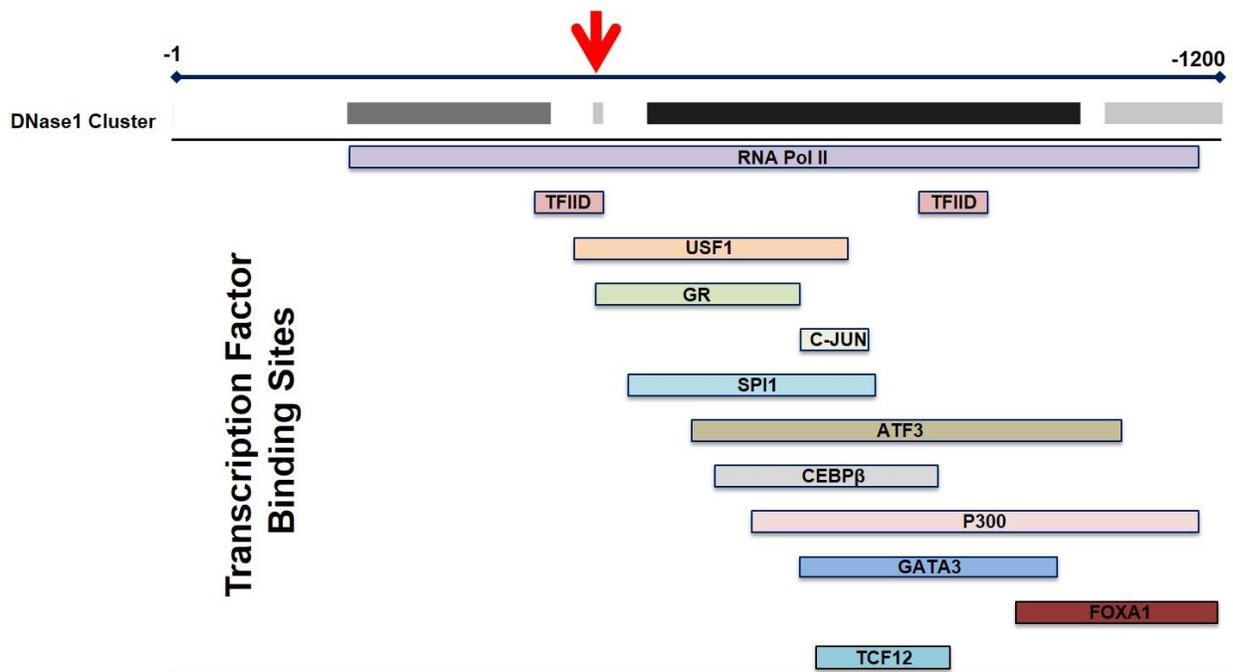


Figure 5-2. Transcription Factor Binding Sites at the EDN1-AS Predicted Promoter Region. These transcription factor binding sites were shown by ChIP assay. The cluster of DNase sensitivity as well as confirmed binding of many transcription factors known to be important in transcription suggest a regulated promoter region at the 3' end of the EDN1 gene. Figure courtesy of Dr. J. R. Richards.

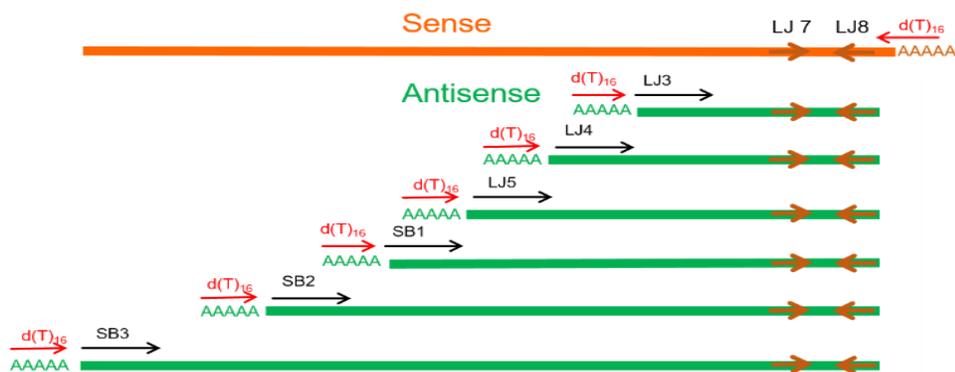


Figure 5-3. Diagram of strand-specific primer placement. Strand-specific primer used to identify EDN1-AS is illustrated here (not to scale). Each primer from LJ3 to SB3 starts progressively farther to the 5' end of EDN1. LJ7 and LJ8 amplify the same piece of cDNA regardless of the strand-specific primer used. Therefore, all bands in the PCR gel are the same size though they represent different sizes of EDN1-AS detected.

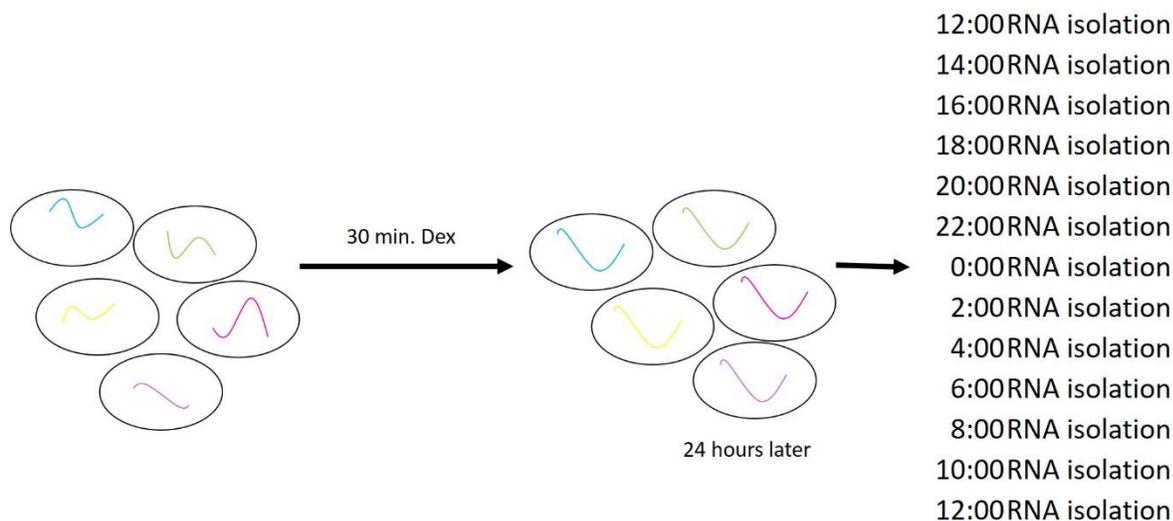


Figure 5-4. Dexamethasone Treatment of HK-2 Cells and RNA Isolation. HK-2 cells were plated in 6 well plates and grown to 80% confluency. Dexamethasone was added to media and removed after 30 minutes. 24 hours later, when cells were synchronized, RNA was isolated every 2 hours for 24 hours and used to make cDNA for later experiments.

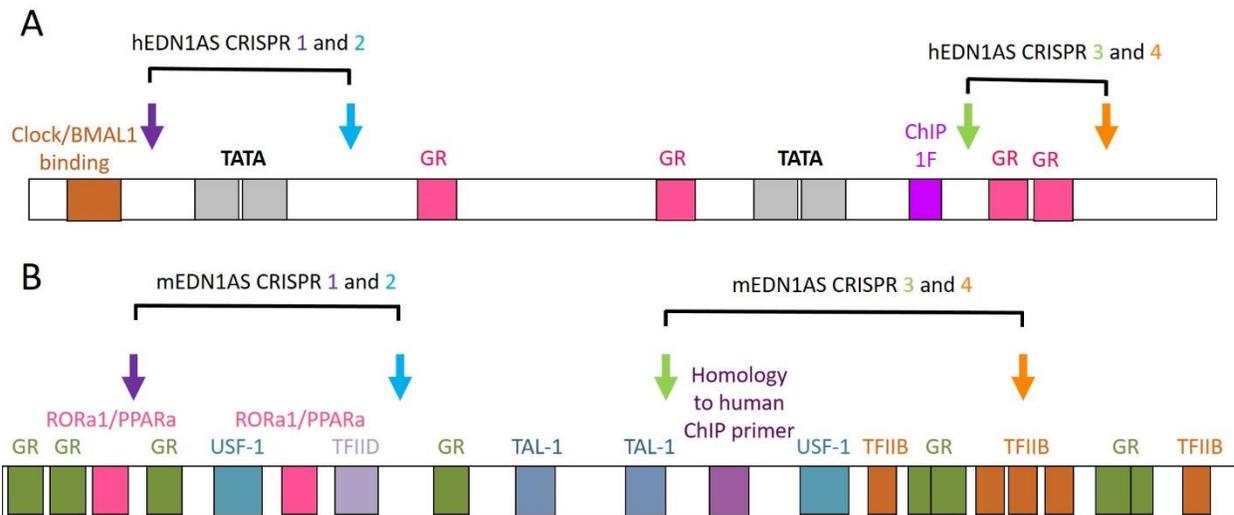


Figure 5-5. Rationalization for gRNA Design in Human EDN1. CRISPR gRNAs were designed in order to attempt to remove important transcription factor binding sites from the promoter region. Two gRNAs were spaced about 100bp apart. A) Human sites were confirmed by CHIP assay and obtained from ENCODE data through the UCSC Genome Browser. B) Mouse sites are predicted and were obtained from ENCODE data through the UCSC Genome Browser.

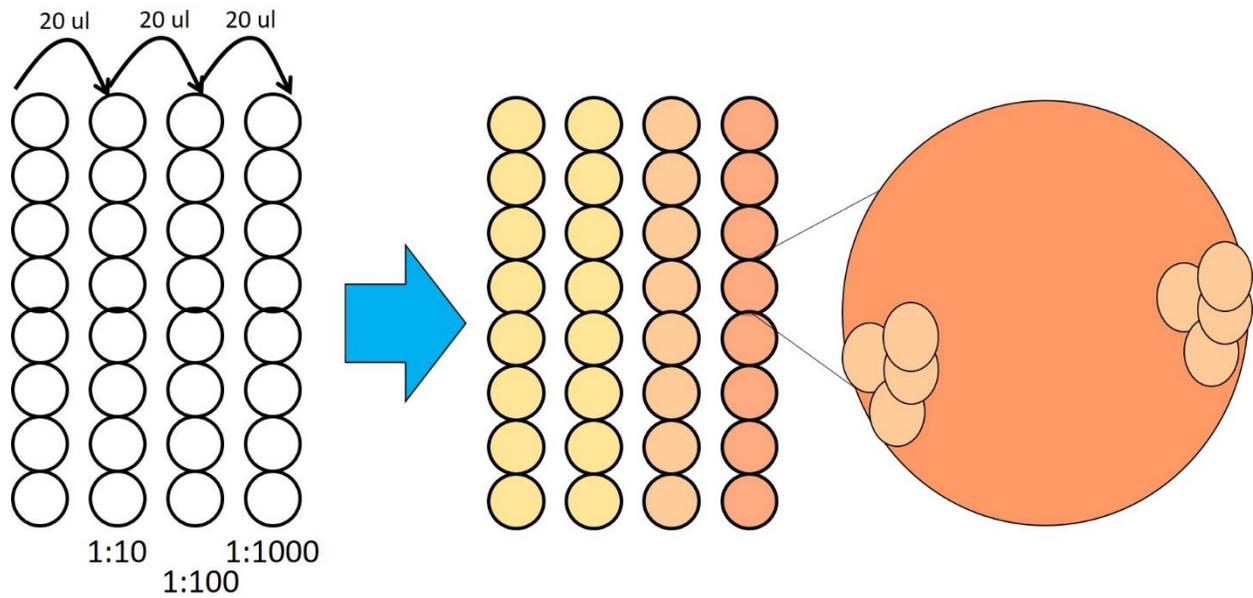


Figure 5-6. 96-well Cloning Method for CRISPR/Cas9 Knockout Cells. Multiple cells from single “colony” isolations were plated along a column of a 96 well plate in 200 μ l of media. 20 μ l was taken and diluted 1:10 in 3 subsequent columns, producing a final dilution of 1:1000. Using media color as an indication of growth, once the first one or two columns showed significant acidification, columns 3 and 4 were observed for colony growth. Individual wells with one to three colonies were isolated with trypsin and replated in a new 96 well plate to attempt to homogenize populations.

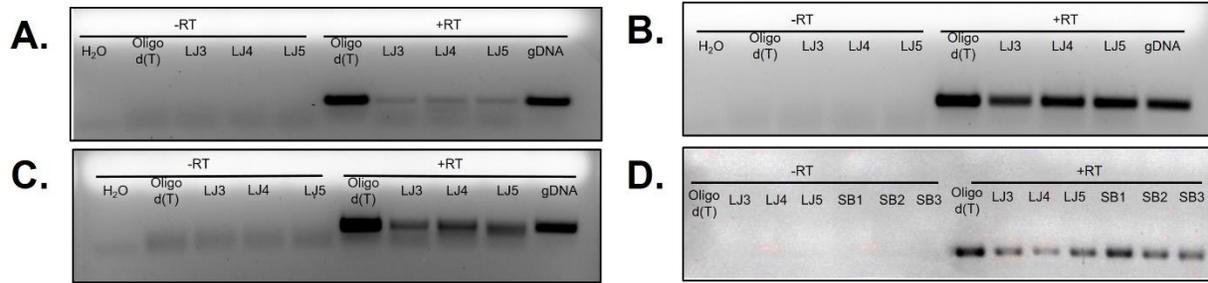


Figure 5-7. EDN1-AS is expressed in several human cell lines and in human kidney. EDN1-AS is expressed in A) In s9 (human bronchoendothelial) cells, B) HMEC (human primary mammary endothelial cells), C) HK-2 (human kidney PT epithelial) cells and D) total human kidney RNA. The first five lanes in A, B, and C correspond to water, oligo d(T), LJ3, LJ4 and LJ5 strand specific primers, respectively, with no reverse transcriptase (RT) as negative controls. The last five lanes in A, B and C correspond to oligo d(T), LJ3, LJ4 and LJ5 strand-specific primers and gDNA as a positive control. In D, lanes correspond to gDNA, LJ3, LJ4, LJ5 and SB1, SB2 and SB3 (which are strand specific primers even more distal to the LJ primers) with RT and the next lanes correspond to the same primers with no RT and the last lane has water as a negative control. The presence of the 165 bp band in the +RT lanes indicates the presence of EDN1-AS.

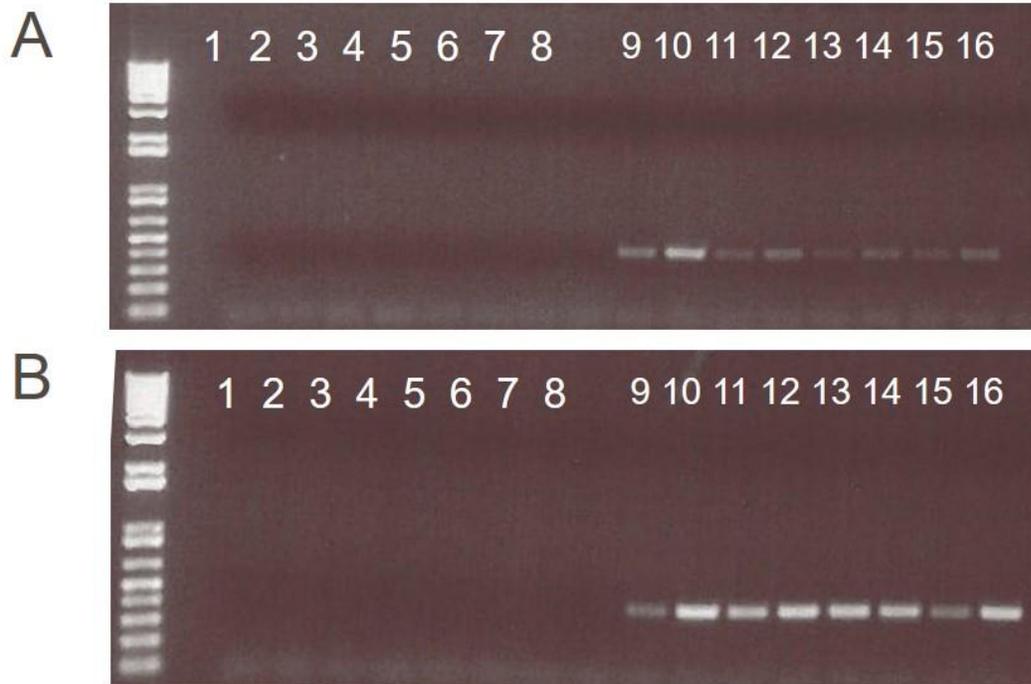


Figure 5-8. EDN1-AS expression is increased in the murine renal outer medulla. Male 129/svs2 mouse kidneys were harvested at midnight and dissected into cortex and outer medulla. RT-PCR was performed as described except with mouse EDN1-AS strand-specific and PCR primers. EDN1-AS was detected in both A) cortex and B) outer medulla. Each lane on the cortex gel corresponds to the same lane and mouse on the outer medulla gel. Lanes 1-8 on each gel are -RT controls and lanes 9-16 on each gel are +RT. A single strand-specific RT primer (mEDN1AS-4) was used so each band represents a different mouse. Levels of EDN1-AS appear to be increased in the outer medulla compared to the cortex for each mouse.



Figure 5-9. EDN1-AS Expression in Mouse Aorta. Aorta collected from C57BL/6J mice on control diet and HS/DOCP was processed and subjected to SS-RT-PCR to probe for EDN1-AS expression. While there were multiple mice that had bands in the –RT lanes (panel A, lanes 4, 8, 9, 10), two did not have bands in –RT (panel A, lanes 3, 6) but showed bands +RT (panel B, lanes 3, 6) (blue boxes), confirming the presence of EDN1-AS in aorta. Panel B lane 11 is a negative water control, lane 12 is a positive gDNA control. The extreme exposure (as indicated by the ladder and gDNA) suggest low expression levels of EDN1-AS in aorta or poor RNA quality.

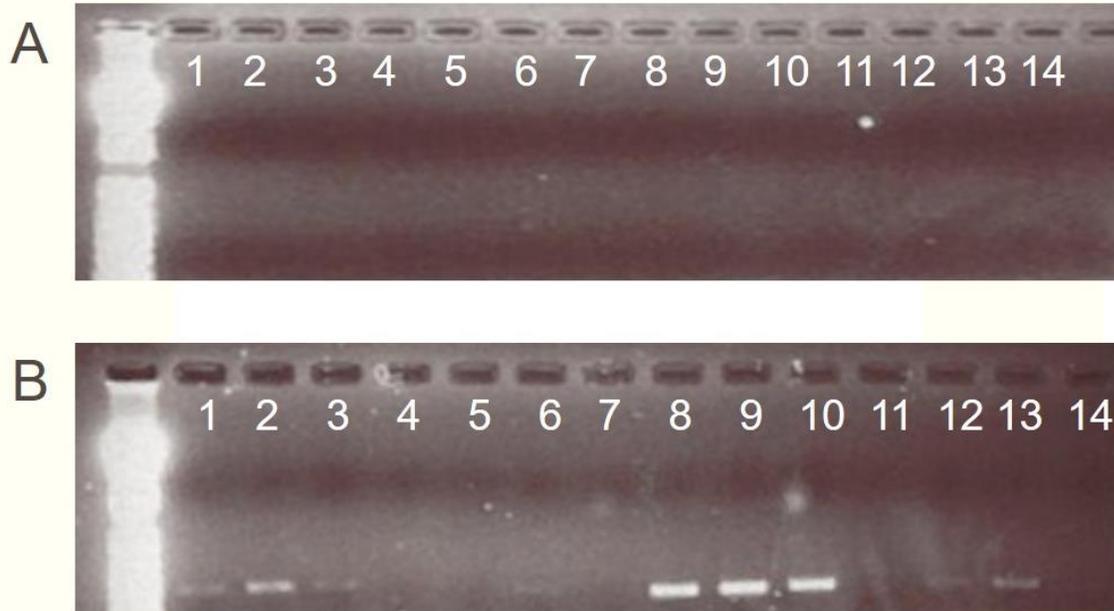


Figure 5-10. EDN1-AS expression may exhibit sex differences in murine renal cortex. Male and female 129/svs1s4 mouse kidneys were harvested at midnight and cortex was dissected and processed for EDN1-AS as described. A) -RT controls were negative while B) +RT samples showed varying amounts of EDN1-AS. In both panels, lanes 1-7 are male mice and lanes 8-14 are female. It appears that females may have higher levels of EDN1-AS in kidney cortex, however the estrous cycles were not synchronized which may account for some variation. Each band represents an individual mouse.



Figure 5-11. EDN1-AS expression is regulated by aldosterone in mmIMCD3 cells. mmIMCD3 (a model of the murine inner medullary CD) cells were treated with 100 nM aldosterone in three separate experiments. Cells were harvested and RNA was collected with Trizol. After DNase treatment, strand-specific RT was performed as previously described. On the left side of Figure 6 are -RT controls, which have no visible bands. The samples on the right had +RT and bands are clearly visible. Vehicle (ethanol) and aldosterone treated samples alternate and it is apparent that there is an increase in EDN1-AS expression with all three aldosterone treatments. Thus, it appears that EDN1-AS expression is positively regulated by aldosterone in vitro.

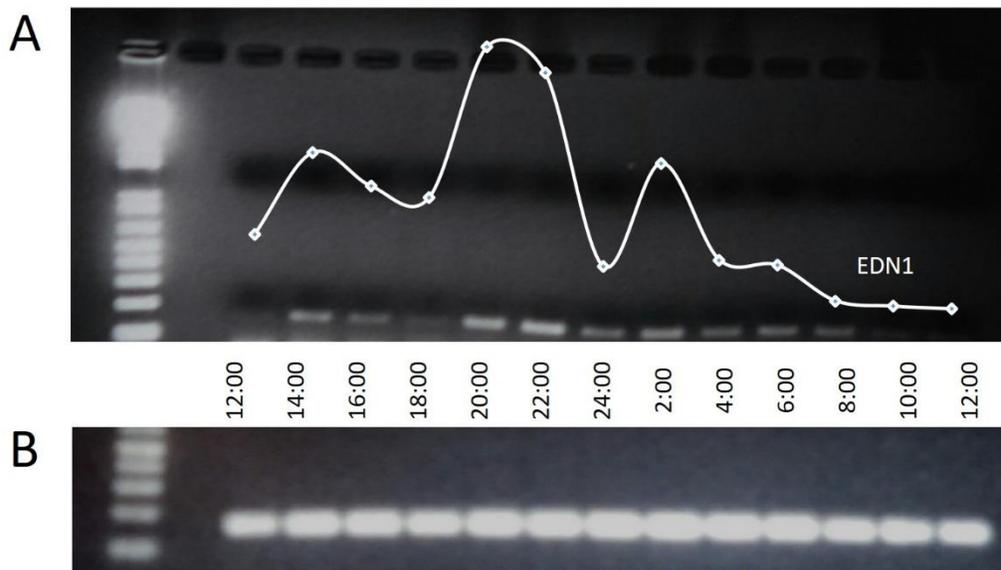


Figure 5-12. EDN1-AS is regulated by the circadian clock. HK-2 cells were synchronized with 100 nM dexamethasone for 30 minutes, after which media was changed. 24 hours later, RNA from these cells was isolated every 2 hours for 24 hours starting at noon (12:00). A) Strand-specific RT using LJ4 and PCR reactions using LJ7 and LJ8 was done to assess EDN1-AS expression. cDNA was also made using random hexamers to assay EDN1 expression (real time curve, panel A). Expression of EDN1-AS is in phase with EDN1 mRNA. B) GAPDH expression did not change over time.

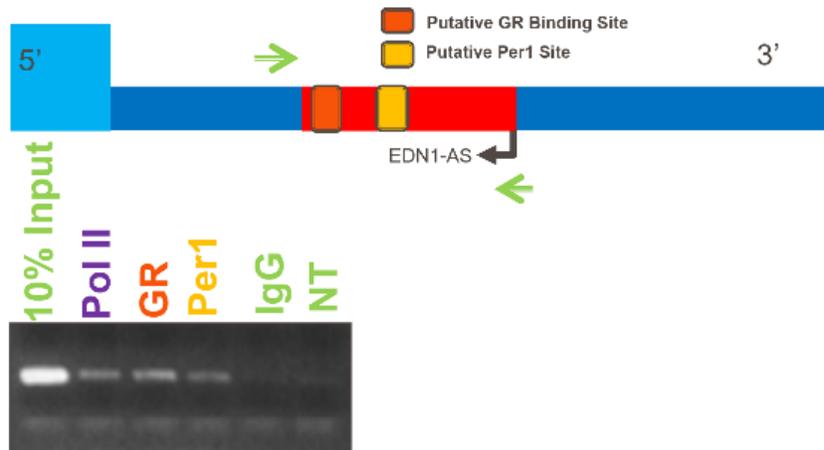


Figure 5-13. RNA polymerase II, GR and Per1 are present at the EDN1-AS promoter. Preliminary ChIP data show the binding of RNA polymerase II, the glucocorticoid receptor (GR) and the circadian clock protein Per1 to the EDN1-AS promoter region. Chromatin/protein interactions were fixed in HK-2 cells and sheared chromatin was immunoprecipitated with antibodies against Pol II, GR, Per1 and rabbit IgG (as a negative control). PCR with primers encompassing the EDN1-AS promoter (indicated by green arrows) gave positive signals for all three antibodies and no signal for the negative control. Data provided by the Cain lab, courtesy of J. Richards.

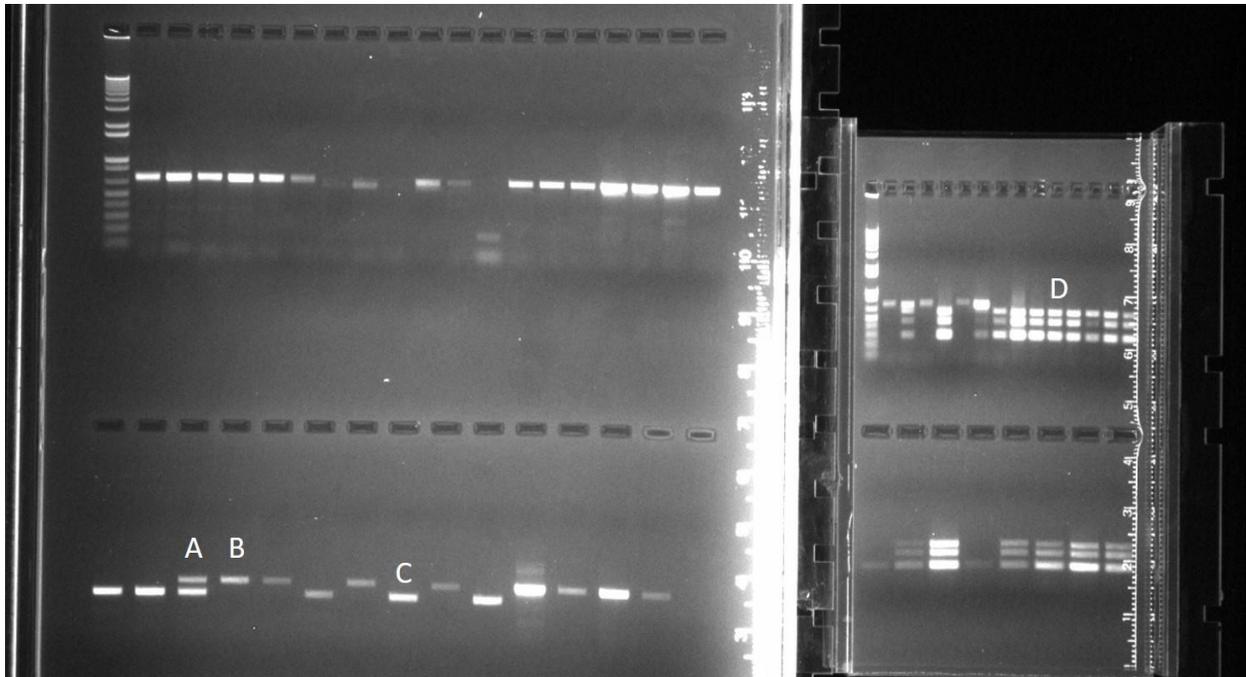


Figure 5-14. Representative Genotyping Gel for CRISPR/Cas9 Knockout. HK-2 cells were genotyped for EDN1-AS promoter region deletion in the gel on the left side showing A) heterozygous KO, B) WT and C) homozygous KO cells. On the right side gel, mIMCD3 cells were genotyped, displaying bands of multiple sizes (D).

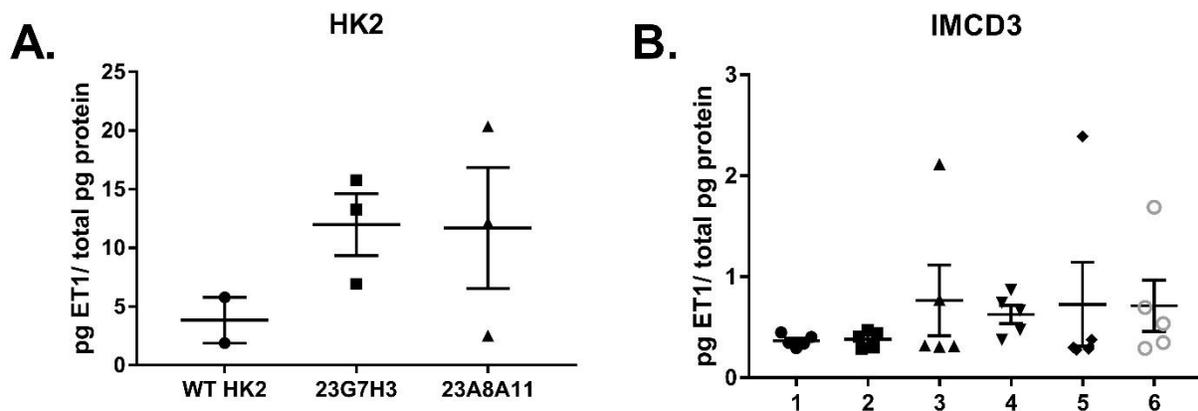


Figure 5-15. ET-1 ELISA of Media from CRISPR Cell Lines. ET-1 ELISA of media from HK-2 and mIMCD3 cells was done to assess differences due to KO of the promoter region of EDN1-AS. A) Levels of ET-1 in WT and homozygous KO HK-2 lines. B) Levels of ET-1 in WT (1-3) or heterozygous KO (4-6) mIMCD3 cell lines.

Table 5-1. Cut size and expected amplicon size for all gRNA combinations.

Human Primers 1F and 2R			Mouse Primers 1F and 2R		
gRNAs	cut size	amplicon size	gRNAs	cut size	amplicon size
none	0	689	none	0	621
1 and 2	77	612	1 and 2	65	556
1 and 3	366	323	1 and 3	394	227
1 and 4	489	200	1 and 4	513	108
2 and 3	289	400	2 and 3	329	292
2 and 4	412	277	2 and 4	448	173
3 and 4	123	566	3 and 4	119	502

CHAPTER 6 CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The contribution of Per1 to the regulation of salt and water reabsorption by the nephron is clear from previous work in our lab. The work presented in Chapter 2 showing that Per1 regulates sodium transport in the PT added to the previous work and showed that Per1 regulates sodium transport throughout the entire nephron. Additionally, the work suggested that this regulation was done in a coordinate manner, with Per1 positively regulating positive regulators of sodium reabsorption and negatively regulating negative regulators of sodium reabsorption. All of the cell culture data fit nicely with experiments in WT and Per1 KO mice on a 129/svs2 background.

However, when telemetry data was analyzed from WT and Per1 KO mice on a C57BL/6J background, the same baseline phenotypes were not observed as the mice on a 129sv/s2 strain. While Per1 KO mice on a 129sv/s2 background had considerably lower BP (BP) compared to WT mice, Per1 KO mice on a C57BL/6J background had significantly higher BP than WT mice. After an initial panic, it became clear, and after some thought not all that surprising, that strain differences in background genetics of the mice could be at play. After all, two different humans could display two different phenotypes after the same insult and nobody would bat an eye because humans are not inbred like the laboratory mice that are used.

Furthermore, these mice who were at first a problem became a great source of information when their BP on a HS/DOCP diet was examined. Normally, C57 mice are hardy little creatures and are difficult to stress. Hypertension is usually induced in this mice not only by a high salt diet plus mineralocorticoid (like DOCP) but also by infusion

of Angiotensin II or uninephrectomy. Without this, the mice show no change in BP, as we see in the male WT mice in Chapter 3. However, without Per1 these mice develop harmful nondipping on a HS/DOCP diet. Thus, simply by removing Per1 and without the need for nephrectomy or infusion of a hypertensive agent, a significant phenotype was created in these mice. This is a powerful example of the importance of Per1 to sodium and BP regulation.

To compliment these studies, we examined the role of Per1 in HS/DOCP diet in female mice, discussed in Chapter 4. In general, females are more protected from harmful effects of protocols like this and indeed, the female mice did not display a nondipping phenotype like the males. This suggests a protective effect in females which may be linked to sex hormones as many of the general protection seen in females is lost after menopause. While this was a limited study, both by the number of animals used and the experimental procedures performed, it was an important first step in the analysis of sex differences in the regulation of BP by the clock. There is a decided lack of studies investigating sex differences in general but also specifically with regard to the circadian control of BP.

The last chapter of this dissertation deals with antisense lncRNAs and Endothelin-1 which seem to be unrelated to the rest of the work at first glance. However, Endothelin-1 is the most potent natural vasoconstrictor known and has diuretic effects in the nephron. It is also regulated by Per1. Early evidence also indicates that Per1 is involved in the regulation of EDN1-AS as well, shown by Per1 residence at the promoter region of EDN1-AS. Thus, if EDN1-AS is later shown to have regulatory function and be regulated itself, there is a high likelihood that Per1 will be strongly

involved. Although there are many pieces of evidence supporting the importance and regulation of EDN1-AS, many of the data is preliminary and needs to be confirmed with more experiments.

Thus, Per1 is clearly an important modulator of BP through regulation of salt and water reabsorption by the nephron. Not only is Per1 implicated in normal regulation of BP, but this work has shown it may also play a role in pathological states as well. Additionally, preliminary but convincing data suggest that Per1 also regulates EDN1-AS which in turn may regulated Endothelin-1.

Future Directions

There appears to be different regulatory mechanisms of Per1 based on model system and strain as well as sex. This provides numerous avenues of exploration. Unfortunately, both strain differences and sex differences are not very broadly studied even though the human race is made up of largely non-inbred humans, about half of which are female. In order to have a representative body of knowledge, this gap needs to be filled. cursory examination of the Jackson Lab Mouse Phenome Database shows a wide range of baseline systolic BP from 75 mmHg to 130 mmHg in 23 strains³¹⁸⁻³²⁰. Thus, it is not all that surprising that C57BL/6J mice have an opposite phenotype from 129sv/s2 mice and that mice on a mixed substrain 129sv/s1s4 background have a slightly different phenotype than both. While at first these conflicting results are frustrating, in reality they provide a way to study very specific aspects of Per1 action since the genetic differences between the strains will point the way. Ideally, genome wide analysis of Per1 binding sites would provide a list of genes to investigate first. If there are any promising candidates, further study of expression differences in WT and

Per1 KO mice of each strain could suggest a mechanism for the observed strain differences. This could eventually be applied to personalized medicine based on genotype or certain single nucleotide polymorphisms (SNPs).

Sex differences provide a very similar avenue to explore specific aspects of Per1 regulation. There is a reason that males develop nondipping and females do not. Exploration and elucidation of this reason could provide very useful information when it comes to personalized treatments for conditions in which Per1 is not regulated properly. Repeating the HS/DOCP experiment with sham and ovariectomized mice would be the first step in understanding the role of sex hormones in the observed phenotypes. If ovariectomy shows no difference, mice could be allowed to age naturally in order to determine if age itself plays a role. It would also be interesting to see if there are any estrogen receptor binding sites on any genes related to sodium regulation in the nephron and see if mutation of these sites produces an observable phenotype.

It is also important to continue work on nondipping in the male mice. This is a problem in humans and is associated with increased risk of cardiovascular events. There is a need for more information on the etiology and risk conferred by nondipping. Simultaneously, there is a need to modulate nondipping and restore a proper circadian rhythm to BP in nondippers. These mice represent a novel way to approach this problem and further study of differences not only between WT and KO males but between females as well could shed light on the mechanism of nondipping.

Finally, EDN1-AS shows promise as yet another target of treatment of various diseases. While all of the data gathered so far is quite preliminary, when taken together it becomes more convincing. If EDN1-AS is subsequently shown to regulate EDN1

expression, it could be a major target not only in the kidney but in multiple organs and tissues since EDN1 is so ubiquitously expressed. All of the experiments presented here should be repeated to add confidence. CRISPR/Cas9 cloning is planned to be repeated in the lab shortly with a lipofectamine method instead of the viral method shown here. It is the hope that this method will be more effective and produce clones in a shorter amount of time in order to reduce passage number and the time that cells are alive before experiments can be done. Once clones are created, ET-1 expression levels can be assed at baseline between WT and KO cells as well as in response to different treatments like aldosterone, glucose or albumin. In the future, if EDN1-AS is proven to be all that is hoped, a mouse model can be made with KO of EDN1-AS to determine the tissue specific effects of this lncRNA.

LIST OF REFERENCES

1. Solocinski K, Gumz ML. The circadian clock in the regulation of renal rhythms. *J Biol Rhythms*. 2015;30:470-486
2. Richards J, Gumz ML. Advances in understanding the peripheral circadian clocks. *FASEB J*. 2012;26:3602-3613
3. Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. *Annu Rev Physiol*. 2010;72:517-549
4. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends in cell biology*. 2014;24:90-99
5. Badura L, Swanson T, Adamowicz W, Adams J, Cianfrogna J, Fisher K, Holland J, Kleiman R, Nelson F, Reynolds L, St Germain K, Schaeffer E, Tate B, Sprouse J. An inhibitor of casein kinase i epsilon induces phase delays in circadian rhythms under free-running and entrained conditions. *J Pharmacol Exp Ther*. 2007;322:730-738
6. Lee JW, Hirota T, Peters EC, Garcia M, Gonzalez R, Cho CY, Wu X, Schultz PG, Kay SA. A small molecule modulates circadian rhythms through phosphorylation of the period protein. *Angewandte Chemie*. 2011;50:10608-10611
7. Koike N, Yoo SH, Huang HC, Kumar V, Lee C, Kim TK, Takahashi JS. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science*. 2012;338:349-354
8. Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB. A circadian gene expression atlas in mammals: Implications for biology and medicine. *Proc Natl Acad Sci U S A*. 2014;111:16219-16224
9. Moore-Ede MC. Physiology of the circadian timing system: Predictive versus reactive homeostasis. *Am J Physiol*. 1986;250:R737-752
10. Ede MC, Faulkner MH, Tredre BE. An intrinsic rhythm of urinary calcium excretion and the specific effect of bedrest on the excretory pattern. *Clin Sci*. 1972;42:433-445
11. Palmer LG, Schnermann J. Integrated control of na transport along the nephron. *Clin J Am Soc Nephrol*. 2015;10:676-687

12. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB, American Heart Association Statistics C, Stroke Statistics S. Executive summary: Heart disease and stroke statistics--2014 update: A report from the american heart association. *Circulation*. 2014;129:399-410
13. Saifur Rohman M, Emoto N, Nonaka H, Okura R, Nishimura M, Yagita K, van der Horst GT, Matsuo M, Okamura H, Yokoyama M. Circadian clock genes directly regulate expression of the na(+)/h(+) exchanger nhe3 in the kidney. *Kidney Int*. 2005;67:1410-1419
14. Pizarro A, Hayer K, Lahens NF, Hogenesch JB. Circadb: A database of mammalian circadian gene expression profiles. *Nucleic Acids Res*. 2013;41:D1009-1013
15. Krid H, Dorison A, Salhi A, Cheval L, Crambert G. Expression profile of nuclear receptors along male mouse nephron segments reveals a link between errbeta and thick ascending limb function. *PLoS One*. 2012;7:e34223
16. Richards J, Ko B, All S, Cheng KY, Hoover RS, Gumz ML. A role for the circadian clock protein per1 in the regulation of the nacl co-transporter (ncc) and the with-no-lysine kinase (wnk) cascade in mouse distal convoluted tubule cells. *J Biol Chem*. 2014;289:11791-11806
17. Susa K, Sohara E, Isobe K, Chiga M, Rai T, Sasaki S, Uchida S. Wnk-osr1/spak-ncc signal cascade has circadian rhythm dependent on aldosterone. *Biochem Biophys Res Commun*. 2012;427:743-747
18. Zuber AM, Centeno G, Pradervand S, Nikolaeva S, Maquelin L, Cardinaux L, Bonny O, Firsov D. Molecular clock is involved in predictive circadian adjustment of renal function. *Proc Natl Acad Sci U S A*. 2009;106:16523-16528
19. Stow LR, Richards J, Cheng KY, Lynch IJ, Jeffers LA, Greenlee MM, Cain BD, Wingo CS, Gumz ML. The circadian protein period 1 contributes to blood pressure control and coordinately regulates renal sodium transport genes. *Hypertension*. 2012;59:1151-1156
20. Gumz ML, Stow LR, Lynch IJ, Greenlee MM, Rudin A, Cain BD, Weaver DR, Wingo CS. The circadian clock protein period 1 regulates expression of the renal epithelial sodium channel in mice. *J Clin Invest*. 2009;119:2423-2434
21. Mills JN, Stanbury SW. Persistent 24-hour renal excretory rhythm on a 12-hour cycle of activity. *The Journal of physiology*. 1952;117:22-37

22. Moore Ede MC, Brennan MF, Ball MR. Circadian variation of intercompartmental potassium fluxes in man. *J Appl Physiol.* 1975;38:163-170
23. Koopman MG, Koomen GC, Krediet RT, de Moor EA, Hoek FJ, Arisz L. Circadian rhythm of glomerular filtration rate in normal individuals. *Clin Sci (Lond).* 1989;77:105-111
24. Pons M, Forpomes O, Espagnet S, Cambar J. Relationship between circadian changes in renal hemodynamics and circadian changes in urinary glycosaminoglycan excretion in normal rats. *Chronobiology international.* 1996;13:349-358
25. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature.* 2002;418:935-941
26. Stow LR, Gumz ML. The circadian clock in the kidney. *J Am Soc Nephrol.* 2011;22:598-604
27. Hilfenhaus M. Circadian rhythm of the renin-angiotensin-aldosterone system in the rat. *Archives of toxicology.* 1976;36:305-316
28. Jensen LW, Pedersen EB. Nocturnal blood pressure and relation to vasoactive hormones and renal function in hypertension and chronic renal failure. *Blood pressure.* 1997;6:332-342
29. Morawska-Barszczewska J, Guzek JW, Kaczorowska-Skora J. Cholecystokinin octapeptide and the daily rhythm of vasopressin and oxytocin release. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association.* 1996;104:164-171
30. Stepien M, Witte K, Lemmer B. Chronobiologic evaluation of angiotensin-converting enzyme activity in serum and lung tissue from normotensive and spontaneously hypertensive rats. *Chronobiology international.* 1993;10:331-337
31. Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. *Cell.* 2001;104:545-556
32. Moon JY. Recent update of renin-angiotensin-aldosterone system in the pathogenesis of hypertension. *Electrolyte Blood Press.* 2013;11:41-45
33. Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin-angiotensin system: From physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev.* 2007;59:251-287
34. Prieto MC, Gonzalez AA, Navar LG. Evolving concepts on regulation and function of renin in distal nephron. *Pflugers Arch.* 2013;465:121-132

35. Rittig S, Matthiesen TB, Pedersen EB, Djurhuus JC. Circadian variation of angiotensin ii and aldosterone in nocturnal enuresis: Relationship to arterial blood pressure and urine output. *The Journal of urology*. 2006;176:774-780
36. Portaluppi F, Bagni B, degli Uberti E, Montanari L, Cavallini R, Trasforini G, Margutti A, Ferlini M, Zanella M, Parti M. Circadian rhythms of atrial natriuretic peptide, renin, aldosterone, cortisol, blood pressure and heart rate in normal and hypertensive subjects. *Journal of hypertension*. 1990;8:85-95
37. Kool MJ, Wijnen JA, Derkx FH, Struijker Boudier HA, Van Bortel LM. Diurnal variation in prorenin in relation to other humoral factors and hemodynamics. *American journal of hypertension*. 1994;7:723-730
38. Vesely DL, Sothorn RB, Scheving LE, Bremner FW, Third JL, McCormick JB, Dawson S, Kahn S, Augustine G, Ryan M, Greco J, Nemchausky BA, Shirazi P, Kanabrocki EL. Circadian relationships between circulating atrial natriuretic peptides and serum calcium and phosphate in healthy humans. *Metabolism: clinical and experimental*. 1996;45:1021-1028
39. Winters CJ, Sallman AL, Vesely DL. Circadian rhythm of prohormone atrial natriuretic peptides 1-30, 31-67 and 99-126 in man. *Chronobiology international*. 1988;5:403-409
40. Vesely DL. Urodilatin: A better natriuretic peptide? *Current heart failure reports*. 2007;4:147-152
41. Drummer C, Fiedler F, Konig A, Gerzer R. Urodilatin, a kidney-derived natriuretic factor, is excreted with a circadian rhythm and is stimulated by saline infusion in man. *Journal of the American Society of Nephrology : JASN*. 1991;1:1109-1113
42. Choi MR, Kouyoumdzian NM, Rukavina Mikusic NL, Kravetz MC, Roson MI, Rodriguez Fermepin M, Fernandez BE. Renal dopaminergic system: Pathophysiological implications and clinical perspectives. *World journal of nephrology*. 2015;4:196-212
43. Castaneda TR, de Prado BM, Prieto D, Mora F. Circadian rhythms of dopamine, glutamate and gaba in the striatum and nucleus accumbens of the awake rat: Modulation by light. *Journal of pineal research*. 2004;36:177-185
44. Challet E, Dumont S, Mehdi MK, Allemann C, Bousser T, Gourmelen S, Sage-Ciocca D, Hicks D, Pevet P, Claustrat B. Aging-like circadian disturbances in folate-deficient mice. *Neurobiol Aging*. 2013;34:1589-1598
45. Ozgur S, Sancar A. Purification and properties of human blue-light photoreceptor cryptochrome 2. *Biochemistry*. 2003;42:2926-2932
46. Fournier I, Ploye F, Cottet-Emard JM, Brun J, Claustrat B. Folate deficiency alters melatonin secretion in rats. *The Journal of nutrition*. 2002;132:2781-2784

47. Weinert D. Age-dependent changes of the circadian system. *Chronobiology international*. 2000;17:261-283
48. Walker BR, Campbell JC, Fraser R, Stewart PM, Edwards CR. Mineralocorticoid excess and inhibition of 11 beta-hydroxysteroid dehydrogenase in patients with ectopic acth syndrome. *Clin Endocrinol (Oxf)*. 1992;37:483-492
49. Bonny O, Vinciguerra M, Gumz ML, Mazzoccoli G. Molecular bases of circadian rhythmicity in renal physiology and pathology. *Nephrol Dial Transplant*. 2013;28:2421-2431
50. Cooke CR, Whelton PK, Moore MA, Caputo RA, Bledsoe T, Walker WG. Dissociation of the diurnal variation of aldosterone and cortisol in anephric subjects. *Kidney Int*. 1979;15:669-675
51. Stefanovic V, Ivic M, Mitic M. Diurnal urinary excretion of cortisol and aldosterone in kidney graft recipients. *Pathol Biol (Paris)*. 1995;43:766-771
52. Kino T. Circadian rhythms of glucocorticoid hormone actions in target tissues: Potential clinical implications. *Sci Signal*. 2012;5:pt4
53. Leliavski A, Dumbell R, Ott V, Oster H. Adrenal clocks and the role of adrenal hormones in the regulation of circadian physiology. *J Biol Rhythms*. 2015;30:20-34
54. Gava AL, Freitas FP, Balarini CM, Vasquez EC, Meyrelles SS. Effects of 5/6 nephrectomy on renal function and blood pressure in mice. *International journal of physiology, pathophysiology and pharmacology*. 2012;4:167-173
55. Huang XM, Chen WL, Yuan JP, Yang YH, Mei QH, Huang LX. Altered diurnal variation and localization of clock proteins in the remnant kidney of 5/6 nephrectomy rats. *Nephrology (Carlton)*. 2013;18:555-562
56. Grisk O, Kloting I, Exner J, Spiess S, Schmidt R, Junghans D, Lorenz G, Rettig R. Long-term arterial pressure in spontaneously hypertensive rats is set by the kidney. *J Hypertens*. 2002;20:131-138
57. Fang Z, Carlson SH, Peng N, Wyss JM. Circadian rhythm of plasma sodium is disrupted in spontaneously hypertensive rats fed a high-nacl diet. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2000;278:R1490-1495
58. Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell*. 2000;103:1009-1017

59. Alvarez JD, Hansen A, Ord T, Bebas P, Chappell PE, Giebultowicz JM, Williams C, Moss S, Sehgal A. The circadian clock protein *bmal1* is necessary for fertility and proper testosterone production in mice. *Journal of biological rhythms*. 2008;23:26-36
60. Sun Y, Yang Z, Niu Z, Wang W, Peng J, Li Q, Ma MY, Zhao Y. The mortality of *mop3* deficient mice with a systemic functional failure. *Journal of biomedical science*. 2006;13:845-851
61. Curtis AM, Cheng Y, Kapoor S, Reilly D, Price TS, Fitzgerald GA. Circadian variation of blood pressure and the vascular response to asynchronous stress. *Proc Natl Acad Sci U S A*. 2007;104:3450-3455
62. Izumo M, Pejchal M, Schook AC, Lange RP, Walisser JA, Sato TR, Wang X, Bradfield CA, Takahashi JS. Differential effects of light and feeding on circadian organization of peripheral clocks in a forebrain *bmal1* mutant. *eLife*. 2014;3
63. Tokonami N, Mordasini D, Pradervand S, Centeno G, Jouffe C, Maillard M, Bonny O, Gachon F, Gomez RA, Sequeira-Lopez ML, Firsov D. Local renal circadian clocks control fluid-electrolyte homeostasis and bp. *J Am Soc Nephrol*. 2014;25:1430-1439
64. Nikolaeva S, Pradervand S, Centeno G, Zavadova V, Tokonami N, Maillard M, Bonny O, Firsov D. The circadian clock modulates renal sodium handling. *J Am Soc Nephrol*. 2012;23:1019-1026
65. Doi M, Takahashi Y, Komatsu R, Yamazaki F, Yamada H, Haraguchi S, Emoto N, Okuno Y, Tsujimoto G, Kanematsu A, Ogawa O, Todo T, Tsutsui K, van der Horst GT, Okamura H. Salt-sensitive hypertension in circadian clock-deficient *cry*-null mice involves dysregulated adrenal *hsd3b6*. *Nat Med*. 2010;16:67-74
66. Gachon F, Olela FF, Schaad O, Descombes P, Schibler U. The circadian par-domain basic leucine zipper transcription factors *dbp*, *tef*, and *hlf* modulate basal and inducible xenobiotic detoxification. *Cell Metab*. 2006;4:25-36
67. Gachon F, Fonjallaz P, Damiola F, Gos P, Kodama T, Zakany J, Duboule D, Petit B, Tafti M, Schibler U. The loss of circadian par bzip transcription factors results in epilepsy. *Genes & development*. 2004;18:1397-1412
68. Wang Q, Maillard M, Schibler U, Burnier M, Gachon F. Cardiac hypertrophy, low blood pressure, and low aldosterone levels in mice devoid of the three circadian par bzip transcription factors *dbp*, *hlf*, and *tef*. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2010;299:R1013-1019
69. Vukolic A, Antic V, Van Vliet BN, Yang Z, Albrecht U, Montani JP. Role of mutation of the circadian clock gene *per2* in cardiovascular circadian rhythms. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2010;298:R627-634

70. Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, Bradley A. The mper2 gene encodes a functional component of the mammalian circadian clock. *Nature*. 1999;400:169-173
71. Bibikov SI, Biran R, Rudd KE, Parkinson JS. A signal transducer for aerotaxis in escherichia coli. *Journal of bacteriology*. 1997;179:4075-4079
72. Hill S, Austin S, Eydmann T, Jones T, Dixon R. Azotobacter vinelandii nifl is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93:2143-2148
73. Albrecht U, Zheng B, Larkin D, Sun ZS, Lee CC. Mper1 and mper2 are essential for normal resetting of the circadian clock. *J Biol Rhythms*. 2001;16:100-104
74. Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, Honma K. Dec1 and dec2 are regulators of the mammalian molecular clock. *Nature*. 2002;419:841-844
75. Wu T, Ni Y, Zhuge F, Sun L, Xu B, Kato H, Fu Z. Significant dissociation of expression patterns of the basic helix-loop-helix transcription factors dec1 and dec2 in rat kidney. *The Journal of experimental biology*. 2011;214:1257-1263
76. Noshiro M, Furukawa M, Honma S, Kawamoto T, Hamada T, Honma K, Kato Y. Tissue-specific disruption of rhythmic expression of dec1 and dec2 in clock mutant mice. *J Biol Rhythms*. 2005;20:404-418
77. Sun H, Lu B, Li RQ, Flavell RA, Taneja R. Defective t cell activation and autoimmune disorder in stra13-deficient mice. *Nature immunology*. 2001;2:1040-1047
78. Gumz ML, Popp MP, Wingo CS, Cain BD. Early transcriptional effects of aldosterone in a mouse inner medullary collecting duct cell line. *Am J Physiol Renal Physiol*. 2003;285:F664-673
79. Rossier BC. Epithelial sodium channel (enac) and the control of blood pressure. *Current opinion in pharmacology*. 2014;15:33-46
80. Richards J, Jeffers LA, All SC, Cheng KY, Gumz ML. Role of per1 and the mineralocorticoid receptor in the coordinate regulation of alphaenac in renal cortical collecting duct cells. *Front Physiol*. 2013;4:253
81. Richards J, Greenlee MM, Jeffers LA, Cheng KY, Guo L, Eaton DC, Gumz ML. Inhibition of alphaenac expression and enac activity following blockade of the circadian clock-regulatory kinases ck1delta/epsilon. *Am J Physiol Renal Physiol*. 2012;303:F918-927

82. Lubarski I, Pihakaski-Maunsbach K, Karlsh SJ, Maunsbach AB, Garty H. Interaction with the na,k-atpase and tissue distribution of fxyd5 (related to ion channel). *J Biol Chem.* 2005;280:37717-37724
83. Debonneville C, Staub O. Participation of the ubiquitin-conjugating enzyme ube2e3 in nedd4-2-dependent regulation of the epithelial na⁺ channel. *Mol Cell Biol.* 2004;24:2397-2409
84. Lee IH, Campbell CR, Song SH, Day ML, Kumar S, Cook DI, Dinudom A. The activity of the epithelial sodium channels is regulated by caveolin-1 via a nedd4-2-dependent mechanism. *J Biol Chem.* 2009;284:12663-12669
85. Bugaj V, Pochynyuk O, Mironova E, Vandewalle A, Medina JL, Stockand JD. Regulation of the epithelial na⁺ channel by endothelin-1 in rat collecting duct. *Am J Physiol Renal Physiol.* 2008;295:F1063-1070
86. Gallego MS, Ling BN. Regulation of amiloride-sensitive na⁺ channels by endothelin-1 in distal nephron cells. *The American journal of physiology.* 1996;271:F451-460
87. Lynch IJ, Welch AK, Kohan DE, Cain BD, Wingo CS. Endothelin-1 inhibits sodium reabsorption by et(a) and et(b) receptors in the mouse cortical collecting duct. *Am J Physiol Renal Physiol.* 2013;305:F568-573
88. Richards J, Welch AK, Barilovits SJ, All S, Cheng KY, Wingo CS, Cain BD, Gumz ML. Tissue-specific and time-dependent regulation of the endothelin axis by the circadian clock protein per1. *Life Sci.* 2014;118:255-262
89. Dhaun N, Moorhouse R, MacIntyre IM, Melville V, Oosthuyzen W, Kimmitt RA, Brown KE, Kennedy ED, Goddard J, Webb DJ. Diurnal variation in blood pressure and arterial stiffness in chronic kidney disease: The role of endothelin-1. *Hypertension.* 2014;64:296-304
90. Ohkubo T, Hozawa A, Yamaguchi J, Kikuya M, Ohmori K, Michimata M, Matsubara M, Hashimoto J, Hoshi H, Araki T, Tsuji I, Satoh H, Hisamichi S, Imai Y. Prognostic significance of the nocturnal decline in blood pressure in individuals with and without high 24-h blood pressure: The ohasama study. *Journal of hypertension.* 2002;20:2183-2189
91. Hermida RC, Ayala DE, Mojon A, Fernandez JR. Influence of circadian time of hypertension treatment on cardiovascular risk: Results of the mapec study. *Chronobiology international.* 2010;27:1629-1651
92. Kario K, Shimada K. Risers and extreme-dippers of nocturnal blood pressure in hypertension: Antihypertensive strategy for nocturnal blood pressure. *Clin Exp Hypertens.* 2004;26:177-189

93. Richards J, Cheng KY, All S, Skopis G, Jeffers L, Jeanette Lynch I, Wingo CS, Gumz ML. A role for the circadian clock protein per1 in the regulation of aldosterone levels and renal na⁺ retention. *Am J Physiol Renal Physiol*. 2013;305:F1697-1704
94. Richards J, All S, Skopis G, Cheng KY, Compton B, Srialluri N, Stow L, Jeffers LA, Gumz ML. Opposing actions of per1 and cry2 in the regulation of per1 target gene expression in the liver and kidney. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2013;305:R735-747
95. Simard J, Ricketts ML, Gingras S, Soucy P, Feltus FA, Melner MH. Molecular biology of the 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family. *Endocrine reviews*. 2005;26:525-582
96. Richards J, Cheng KY, All S, Skopis G, Jeffers L, Lynch IJ, Wingo CS, Gumz ML. A role for the circadian clock protein per1 in the regulation of aldosterone levels and renal na⁺ retention. *Am J Physiol Renal Physiol*. 2013;305:F1697-1704
97. Oster H, Baeriswyl S, Van Der Horst GT, Albrecht U. Loss of circadian rhythmicity in aging mper1^{-/-}-mcry2^{-/-} mutant mice. *Genes Dev*. 2003;17:1366-1379
98. Ohta H, Honma S, Abe H, Honma K. Effects of nursing mothers on rper1 and rper2 circadian expressions in the neonatal rat suprachiasmatic nuclei vary with developmental stage. *The European journal of neuroscience*. 2002;15:1953-1960
99. Nishide SY, Hashimoto K, Nishio T, Honma K, Honma S. Organ-specific development characterizes circadian clock gene per2 expression in rats. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2014;306:R67-74
100. Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y, Iino M, Hashimoto S. System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nature genetics*. 2005;37:187-192
101. He PJ, Hirata M, Yamauchi N, Hashimoto S, Hattori MA. The disruption of circadian clockwork in differentiating cells from rat reproductive tissues as identified by in vitro real-time monitoring system. *The Journal of endocrinology*. 2007;193:413-420
102. Yamazaki S, Yoshikawa T, Biscoe EW, Numano R, Gallsby LM, Soulsby S, Papadimas E, Pezuk P, Doyle SE, Tei H, Sakaki Y, Block GD, Menaker M. Ontogeny of circadian organization in the rat. *J Biol Rhythms*. 2009;24:55-63
103. Dolatshad H, Cary AJ, Davis FC. Differential expression of the circadian clock in maternal and embryonic tissues of mice. *PLoS one*. 2010;5:e9855

104. Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepkas SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS. Period2::Luciferase real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A*. 2004;101:5339-5346
105. Meszaros K, Pruess L, Szabo AJ, Gondan M, Ritz E, Schaefer F. Development of the circadian clockwork in the kidney. *Kidney Int*. 2014;86:915-922
106. Wu T, Fu O, Yao L, Sun L, Zhuge F, Fu Z. Differential responses of peripheral circadian clocks to a short-term feeding stimulus. *Molecular biology reports*. 2012;39:9783-9789
107. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev*. 2000;14:2950-2961
108. Ferraris RP. Dietary and developmental regulation of intestinal sugar transport. *The Biochemical journal*. 2001;360:265-276
109. Oike H, Nagai K, Fukushima T, Ishida N, Kobori M. High-salt diet advances molecular circadian rhythms in mouse peripheral tissues. *Biochemical and biophysical research communications*. 2010;402:7-13
110. Wu T, Ni Y, Dong Y, Xu J, Song X, Kato H, Fu Z. Regulation of circadian gene expression in the kidney by light and food cues in rats. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2010;298:R635-641
111. Ohnishi N, Tahara Y, Kuriki D, Haraguchi A, Shibata S. Warm water bath stimulates phase-shifts of the peripheral circadian clocks in per2::Luciferase mouse. *PLoS One*. 2014;9:e100272
112. Segel IH. *Enzyme kinetics: Behavior and analysis of rapid steady state and steady-state enzyme systems*. New York: Wiley; 1975.
113. Buhr ED, Yoo SH, Takahashi JS. Temperature as a universal resetting cue for mammalian circadian oscillators. *Science*. 2010;330:379-385
114. Curie T, Maret S, Emmenegger Y, Franken P. In vivo imaging of the central and peripheral effects of sleep deprivation and suprachiasmatic nuclei lesion on period-2 protein in mice. *Sleep*. 2015
115. Gumz ML, Rabinowitz L. Role of circadian rhythms in potassium homeostasis. *Semin Nephrol*. 2013;33:229-236
116. Gumz ML, Rabinowitz L, Wingo CS. An integrated view of potassium homeostasis. *N Engl J Med*. 2015;373:60-72

117. Zicha J, Dobesova Z, Behuliak M, Kunes J, Vaneckova I. Preventive dietary potassium supplementation in young salt-sensitive dahl rats attenuates development of salt hypertension by decreasing sympathetic vasoconstriction. *Acta physiologica*. 2011;202:29-38
118. Kanbay M, Bayram Y, Solak Y, Sanders PW. Dietary potassium: A key mediator of the cardiovascular response to dietary sodium chloride. *Journal of the American Society of Hypertension : JASH*. 2013;7:395-400
119. Salhi A, Centeno G, Firsov D, Crambert G. Circadian expression of h,k-atpase type 2 contributes to the stability of plasma k+ levels. *FASEB J*. 2012
120. Hermida RC, Ayala DE, Smolensky MH, Mojon A, Fernandez JR, Crespo JJ, Moya A, Rios MT, Portaluppi F. Chronotherapy improves blood pressure control and reduces vascular risk in ckd. *Nat Rev Nephrol*. 2013;9:358-368
121. Viljoen M, Steyn ME, van Rensburg BW, Reinach SG. Melatonin in chronic renal failure. *Nephron*. 1992;60:138-143
122. Kim TW, Jeong JH, Hong SC. The impact of sleep and circadian disturbance on hormones and metabolism. *International journal of endocrinology*. 2015;2015:591729
123. Parker KP. Sleep disturbances in dialysis patients. *Sleep medicine reviews*. 2003;7:131-143
124. Koch BC, Nagtegaal JE, Hagen EC, Wee PM, Kerkhof GA. Different melatonin rhythms and sleep-wake rhythms in patients on peritoneal dialysis, daytime hemodialysis and nocturnal hemodialysis. *Sleep Med*. 2010;11:242-246
125. Kamperis K, Hagstroem S, Radvanska E, Rittig S, Djurhuus JC. Excess diuresis and natriuresis during acute sleep deprivation in healthy adults. *American journal of physiology. Renal physiology*. 2010;299:F404-411
126. Masri S, Kinouchi K, Sassone-Corsi P. Circadian clocks, epigenetics, and cancer. *Current opinion in oncology*. 2015;27:50-56
127. Mazzoccoli G, De Cata A, Piepoli A, Vinciguerra M. The circadian clock and the hypoxic response pathway in kidney cancer. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2014;35:1-7
128. Mazzoccoli G, Piepoli A, Carella M, Panza A, Paziienza V, Benegiamo G, Palumbo O, Ranieri E. Altered expression of the clock gene machinery in kidney cancer patients. *Biomed Pharmacother*. 2012;66:175-179
129. Richards J, Gumz ML. Mechanism of the circadian clock in physiology. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2013;304:R1053-1064

130. Richards J, Gumz ML. Advances in understanding the peripheral circadian clocks. *FASEB J.* 2012
131. Albrecht U, Eichele G. The mammalian circadian clock. *Curr Opin Genet Dev.* 2003;13:271-277
132. Lee HM, Chen R, Kim H, Etchegaray JP, Weaver DR, Lee C. The period of the circadian oscillator is primarily determined by the balance between casein kinase 1 and protein phosphatase 1. *Proc Natl Acad Sci U S A.* 2011;108:16451-16456
133. Richards J, Greenlee MM, Jeffers LA, Cheng KY, Guo L, Eaton DC, Gumz ML. Inhibition of alphaenac expression and enac activity following blockade of the circadian clock-regulatory kinases ckidelta/{varepsilon}. *American journal of physiology.* 2012
134. Richards J, All SC, Skopis G, Cheng KY, Compton B, Srialluri N, Stow LR, Jeffers LA, Gumz ML. Opposing actions of per1 and cry2 in the regulation of per1 target gene expression in the liver and kidney. *Am J Physiol Regul Integr Comp Physiol.* 2013
135. Gumz ML, Cheng KY, Lynch IJ, Stow LR, Greenlee MM, Cain BD, Wingo CS. Regulation of alphaenac expression by the circadian clock protein period 1 in mpkccd(c14) cells. *Biochim Biophys Acta.* 2010;1799:622-629
136. Bose S, Boockfor FR. Episodes of prolactin gene expression in gh3 cells are dependent on selective promoter binding of multiple circadian elements. *Endocrinology.* 2010;151:2287-2296
137. Chen R, Schirmer A, Lee Y, Lee H, Kumar V, Yoo SH, Takahashi JS, Lee C. Rhythmic per abundance defines a critical nodal point for negative feedback within the circadian clock mechanism. *Mol Cell.* 2009;36:417-430
138. Richards J, Ko B, All S, Cheng KY, Hoover RS, Gumz ML. A role for the circadian clock protein per1 in the regulation of the nacl co-transporter (ncc) and the with-no-lysine kinase (wnk) cascade in mouse distal convoluted tubule cells. *J Biol Chem.* 2014
139. Alexander RT, Dimke H, Cordat E. Proximal tubular nhes: Sodium, protons and calcium? *American journal of physiology.* 2013;305:F229-236
140. Wallner EI, Wada J, Tramonti G, Lin S, Kanwar YS. Status of glucose transporters in the mammalian kidney and renal development. *Ren Fail.* 2001;23:301-310
141. Nishinaga H, Komatsu R, Doi M, Fustin JM, Yamada H, Okura R, Yamaguchi Y, Matsuo M, Emoto N, Okamura H. Circadian expression of the na⁺/h⁺ exchanger nhe3 in the mouse renal medulla. *Biomed Res.* 2009;30:87-93

142. Bhutta HY, Deelman TE, Ashley SW, Rhoads DB, Tavakkoli A. Disrupted circadian rhythmicity of the intestinal glucose transporter *sglt1* in Zucker diabetic fatty rats. *Dig Dis Sci*. 2013;58:1537-1545
143. Balakrishnan A, Stearns AT, Ashley SW, Rhoads DB, Tavakkolizadeh A. Per1 modulates *sglt1* transcription in vitro independent of e-box status. *Dig Dis Sci*. 2012;57:1525-1536
144. Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of *mper1*, *mper2*, and *mper3* in the *scn* circadian clock. *Neuron*. 2001;30:525-536
145. Meldrum KK, Zhang H, Hile KL, Moldower LL, Dong Z, Meldrum DR. Profibrotic effect of interleukin-18 in HK-2 cells is dependent on stimulation of the toll-like receptor 4 (*tlr4*) promoter and increased *tlr4* expression. *J Biol Chem*. 2012;287:40391-40399
146. Matsui F, Rhee A, Hile KL, Zhang H, Meldrum KK. IL-18 induces profibrotic renal tubular cell injury via *stat3* activation. *American journal of physiology*. 2013;305:F1014-F1021
147. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Methods*. 2001;25:402-408
148. Farre D, Roset R, Huerta M, Adsuara JE, Rosello L, Alba MM, Messeguer X. Identification of patterns in biological sequences at the Algen server: Promo and Malgen. *Nucleic Acids Res*. 2003;31:3651-3653
149. Messeguer X, Escudero R, Farre D, Nunez O, Martinez J, Alba MM. Promo: Detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics*. 2002;18:333-334
150. Khundmiri SJ, Dean WL, McLeish KR, Lederer ED. Parathyroid hormone-mediated regulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ requires ERK-dependent translocation of protein kinase α . *J Biol Chem*. 2005;280:8705-8713
151. Holthouser KA, Mandal A, Merchant ML, Schelling JR, Delamere NA, Valdes RR, Jr., Tyagi SC, Lederer ED, Khundmiri SJ. Ouabain stimulates $\text{Na}^+\text{-K}^+\text{-ATPase}$ through a sodium/hydrogen exchanger-1 (*nhe-1*)-dependent mechanism in human kidney proximal tubule cells. *American journal of physiology*. 2010;299:F77-90
152. Yan Y, Haller S, Shapiro A, Malhotra N, Tian J, Xie Z, Malhotra D, Shapiro JI, Liu J. Ouabain-stimulated trafficking regulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ and *nhe3* in renal proximal tubule cells. *Mol Cell Biochem*. 2012;367:175-183

153. Lipson KE, Baserga R. Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe. *Proc Natl Acad Sci U S A*. 1989;86:9774-9777
154. Chen H, Kilberg MS. Alignment of the transcription start site coincides with increased transcriptional activity from the human asparagine synthetase gene following amino acid deprivation of hepg2 cells. *J Nutr*. 2006;136:2463-2467
155. Salyer SA, Parks J, Barati MT, Lederer ED, Clark BJ, Klein JD, Khundmiri SJ. Aldosterone regulates na(+), k(+) atpase activity in human renal proximal tubule cells through mineralocorticoid receptor. *Biochim Biophys Acta*. 2013;1833:2143-2152
156. Bonny O, Firsov D. Circadian regulation of renal function and potential role in hypertension. *Current opinion in nephrology and hypertension*. 2013;22:439-444
157. Durgan DJ, Young ME. The cardiomyocyte circadian clock: Emerging roles in health and disease. *Circ Res*. 2010;106:647-658
158. Sheward WJ, Naylor E, Knowles-Barley S, Armstrong JD, Brooker GA, Seckl JR, Turek FW, Holmes MC, Zee PC, Harmar AJ. Circadian control of mouse heart rate and blood pressure by the suprachiasmatic nuclei: Behavioral effects are more significant than direct outputs. *PLoS One*. 2010;5:e9783
159. Suzuki J, Ogawa M, Tamura N, Maejima Y, Takayama K, Maemura K, Honda K, Hirata Y, Nagai R, Isobe M. A critical role of sympathetic nerve regulation for the treatment of impaired daily rhythm in hypertensive dahl rats. *Hypertens Res*. 2010;33:1060-1065
160. Anea CB, Zhang M, Stepp DW, Simkins GB, Reed G, Fulton DJ, Rudic RD. Vascular disease in mice with a dysfunctional circadian clock. *Circulation*. 2009;119:1510-1517
161. Anea CB, Cheng B, Sharma S, Kumar S, Caldwell RW, Yao L, Ali MI, Merloiu AM, Stepp DW, Black SM, Fulton DJ, Rudic RD. Increased superoxide and endothelial no synthase uncoupling in blood vessels of bmal1-knockout mice. *Circ Res*. 2012;111:1157-1165
162. Kunieda T, Minamino T, Miura K, Katsuno T, Tateno K, Miyauchi H, Kaneko S, Bradfield CA, FitzGerald GA, Komuro I. Reduced nitric oxide causes age-associated impairment of circadian rhythmicity. *Circ Res*. 2008;102:607-614
163. Marques FZ, Campaign AE, Tomaszewski M, Zukowska-Szczechowska E, Yang YH, Charchar FJ, Morris BJ. Gene expression profiling reveals renin mrna overexpression in human hypertensive kidneys and a role for micornas. *Hypertension*. 2011;58:1093-1098

164. Schnermann J. Sodium transport deficiency and sodium balance in gene-targeted mice. *Acta physiologica Scandinavica*. 2001;173:59-66
165. Noonan WT, Woo AL, Nieman ML, Prasad V, Schultheis PJ, Shull GE, Lorenz JN. Blood pressure maintenance in nhe3-deficient mice with transgenic expression of nhe3 in small intestine. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R685-691
166. Liu M, Takahashi H, Morita Y, Maruyama S, Mizuno M, Yuzawa Y, Watanabe M, Toriyama T, Kawahara H, Matsuo S. Non-dipping is a potent predictor of cardiovascular mortality and is associated with autonomic dysfunction in haemodialysis patients. *Nephrol Dial Transplant*. 2003;18:563-569
167. Fagard RH, Thijs L, Staessen JA, Clement DL, De Buyzere ML, De Bacquer DA. Night-day blood pressure ratio and dipping pattern as predictors of death and cardiovascular events in hypertension. *J Hum Hypertens*. 2009;23:645-653
168. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB, American Heart Association Statistics C, Stroke Statistics S. Heart disease and stroke statistics--2015 update: A report from the american heart association. *Circulation*. 2015;131:e29-322
169. Richards J, Diaz AN, Gumz ML. Clock genes in hypertension: Novel insights from rodent models. *Blood Press Monit*. 2014;19:249-254
170. Pati P, Fulton DJ, Bagi Z, Chen F, Wang Y, Kitchens J, Cassis LA, Stepp DW, Rudic RD. Low-salt diet and circadian dysfunction synergize to induce angiotensin ii-dependent hypertension in mice. *Hypertension*. 2016;67:661-668
171. Solocinski K, Richards J, All SC, Cheng KY, Khundmiri SJ, Gumz ML. Transcriptional regulation of nhe3 and sglt1 by the circadian clock protein per1 in proximal tubule cells. *Am J Physiol Renal Physiol*. 2015:ajrenal 00197 02014
172. Atlas SA. The renin-angiotensin aldosterone system: Pathophysiological role and pharmacologic inhibition. *J Manag Care Pharm*. 2007;13:9-20
173. Pendergast JS, Friday RC, Yamazaki S. Endogenous rhythms in period1 mutant suprachiasmatic nuclei in vitro do not represent circadian behavior. *J Neurosci*. 2009;29:14681-14686
174. Butz GM, Davisson RL. Long-term telemetric measurement of cardiovascular parameters in awake mice: A physiological genomics tool. *Physiol Genomics*. 2001;5:89-97

175. Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. Metacycle: An integrated R package to evaluate periodicity in large scale data. *Bioinformatics*. 2016
176. Crowley SD, Coffman TM. Recent advances involving the renin-angiotensin system. *Exp Cell Res*. 2012;318:1049-1056
177. Hyndman KA, Bugaj V, Mironova E, Stockand JD, Pollock JS. Nos1-dependent negative feedback regulation of the epithelial sodium channel in the collecting duct. *Am J Physiol Renal Physiol*. 2015;308:F244-251
178. Chavez-Canales M, Zhang C, Soukaseum C, Moreno E, Pacheco-Alvarez D, Vidal-Petiot E, Castaneda-Bueno M, Vazquez N, Rojas-Vega L, Meermeier NP, Rogers S, Jeunemaitre X, Yang CL, Ellison DH, Gamba G, Hadchouel J. Wnk-spak-ncc cascade revisited: Wnk1 stimulates the activity of the Na-Cl cotransporter via Spak, an effect antagonized by Wnk4. *Hypertension*. 2014;64:1047-1053
179. Refinetti R, Lissen GC, Halberg F. Procedures for numerical analysis of circadian rhythms. *Biol Rhythm Res*. 2007;38:275-325
180. Richards J, Jeffers LA, All SC, Cheng KY, Gumz ML. A role for the circadian clock protein Per1 in the regulation of NHE3 and SGLT1 in human proximal tubule cells *FASEB J*. . 2014;28:Abstract 1109.1104
181. Korshunov V. Heart rate and blood pressure in males of 58 strains of mice. *Mouse Phenome Database web site*. 2016;MPD:45302
182. Cechova S, Zeng Q, Billaud M, Mutchler S, Rudy CK, Straub AC, Chi L, Chan FR, Hu J, Griffiths R, Howell NL, Madsen K, Jensen BL, Palmer LA, Carey RM, Sung SS, Malakauskas SM, Isakson BE, Le TH. Loss of collectrin, an angiotensin-converting enzyme 2 homolog, uncouples endothelial nitric oxide synthase and causes hypertension and vascular dysfunction. *Circulation*. 2013;128:1770-1780
183. Hartner A, Cordasic N, Klanke B, Veelken R, Hilgers KF. Strain differences in the development of hypertension and glomerular lesions induced by deoxycorticosterone acetate salt in mice. *Nephrol Dial Transplant*. 2003;18:1999-2004
184. Ma LJ, Fogo AB. Model of robust induction of glomerulosclerosis in mice: Importance of genetic background. *Kidney Int*. 2003;64:350-355
185. Brosius FC, 3rd, Alpers CE, Bottinger EP, Breyer MD, Coffman TM, Gurley SB, Harris RC, Kakoki M, Kretzler M, Leiter EH, Levi M, McIndoe RA, Sharma K, Smithies O, Susztak K, Takahashi N, Takahashi T, Animal Models of Diabetic Complications C. Mouse models of diabetic nephropathy. *J Am Soc Nephrol*. 2009;20:2503-2512

186. Viera AJ, Zhu S, Hinderliter AL, Shimbo D, Person SD, Jacobs DR, Jr. Diurnal blood pressure pattern and development of prehypertension or hypertension in young adults: The cardia study. *J Am Soc Hypertens*. 2011;5:48-55
187. Ivy JR, Oosthuyzen W, Peltz TS, Howarth AR, Hunter RW, Dhaun N, Al-Dujaili EA, Webb DJ, Dear JW, Flatman PW, Bailey MA. Glucocorticoids induce nondipping blood pressure by activating the thiazide-sensitive cotransporter. *Hypertension*. 2016;67:1029-1037
188. Masuki S, Todo T, Nakano Y, Okamura H, Nose H. Reduced alpha-adrenoceptor responsiveness and enhanced baroreflex sensitivity in cry-deficient mice lacking a biological clock. *J Physiol*. 2005;566:213-224
189. Mohammed-Ali Z, Cruz GL, Lu C, Carlisle RE, Werner KE, Ask K, Dickhout JG. Development of a model of chronic kidney disease in the c57bl/6 mouse with properties of progressive human ckd. *Biomed Res Int*. 2015;2015:172302
190. Friedman O, Logan AG. Nocturnal blood pressure profiles among normotensive, controlled hypertensive and refractory hypertensive subjects. *Can J Cardiol*. 2009;25:e312-316
191. Cakici M, Dogan A, Oylumlu M, Uckardes F, Davutoglu V. Impaired circadian rhythm of blood pressure in normotensive patients with rheumatic mitral valve stenosis. *Blood Press Monit*. 2014;19:76-80
192. Birkenhager AM, van den Meiracker AH. Causes and consequences of a non-dipping blood pressure profile. *Neth J Med*. 2007;65:127-131
193. Higashi Y, Oshima T, Ozono R, Nakano Y, Matsuura H, Kambe M, Kajiyama G. Nocturnal decline in blood pressure is attenuated by nacl loading in salt-sensitive patients with essential hypertension: Noninvasive 24-hour ambulatory blood pressure monitoring. *Hypertension*. 1997;30:163-167
194. Uzu T, Ishikawa K, Fujii T, Nakamura S, Inenaga T, Kimura G. Sodium restriction shifts circadian rhythm of blood pressure from nondipper to dipper in essential hypertension. *Circulation*. 1997;96:1859-1862
195. Martino TA, Young ME. Influence of the cardiomyocyte circadian clock on cardiac physiology and pathophysiology. *J Biol Rhythms*. 2015;30:183-205
196. Su W, Xie Z, Guo Z, Duncan MJ, Lutshumba J, Gong MC. Altered clock gene expression and vascular smooth muscle diurnal contractile variations in type 2 diabetic db/db mice. *Am J Physiol Heart Circ Physiol*. 2012;302:H621-633
197. Gavish B, Izzo JL, Jr. Arterial stiffness: Going a step beyond. *Am J Hypertens*. 2016

198. Uzu T, Kimura G. Diuretics shift circadian rhythm of blood pressure from nondipper to dipper in essential hypertension. *Circulation*. 1999;100:1635-1638
199. Gatzka CD, Schobel HP, Klingbeil AU, Neumayer HH, Schmieder RE. Normalization of circadian blood pressure profiles after renal transplantation. *Transplantation*. 1995;59:1270-1274
200. Woon PY, Kaisaki PJ, Braganca J, Bihoreau MT, Levy JC, Farrall M, Gauguier D. Aryl hydrocarbon receptor nuclear translocator-like (bmal1) is associated with susceptibility to hypertension and type 2 diabetes. *Proc Natl Acad Sci U S A*. 2007;104:14412-14417
201. Portaluppi F, Vergnani L, Manfredini R, degli Uberti EC, Fersini C. Time-dependent effect of isradipine on the nocturnal hypertension in chronic renal failure. *Am J Hypertens*. 1995;8:719-726
202. Hermida RC, Ayala DE, Fernandez JR, Calvo C. Chronotherapy improves blood pressure control and reverts the nondipper pattern in patients with resistant hypertension. *Hypertension*. 2008;51:69-76
203. Coats AJ. Benefits of ambulatory blood pressure monitoring in the design of antihypertensive drug trials. *Blood Press Monit*. 1996;1:157-160
204. Syrseloudis D, Andrikou I, Andrikou E, Dimitriadis K, Stefanadis C. Ambulatory blood pressure monitoring in resistant hypertension. *Int J Hypertens*. 2011;2011:285612
205. de la Sierra A, Banegas JR, Segura J, Gorostidi M, Ruilope LM, Investigators CE. Ambulatory blood pressure monitoring and development of cardiovascular events in high-risk patients included in the spanish abpm registry: The cardiorisc event study. *J Hypertens*. 2012;30:713-719
206. Batchu SN, Smolock EM, Dyachenko IA, Murashev AN, Korshunov VA. Autonomic dysfunction determines stress-induced cardiovascular and immune complications in mice. *J Am Heart Assoc*. 2015;4
207. Whitesall SE, Hoff JB, Vollmer AP, D'Alecy LG. Comparison of simultaneous measurement of mouse systolic arterial blood pressure by radiotelemetry and tail-cuff methods. *Am J Physiol Heart Circ Physiol*. 2004;286:H2408-2415
208. Law MR, Morris JK, Wald NJ. Use of blood pressure lowering drugs in the prevention of cardiovascular disease: Meta-analysis of 147 randomised trials in the context of expectations from prospective epidemiological studies. *BMJ*. 2009;338:b1665
209. Murphy BP, Stanton T, Dunn FG. Hypertension and myocardial ischemia. *Med Clin North Am*. 2009;93:681-695

210. Merai R, Siegel C, Rakotz M, Basch P, Wright J, Wong B, Dhsc, Thorpe P. Cdc grand rounds: A public health approach to detect and control hypertension. *MMWR Morb Mortal Wkly Rep.* 2016;65:1261-1264
211. Weinberger MH, Miller JZ, Luft FC, Grim CE, Fineberg NS. Definitions and characteristics of sodium sensitivity and blood pressure resistance. *Hypertension.* 1986;8:1127-134
212. Morimoto A, Uzu T, Fujii T, Nishimura M, Kuroda S, Nakamura S, Inenaga T, Kimura G. Sodium sensitivity and cardiovascular events in patients with essential hypertension. *Lancet.* 1997;350:1734-1737
213. Weinberger MH, Fineberg NS, Fineberg SE, Weinberger M. Salt sensitivity, pulse pressure, and death in normal and hypertensive humans. *Hypertension.* 2001;37:429-432
214. Crowley SD, Coffman TM. In hypertension, the kidney rules. *Curr Hypertens Rep.* 2007;9:148-153
215. Curtis JJ, Luke RG, Dustan HP, Kashgarian M, Whelchel JD, Jones P, Diethelm AG. Remission of essential hypertension after renal transplantation. *The New England journal of medicine.* 1983;309:1009-1015
216. Dahl LK, Heine M. Primary role of renal homografts in setting chronic blood pressure levels in rats. *Circulation research.* 1975;36:692-696
217. Gambling L, Dunford S, Wilson CA, McArdle HJ, Baines DL. Estrogen and progesterone regulate alpha, beta, and gammaenac subunit mrna levels in female rat kidney. *Kidney Int.* 2004;65:1774-1781
218. Solocinski K, Holzworth M, Wen X, Cheng KY, Lynch IJ, Cain BD, Wingo CS, Gumz ML. Desoxycorticosterone pivalate-salt treatment leads to non-dipping hypertension in per1 knockout mice. *Acta Physiol (Oxf).* 2017;220:72-82
219. Uzu T, Nishimura M, Fujii T, Takeji M, Kuroda S, Nakamura S, Inenaga T, Kimura G. Changes in the circadian rhythm of blood pressure in primary aldosteronism in response to dietary sodium restriction and adrenalectomy. *J Hypertens.* 1998;16:1745-1748
220. Meneton P, Galan P, Bertrais S, Heudes D, Hercberg S, Menard J. High plasma aldosterone and low renin predict blood pressure increase and hypertension in middle-aged caucasian populations. *J Hum Hypertens.* 2008;22:550-558
221. Durukan M, Guray U, Aksu T, Guray Y, Demirkan B, Korkmaz S. Low plasma renin activity and high aldosterone/renin ratio are associated with untreated isolated systolic hypertension. *Blood pressure.* 2012;21:320-325

222. Domanski MJ, Davis BR, Pfeffer MA, Kastantin M, Mitchell GF. Isolated systolic hypertension : Prognostic information provided by pulse pressure. *Hypertension*. 1999;34:375-380
223. Smulyan H, Mookherjee S, Safar ME. The two faces of hypertension: Role of aortic stiffness. *J Am Soc Hypertens*. 2016;10:175-183
224. Franklin SS, Pio JR, Wong ND, Larson MG, Leip EP, Vasan RS, Levy D. Predictors of new-onset diastolic and systolic hypertension: The framingham heart study. *Circulation*. 2005;111:1121-1127
225. Sandberg K, Ji H. Sex differences in primary hypertension. *Biol Sex Differ*. 2012;3:7
226. Van Liere EJ, Stickney JC, Marsh DF. Sex differences in blood pressure of dogs. *Science*. 1949;109:489
227. Ruiz-Feria CA, Zhang D, Nishimura H. Age- and sex-dependent changes in pulse pressure in fowl aorta. *Comp Biochem Physiol A Mol Integr Physiol*. 2004;137:311-320
228. Ganten U, Schroder G, Witt M, Zimmermann F, Ganten D, Stock G. Sexual dimorphism of blood pressure in spontaneously hypertensive rats: Effects of anti-androgen treatment. *J Hypertens*. 1989;7:721-726
229. Maris ME, Melchert RB, Joseph J, Kennedy RH. Gender differences in blood pressure and heart rate in spontaneously hypertensive and wistar-kyoto rats. *Clin Exp Pharmacol Physiol*. 2005;32:35-39
230. Xue B, Pamidimukkala J, Hay M. Sex differences in the development of angiotensin ii-induced hypertension in conscious mice. *Am J Physiol Heart Circ Physiol*. 2005;288:H2177-2184
231. Ji H, Zheng W, Wu X, Liu J, Ecelbarger CM, Watkins R, Arnold AP, Sandberg K. Sex chromosome effects unmasked in angiotensin ii-induced hypertension. *Hypertension*. 2010;55:1275-1282
232. Tatchum-Talom R, Eyster KM, Martin DS. Sexual dimorphism in angiotensin ii-induced hypertension and vascular alterations. *Can J Physiol Pharmacol*. 2005;83:413-422
233. Tostes Passaglia RC, David FL, Fortes ZB, Nigro D, Scivoletto R, Catelli De Carvalho MH. Deoxycorticosterone acetate-salt hypertensive rats display gender-related differences in et(b) receptor-mediated vascular responses. *Br J Pharmacol*. 2000;130:1092-1098

234. Sullivan JC, Bhatia K, Yamamoto T, Elmarakby AA. Angiotensin (1-7) receptor antagonism equalizes angiotensin ii-induced hypertension in male and female spontaneously hypertensive rats. *Hypertension*. 2010;56:658-666
235. Johnston JG, Speed JS, Jin C, Pollock DM. Loss of endothelin b receptor function impairs sodium excretion in a time- and sex-dependent manner. *Am J Physiol Renal Physiol*. 2016;311:F991-F998
236. Lange DL, Haywood JR, Hinojosa-Laborde C. Role of the adrenal medullae in male and female doca-salt hypertensive rats. *Hypertension*. 1998;31:403-408
237. Sampson AK, Moritz KM, Jones ES, Flower RL, Widdop RE, Denton KM. Enhanced angiotensin ii type 2 receptor mechanisms mediate decreases in arterial pressure attributable to chronic low-dose angiotensin ii in female rats. *Hypertension*. 2008;52:666-671
238. Venegas-Pont M, Sartori-Valinotti JC, Glover PH, Reckelhoff JF, Ryan MJ. Sexual dimorphism in the blood pressure response to angiotensin ii in mice after angiotensin-converting enzyme blockade. *Am J Hypertens*. 2010;23:92-96
239. Xue B, Johnson AK, Hay M. Sex differences in angiotensin ii- induced hypertension. *Braz J Med Biol Res*. 2007;40:727-734
240. Cambotti LJ, Cole FE, Gerall AA, Frohlich ED, MacPhee AA. Neonatal gonadal hormones and blood pressure in the spontaneously hypertensive rat. *Am J Physiol*. 1984;247:E258-264
241. Iams SG, Wexler BC. Retardation in the development of spontaneous hypertension in sh rats by gonadectomy. *J Lab Clin Med*. 1977;90:997-1003
242. Ouchi Y, Share L, Crofton JT, Iitake K, Brooks DP. Sex difference in the development of deoxycorticosterone-salt hypertension in the rat. *Hypertension*. 1987;9:172-177
243. Routledge FS, McFetridge-Durdle JA, Dean CR. Stress, menopausal status and nocturnal blood pressure dipping patterns among hypertensive women. *Can J Cardiol*. 2009;25:e157-163
244. Mercurio G, Zoncu S, Piano D, Pilia I, Lao A, Melis GB, Cherchi A. Estradiol-17beta reduces blood pressure and restores the normal amplitude of the circadian blood pressure rhythm in postmenopausal hypertension. *Am J Hypertens*. 1998;11:909-913
245. Chang CT, Sun CY, Pong CY, Chen YC, Lin GP, Chang TC, Wu MS. Interaction of estrogen and progesterone in the regulation of sodium channels in collecting tubular cells. *Chang Gung Med J*. 2007;30:305-312

246. Gohar EY, Yusuf C, Pollock DM. Ovarian hormones modulate endothelin a and b receptor expression. *Life Sci.* 2016;159:148-152
247. Fang Z, Carlson SH, Chen YF, Oparil S, Wyss JM. Estrogen depletion induces nacl-sensitive hypertension in female spontaneously hypertensive rats. *American journal of physiology. Regulatory, integrative and comparative physiology.* 2001;281:R1934-1939
248. Hinojosa-Laborde C, Lange DL, Haywood JR. Role of female sex hormones in the development and reversal of dahl hypertension. *Hypertension.* 2000;35:484-489
249. Gallego MS, Ling BN. Regulation of amiloride-sensitive na⁺ channels by endothelin-1 in distal nephron cells. *Am J Physiol.* 1996;271:F451-460.
250. Jin C, Speed JS, Hyndman KA, O'Connor PM, Pollock DM. Sex differences in et-1 receptor expression and ca²⁺ signaling in the imcd. *Am J Physiol Renal Physiol.* 2013;305:F1099-1104
251. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 1988;332:411-415
252. Kohan DE, Rossi NF, Inscho EW, Pollock DM. Regulation of blood pressure and salt homeostasis by endothelin. *Physiol Rev.* 2011;91:1-77
253. Stow LR, Jacobs ME, Wingo CS, Cain BD. Endothelin-1 gene regulation. *FASEB J.* 2011;25:16-28
254. Saleh MA, Boesen EI, Pollock JS, Savin VJ, Pollock DM. Endothelin-1 increases glomerular permeability and inflammation independent of blood pressure in the rat. *Hypertension.* 2010;56:942-949
255. Elmarakby AA, Loomis ED, Pollock JS, Pollock DM. NADPH oxidase inhibition attenuates oxidative stress but not hypertension produced by chronic et-1. *Hypertension.* 2005;45:283-287
256. Kohan DE, Barton M. Endothelin and endothelin antagonists in chronic kidney disease. *Kidney Int.* 2014;86:896-904
257. Yanagisawa M, Masaki T. Molecular biology and biochemistry of the endothelins. *Trends Pharmacol Sci.* 1989;10:374-378
258. Kitamura K, Tanaka T, Kato J, Eto T, Tanaka K. Regional distribution of immunoreactive endothelin in porcine tissue: Abundance in inner medulla of kidney. *Biochem Biophys Res Commun.* 1989;161:348-352

259. Madeddu P, Troffa C, Glorioso N, Pazzola A, Soro A, Manunta P, Tonolo G, Demontis MP, Varoni MV, Anania V. Effect of endothelin on regional hemodynamics and renal function in awake normotensive rats. *J Cardiovasc Pharmacol.* 1989;14:818-825
260. Bugaj V, Mironova E, Kohan DE, Stockand JD. Collecting duct-specific endothelin b receptor knockout increases enac activity. *Am J Physiol Cell Physiol.* 2012;302:C188-194
261. Schneider MP, Ge Y, Pollock DM, Pollock JS, Kohan DE. Collecting duct-derived endothelin regulates arterial pressure and na excretion via nitric oxide. *Hypertension.* 2008;51:1605-1610
262. Rosano L, Spinella F, Bagnato A. Endothelin 1 in cancer: Biological implications and therapeutic opportunities. *Nat Rev Cancer.* 2013;13:637-651
263. Rossi GP, Colonna S, Pavan E, Albertin G, Della Rocca F, Gerosa G, Casarotto D, Sartore S, Pauletto P, Pessina AC. Endothelin-1 and its mrna in the wall layers of human arteries ex vivo. *Circulation.* 1999;99:1147-1155
264. Schiffrin EL. Role of endothelin-1 in hypertension and vascular disease. *Am J Hypertens.* 2001;14:83S-89S
265. Tajmir-Riahi HA. Interaction of purine nucleotides with cobalt-hexammine, cobalt-pentammine and cobalt-tetrammine cations. Evidence for the rigidity of adenosine and flexibility of guanosine and deoxyguanosine sugar conformations. *J Biomol Struct Dyn.* 1991;8:1169-1186
266. Maguire JJ, Davenport AP. Endothelin receptors and their antagonists. *Semin Nephrol.* 2015;35:125-136
267. Dhaun N, Macintyre IM, Melville V, Lilitkarntakul P, Johnston NR, Goddard J, Webb DJ. Blood pressure-independent reduction in proteinuria and arterial stiffness after acute endothelin-a receptor antagonism in chronic kidney disease. *Hypertension.* 2009;54:113-119
268. Mann JF, Green D, Jamerson K, Ruilope LM, Kuranoff SJ, Littke T, Viberti G, Group AS. Avosentan for overt diabetic nephropathy. *J Am Soc Nephrol.* 2010;21:527-535
269. Meguid El Nahas A, Bello AK. Chronic kidney disease: The global challenge. *Lancet.* 2005;365:331-340
270. Bhan A, Mandal SS. Long noncoding rnas: Emerging stars in gene regulation, epigenetics and human disease. *ChemMedChem.* 2014;9:1932-1956
271. Consortium EP. An integrated encyclopedia of DNA elements in the human genome. *Nature.* 2012;489:57-74

272. Galupa R, Heard E. X-chromosome inactivation: New insights into cis and trans regulation. *Curr Opin Genet Dev.* 2015;31:57-66
273. Tollervey JR, Lunyak VV. Epigenetics: Judge, jury and executioner of stem cell fate. *Epigenetics.* 2012;7:823-840
274. Chen LL, Carmichael GG. Decoding the function of nuclear long non-coding rnas. *Curr Opin Cell Biol.* 2010;22:357-364
275. Magistri M, Faghihi MA, St Laurent G, 3rd, Wahlestedt C. Regulation of chromatin structure by long noncoding rnas: Focus on natural antisense transcripts. *Trends Genet.* 2012;28:389-396
276. Bernstein E, Allis CD. Rna meets chromatin. *Genes Dev.* 2005;19:1635-1655
277. Lapidot M, Pilpel Y. Genome-wide natural antisense transcription: Coupling its regulation to its different regulatory mechanisms. *EMBO Rep.* 2006;7:1216-1222
278. Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT. Polycomb proteins targeted by a short repeat rna to the mouse x chromosome. *Science.* 2008;322:750-756
279. Swiezewski S, Liu F, Magusin A, Dean C. Cold-induced silencing by long antisense transcripts of an arabidopsis polycomb target. *Nature.* 2009;462:799-802
280. Li YY, Qin L, Guo ZM, Liu L, Xu H, Hao P, Su J, Shi Y, He WZ, Li YX. In silico discovery of human natural antisense transcripts. *BMC Bioinformatics.* 2006;7:18
281. Bhan A, Mandal SS. Lncrna hotair: A master regulator of chromatin dynamics and cancer. *Biochim Biophys Acta.* 2015;1856:151-164
282. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. The human genome browser at ucsc. *Genome Res.* 2002;12:996-1006
283. Stemmer M, Thumberger T, Del Sol Keyer M, Wittbrodt J, Mateo JL. Cctop: An intuitive, flexible and reliable crispr/cas9 target prediction tool. *PLoS One.* 2015;10:e0124633
284. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for crispr screening. *Nat Methods.* 2014;11:783-784
285. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen T, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F. Genome-scale crispr-cas9 knockout screening in human cells. *Science.* 2014;343:84-87
286. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3--new capabilities and interfaces. *Nucleic Acids Res.* 2012;40:e115

287. Sharma D, Bhattacharya P, Kalia K, Tiwari V. Diabetic nephropathy: New insights into established therapeutic paradigms and novel molecular targets. *Diabetes Res Clin Pract.* 2017;128:91-108
288. Lau EMT, Giannoulatou E, Celermajer DS, Humbert M. Epidemiology and treatment of pulmonary arterial hypertension. *Nat Rev Cardiol.* 2017;14:603-614
289. Roberts L, LaMarca BB, Fournier L, Bain J, Cockrell K, Granger JP. Enhanced endothelin synthesis by endothelial cells exposed to sera from pregnant rats with decreased uterine perfusion. *Hypertension.* 2006;47:615-618
290. Gilbert JS, Ryan MJ, LaMarca BB, Sedeek M, Murphy SR, Granger JP. Pathophysiology of hypertension during preeclampsia: Linking placental ischemia with endothelial dysfunction. *Am J Physiol Heart Circ Physiol.* 2008;294:H541-550
291. Li MW, Mian MO, Barhoumi T, Rehman A, Mann K, Paradis P, Schiffrin EL. Endothelin-1 overexpression exacerbates atherosclerosis and induces aortic aneurysms in apolipoprotein e knockout mice. *Arterioscler Thromb Vasc Biol.* 2013;33:2306-2315
292. Barton M, Haudenschild CC, d'Uscio LV, Shaw S, Munter K, Luscher TF. Endothelin eta receptor blockade restores no-mediated endothelial function and inhibits atherosclerosis in apolipoprotein e-deficient mice. *Proc Natl Acad Sci U S A.* 1998;95:14367-14372
293. Grant K, Loizidou M, Taylor I. Endothelin-1: A multifunctional molecule in cancer. *Br J Cancer.* 2003;88:163-166
294. Kolettis TM, Barton M, Langleben D, Matsumura Y. Endothelin in coronary artery disease and myocardial infarction. *Cardiol Rev.* 2013;21:249-256
295. Davenport AP, Hyndman KA, Dhaun N, Southan C, Kohan DE, Pollock JS, Pollock DM, Webb DJ, Maguire JJ. Endothelin. *Pharmacol Rev.* 2016;68:357-418
296. Kohan DE, Inscho EW, Wesson D, Pollock DM. Physiology of endothelin and the kidney. *Compr Physiol.* 2011;1:883-919
297. Kohan DE. Endothelin, hypertension and chronic kidney disease: New insights. *Curr Opin Nephrol Hypertens.* 2010;19:134-139
298. Neuhofer W, Pittrow D. Endothelin receptor selectivity in chronic kidney disease: Rationale and review of recent evidence. *Eur J Clin Invest.* 2009;39 Suppl 2:50-67
299. Ohkita M, Takaoka M, Matsumura Y. Drug discovery for overcoming chronic kidney disease (ckd): The endothelin et b receptor/nitric oxide system functions as a protective factor in ckd. *J Pharmacol Sci.* 2009;109:7-13

300. Wahlestedt C. Natural antisense and noncoding rna transcripts as potential drug targets. *Drug Discov Today*. 2006;11:503-508
301. Wahlestedt C. Targeting long non-coding rna to therapeutically upregulate gene expression. *Nat Rev Drug Discov*. 2013;12:433-446
302. Cayre A, Rossignol F, Clottes E, Penault-Llorca F. Ahif but not hif-1alpha transcript is a poor prognostic marker in human breast cancer. *Breast Cancer Res*. 2003;5:R223-230
303. Thrash-Bingham CA, Tartof KD. Ahif: A natural antisense transcript overexpressed in human renal cancer and during hypoxia. *J Natl Cancer Inst*. 1999;91:143-151
304. Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, Semple CA, Devon RS, St Clair DM, Muir WJ, Blackwood DH, Porteous DJ. Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum Mol Genet*. 2000;9:1415-1423
305. Faghihi MA, Modarresi F, Khalil AM, Wood DE, Sahagan BG, Morgan TE, Finch CE, St Laurent G, 3rd, Kenny PJ, Wahlestedt C. Expression of a noncoding rna is elevated in alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat Med*. 2008;14:723-730
306. Yu W, Gius D, Onyango P, Muldoon-Jacobs K, Karp J, Feinberg AP, Cui H. Epigenetic silencing of tumour suppressor gene p15 by its antisense rna. *Nature*. 2008;451:202-206
307. Chung DW, Rudnicki DD, Yu L, Margolis RL. A natural antisense transcript at the huntington's disease repeat locus regulates htt expression. *Hum Mol Genet*. 2011;20:3467-3477
308. Modarresi F, Faghihi MA, Lopez-Toledano MA, Fatemi RP, Magistri M, Brothers SP, van der Brug MP, Wahlestedt C. Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. *Nat Biotechnol*. 2012;30:453-459
309. He Y, Vogelstein B, Velculescu VE, Papadopoulos N, Kinzler KW. The antisense transcriptomes of human cells. *Science*. 2008;322:1855-1857
310. Berisha B, Schams D, Miyamoto A. The expression of angiotensin and endothelin system members in bovine corpus luteum during estrous cycle and pregnancy. *Endocrine*. 2002;19:305-312
311. Merki-Feld GS, Imthurn B, Keller PJ. The effect of the menstrual cycle and of ethinylestradiol on nitric oxide, endothelin-1 and homocysteine plasma levels. *Horm Metab Res*. 2000;32:288-293

312. Polderman KH, Stehouwer CD, van Kamp GJ, Schalkwijk CG, Gooren LJ. Modulation of plasma endothelin levels by the menstrual cycle. *Metabolism*. 2000;49:648-650
313. Li W, Notani D, Rosenfeld MG. Enhancers as non-coding rna transcription units: Recent insights and future perspectives. *Nat Rev Genet*. 2016;17:207-223
314. Ashe HL, Monks J, Wijgerde M, Fraser P, Proudfoot NJ. Intergenic transcription and transinduction of the human beta-globin locus. *Genes Dev*. 1997;11:2494-2509
315. Tuan D, Kong S, Hu K. Transcription of the hypersensitive site hs2 enhancer in erythroid cells. *Proc Natl Acad Sci U S A*. 1992;89:11219-11223
316. De Santa F, Barozzi I, Mietton F, Ghisletti S, Polletti S, Tusi BK, Muller H, Ragoussis J, Wei CL, Natoli G. A large fraction of extragenic rna pol ii transcription sites overlap enhancers. *PLoS Biol*. 2010;8:e1000384
317. Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, Harmin DA, Laptewicz M, Barbara-Haley K, Kuersten S, Markenscoff-Papadimitriou E, Kuhl D, Bito H, Worley PF, Kreiman G, Greenberg ME. Widespread transcription at neuronal activity-regulated enhancers. *Nature*. 2010;465:182-187
318. Bogue MA, Churchill GA, Chesler EJ. Collaborative cross and diversity outbred data resources in the mouse phenome database. *Mamm Genome*. 2015;26:511-520
319. Grubb SC, Bult CJ, Bogue MA. Mouse phenome database. *Nucleic Acids Res*. 2014;42:D825-834
320. Bogue MA, Peters LL, Paigen B, Korstanje R, Yuan R, Ackert-Bicknell C, Grubb SC, Churchill GA, Chesler EJ. Accessing data resources in the mouse phenome database for genetic analysis of murine life span and health span. *J Gerontol A Biol Sci Med Sci*. 2016;71:170-177

BIOGRAPHICAL SKETCH

Kristen attended St. Peter the Apostle and St. Joan of Arc for middle and elementary school. She attended South Lake High School in St. Clair Shores, MI. She completed her first two years of undergraduate work at Macomb Community College and finished her Bachelor of Science with a double major in Biochemistry and Biology at Western Michigan University. She then moved to Gainesville, Florida to attend graduate school. She graduated with her Doctor of Philosophy in Biomedical Sciences in December 2017.