Conditional ablation of the prorenin receptor in nephron progenitor cells results in developmental programming of hypertension

Renfang Song, Laura Kidd, Adam Janssen & Ihor V. Yosypiv

1 Department of Pediatrics, Tulane University School of Medicine, New Orleans, Los Angeles
2 Department of Pathology, Tulane University School of Medicine, New Orleans, Los Angeles

Keywords
Developmental programming, hypertension, kidney development, nephrogenesis, nephron progenitor cells, prorenin receptor.

Abstract
Nephron induction during kidney development is driven by reciprocal interactions between progenitor cells (NPCs) of the cap mesenchyme (CM) and the ureteric bud (UB). The prorenin receptor (PRR) is a receptor for renin and prorenin, and an accessory subunit of the vacuolar proton pump V-ATPase. Previously, we demonstrated that conditional ablation of the PRR in Six2+ NPCs in mice (Six2PRR+/−) causes early neonatal death. Here, we identified genes that are regulated by PRR in Six2+ NPCs FACS-isolated from Six2PRR+/− and control kidneys on embryonic day E15.5 using whole-genome expression analysis. Seven genes with expression in CM cells previously shown to direct kidney development, including Notch1, β-catenin, Lef1, Lhx1, Jag1, and p53, were downregulated. The functional groups within the downregulated gene set included genes involved in embryonic and cellular development, renal regeneration, cellular assembly and organization, cell morphology, death and survival.

Double-transgenic Six2PRR+/−/BatGal mice, a reporter strain for β-catenin transcriptional activity, showed decreased β-catenin activity in the UB in vivo. Reduced PRR gene dosage in heterozygous Six2PRR+/− mice was associated with decreased glomerular number, segmental thickening of the glomerular basement membrane with focal podocyte foot process effacement, development of hypertension and increased soluble PRR (sPRR) levels in the urine at 2 months of age. Together, these data demonstrate that NPC PRR performs essential functions during nephrogenesis via control of hierarchy of genes that regulate critical cellular processes. Both reduced nephron endowment and augmented urine sPRR likely contribute to programming of hypertension in Six2PRR+/− mice.

Introduction
Mammalian kidney development is driven by reciprocal inductive interactions between self-renewing Six2+/Cited1+ nephron progenitor cells (NPCs) of the cap mesenchyme (CM) and progenitor cells in the adjacent tips of the ureteric bud (UB) (Kobayashi et al. 2008). NPCs give rise to all segments of the mature nephron from the glomerulus to the connecting tubule (Little and McMahon 2012). NPCs have a limited lifespan and are depleted around 36 weeks of gestation in humans and postnatal day 3 in mice, leading to cessation of nephrogenesis (Hinchliffe et al. 1991). Premature cessation of nephrogenesis results in reduced nephron number and is associated with renal hypoplasia, proteinuria, susceptibility to subsequent hypertension and chronic kidney disease (Brenner et al. 1988; Barker et al. 1989; Bertram et al. 2011). Thus, understanding the mechanisms that determine final nephron number might facilitate prevention of kidney and associated cardiovascular disease.

The prorenin receptor (PRR) is a receptor for prorenin and renin encoded by the ATP6AP2 (ATPase-associated
protein2) gene (subsequently referred to as PRR) located on the X chromosome in humans (Nguyen et al. 2002). PRR is also an accessory protein of the vacuolar proton pump V-ATPase (Ludwig et al. 1998). Global PRR knock-out is lethal in mice, indicating an essential role of the PRR in embryonic development (Sihn et al. 2013; Song et al. 2016). In humans, PRR mutations are associated with a high blood pressure, left ventricular hypertrophy, and X-linked mental retardation (Ramser et al. 2005; Hirose et al. 2009, 2011; Reidy and Rosenblum 2009). Previously, we demonstrated that nephron progenitor PRR is critical for normal kidney development and function. PRR ablation in Six2+ NPCs of the CM results in a marked decrease in the number of developing nephrons at birth and early postnatal death (Song et al. 2016). However, the transcriptome downstream of the nephron progenitor PRR and the role of PRR in programming of blood pressure have not been previously defined.

In this study, we: (1) Identified genes that are regulated by PRR in Six2+ NPCs using a whole-genome expression analysis of RNA in Six2+ cells FACs-isolated from Six2PRR−/− (Mut) mice on embryonic day E15.5 and conducted gene ontology analysis to identify functional groups of differentially expressed genes; (2) Tested the hypothesis that reduced PRR gene dosage in heterozygous Six2PRR+/− mice (Het) is associated with development of hypertension during later life; and (3) Tested the hypothesis that soluble PRR (sPRR), PRR cleavage product generated subcellularly and secreted into the plasma and urine, can contribute to BP programming in Het mice. Our data demonstrate that NPC PRR performs essential functions during nephrogenesis via control of hierarchy of genes that regulate critical cellular processes. Both reduced nephron endowment and augmented urine sPRR likely contribute to programming of hypertension in Het mice.

Materials and Methods

Conditional deletion of PRR from nephron progenitors

PRR-floxed mice were provided by Dr. Atsuhiro Ichihara (Keio University, Tokyo, Japan) (Oshima et al. 2011). To delete PRR conditionally in the CM and its epithelial derivatives, we used the Six2GFPCre TGC transgenic mice, which drives Cre expression in nephron progenitors (Kobayashi et al. 2008), and a floxed allele of the PRR. The resulting Six2Cre+/PRRfloxed/floxed mice represent nephron progenitor-specific PRR-knockout mice (Mut) (Terada et al. 2017). Control mice consisted of Six2Cre+/PRR+/+ littermates (WT). Mice were housed at the animal care facility at Tulane University at 25°C with a 12 h light/dark cycle. Animals were fed a commercial diet (Double M feed Garden & Pet, lab rodent diet 5053, ) and tap water. All experiments involving mice were approved by Tulane Institutional Animal Care and Use Committee.

Fluorescence-Activated Cell Sorting

E15.5 kidneys from Mut and WT mice which express GFP in Cre+ cells were digested in collagenase A (25 mg/10 mL PBS) and pancreatin (100 mg/10 mL PBS) at 37°C for 15 min, dissociated by repetitive pipetting and resuspended in PBS containing 2% FBS and 10 mmol/L EDTA. The resuspended cells were filtered through 40 μm nylon cell membrane (BD Falcon) and kept on ice until Fluorescence-Activated Cell Sorting (FACS). The GFP+ cells were isolated using FACS Vantage and data were subsequently analyzed with Diva software v.5.02 (Becton Dickinson). RNA was isolated using Absolutely RNA Nanoprep kit (Stratagene). qRT-PCR was performed to validate elimination of PRR in FACS-isolated Six2Cre+ cells and revealed a 72% decrease in PRR mRNA levels in Six2+ NPCs FACs-isolated from Mut compared with WT kidneys (0.28 ± 0.001 vs. 1.0 ± 0, P < 0.001) (Fig. 1).

Microarray and gene ontology analysis

Six2Cre+ cells FACs-isolated from mutant and control kidneys were divided into two random pools (mutant: n = 3; control: n = 3 pools) consisting of 10 kidneys each. Isolated RNA was hybridized to Agilent mouse GE4X44K gene expression microarray. Hybridization, scanning, and analysis were done by a core facility of the Tulane Cancer Center. Microarray data of biological triplicates of WT and Mut FACs-isolated cells that pass QC parameters were normalized and analyzed using GeneSpring GX (Agilent, US) 12.0 software. The Benjamini-Hochberg correction for multiple testing was applied to the set of P values generated for the probe coefficients, and probes with adjusted P < 0.05 and fold change values of >1.5 and <1.5 were determined to be statistically significant. Molecular pathway analyses were performed by Ingenuity Pathway Analysis (IPA) version 7.1 (Redwood City, CA). Microarray data are available at GEO under accession number GSE101460.

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR)

SYBR Green quantitative real-time RT-PCR was conducted using MxPro QPCR software (Stratagene). The quantity of each target mRNA expression was normalized by that of GAPDH mRNA expression. RNA samples from...
PCR reaction was performed twice. Blood pressure measurement
Conscious tail-cuff mean (MAP), systolic and diastolic arterial blood pressure was measured in Six2PRR+/C0 (Het, n = 3) and control Six2PRR+/+ (WT, n = 4) mice at 2 months of age (P60) using a Visitech BP2000 system (Visitech Systems, Apex, NC). After 3 days of animal conditioning training, three consecutive cycles (10 recordings/mouse/cycle) of blood pressure readings were obtained on same day. Mean blood pressure values were calculated per each animal and used for statistical analysis.

Measurement of plasma and urine soluble PRR (sPRR)
Urine and plasma sPRR levels were measured on P60 by enzyme-linked immunosorbent assay (ELISA) kit according to the manufactures’ instruction (IBL American, # JP27782).
**Electron microscopy, histopathology, and immunohistochemistry**

P60 Het and WT kidney tissues stored in 3% glutaraldehyde were processed and embedded by the Department of Pathology, Tulane University. Ultimately, 60 nm sections were cut and imaged using a Hitachi H-7100 electron microscope. Glomerular number was counted in Het and WT kidneys on P60 from 3 consecutive H&E-stained sections/kidney adjacent to the longitudinal midplane (n = 3 kidneys/genotype). E13.5-E15.5 4-μm kidney sections from Mut and WT mice were processed for immunofluorescence using anti-amphiphysin (ProteinTech, 1:200), anti-active β-catenin (ABC, Millipore, 1:400), anti-Lotus Tetragonolobus Lectin (LTL) (1:400, Vector Laboratories), and anti-cytokeratin (1:200, Sigma) antibodies. Immunostaining was performed by the immunoperoxidase technique with Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Secondary antibodies were detected with Alexa Fluor dyes (Invitrogen). Specificity of immunostaining was documented by the omission of the primary antibody.

**β-catenin-dependent transcriptional activation in BatGal**

Mut or WT mice were crossed with BatGal mice. BatGal transgenic mice are a reporter strain which drives expression of nuclear β-galactosidase (BatGal) under the control of multimerized LEF/TCF-binding sites (Maretto et al. 2003). For β-gal staining of whole kidneys, E13.5 kidneys (n = 3 kidneys/genotype) were fixed for 60 min in 0.2% Glutaraldehyde, 5 mmol/L EGTA, and 2 mmol/L MgCl2 in 0.1 mol/L phosphate buffer, pH 7.5 on ice, rinsed for 15 min in 0.02% Igepal, 0.01% Sodium Deoxycholate, and 2 mM MgCl2 in 0.1 mol/L phosphate buffer, pH 7.5, and stained by immersion in 1 mg/mL β-gal solution at 37°C in the dark. Reaction was stopped by washing the kidneys with PBS. 14 μm midplane frozen sections of E14.5 kidneys (n = 3 kidneys/genotype) were stained for β-gal (1 mg/mL) at 37°C for 2 h in the dark, fixed for 30 min with 4% PFA, rinsed with water and counterstained with Eosin for 10 min. The intensity of kidney whole mount or kidney section β-gal staining was assessed visually in a blinded fashion. The number of β-gal positive cells per cortical area of kidney section (n = 3 sections per kidney/genotype) was counted.

**In situ hybridization**

Section In situ hybridization (ISH) was performed on E13.5 Mut and WT kidneys as previously described (Song et al. 2016). Two embryonic kidneys per group were examined.

**Statistics**

Statistical analyses were carried out upon all biologic replicates with Student’s t test or a one-way ANOVA, followed by Bonferroni test. Data are presented as Mean ± SEM. A P < 0.05 was considered statistically significant.

**Results**

**Effect of PRR deficiency in Six2 nephron progenitors on Six2 cell transcriptome**

Previously, we demonstrated that mice with conditional deletion of PRR in the Six2 nephron progenitors have a marked decrease in the number of developing nephrons and small cystic kidneys at birth, and early postnatal death (Song et al. 2016). Here, we investigated PRR-directed transcriptome using whole-genome-based analysis of gene expression in Six2 nephron progenitors FACS-isolated from Mut and WT kidneys on E15.5. On E13.5, Mut kidneys are smaller than WT kidneys and demonstrate reduced expression of nephron differentiation marker amphiphysin (Fig. 1). On E15.5, Mut kidneys remain smaller than controls and demonstrate abnormal gene regulation in NPCs (Fig. 1). Analysis of the microarray data by hierarchical cluster analysis revealed a low level of variability among biological replicates (Fig. 1). Of the ~44,000 transcripts represented on the GE4X44K array, data analysis identified 4255 (10.2%) differentially expressed genes [2129 (5.1%) upregulated, 2126 (5.1%) downregulated] (>1.5-fold change, P < 0.05) (Fig. 2). To determine changes in expression levels of individual genes, we identified the top 10 up- and downregulated genes (Tables 1,2). The magnitude of changes in top 10 gene expression was 9–35-fold for downregulated genes and 5–8-fold in upregulated genes.

**Gene ontology analysis suggests abnormal regulation of kidney development and function genes**

Next, we performed a gene ontology analysis to identify functional groups of differentially expressed genes by Ingenuity Pathway Analysis (IPA). The major GO biological processes and molecular functions enriched within top downregulated genes included cancer, cellular assembly and organization, cellular function, development, morphology, cell death and survival (Table 3). These categories are highly consistent with the mutant phenotype.
and a putative role of PRR in controlling the expression of genes involved in nephrogenesis and kidney development (Song et al. 2016). We next investigated the list of up- and downregulated genes for genes which redundant/deficient state is associated with aberrant nephrogenesis. We identified seven downregulated genes that met these criteria (Table 4). The expression of PRR-dependent genes in NPCs was validated by qRT-PCR (Fig. 2). Consistent with predominant morphogenetic events that occur at E15.5, such as nephron formation (nephrogenesis) and differentiation (establishment of specialized cell types marking specific nephron segments), we observed enrichment of IPA terms such as cellular assembly and organization, cell morphology, development, cell death and survival within the downregulated gene set (Table 3). To identify genes that may function during early nephrogenesis, we crossreferenced all downregulated genes found in this study with the microarray expression data in the CM lineage available in GUDMAP (Harding et al. 2011). A total of 238 genes were referenced in GUDMAP as expressed in the E15.5 CM lineage (Harding et al. 2011). Of 2126 genes downregulated in our array, 12 were referenced as expressed in the CM lineage in GUDMAP. Table 5 shows top 10 of these 12 downregulated genes in our array. The majority of downregulated genes belonged to kidney/embryonic development, cell morphogenesis,
immunofluorescence was decreased in the UB of *Mut* functionally active form of 2016). In accord with IPA findings, expression of the (Fig. 2) (Park et al. 2007; Marose et al. 2008; Song et al. downstream of NPC PRR to direct nephrogenesis (Song et al. 2016). IPA analysis of current microarray mesenchyme to epithelial transition (MET) in vitro canonical Wnt/\(\beta\)-catenin signaling is required downstream of NPC PRR for the NPC PRR in nephrogenesis.

**NPC PRR promotes nephrogenesis through canonical Wnt/\(\beta\)-catenin signaling**

Previously, we demonstrated that canonical Wnt/\(\beta\)-catenin signaling is required downstream of NPC PRR for mesenchyme to epithelial transition (MET) in vitro (Song et al. 2016). IPA analysis of current microarray data revealed downregulation of the Wnt/\(\beta\)-catenin pathway in FACS-isolated mutant Six2+ NPCs, consistent with critical role of intact Wnt/\(\beta\)-catenin signaling downstream of NPC PRR to direct nephrogenesis (Fig. 2) (Park et al. 2007; Marose et al. 2008; Song et al. 2016). In accord with IPA findings, expression of the functionally active form of \(\beta\)-catenin protein (ABC) dephosphorylated on \(\text{Ser}37\) or \(\text{Thr}41\) was reduced in the CM of E15.5 *Mut* kidneys (Fig. 3). In addition, ABC immunofluorescence was decreased in the UB of *Mut* compared to WT kidneys (Fig. 3). To provide genetic proof that PRR signaling induces \(\beta\)-catenin transcriptional activity in vivo in NPCs, we tested the hypothesis that metanephroi of double-transgenic *Mut/BatGal*+ mice exhibit decreased \(\beta\)-catenin activity in the CM. Whole mount LacZ staining on E13.5 was apparent only in the UB in both *Mut/BatGal*+ and *WT/BatGal*+ mice and was reduced in the UB of *Mut/BatGal*+ mice (Fig. 3). Given that targeted inactivation of \(\beta\)-catenin in the UB cell lineage in mice causes reduced UB branching, (Bridgewater et al. 2008) a UB phenotype similar to what we observed in *Mut* mice,(Song et al. 2016) our findings of reduced \(\beta\)-catenin activity in the UB of *Mut* mice demonstrate an additional pathological hit in which decreased \(\beta\)-catenin in NPCs of *Mut* kidneys leads to reduced \(\beta\)-catenin activity in the ureteric epithelium. Thus, reduced UB branching observed in *Mut* mice (Song et al. 2016) may result from both decreased nephrogenesis and reduced levels of transcriptionally active \(\beta\)-catenin within the ureteric epithelium. Because overexpression of \(\beta\)-catenin

### Table 1. Top 10 downregulated genes in Six2+ cells FACS-isolated from Six2PRR−/− kidneys.

| Gene name | Fold change | GO biological process | GO molecular function |
|-----------|-------------|-----------------------|----------------------|
| Hoxb8     | −35.6       | Dorsal spinal cord development; cytokine-mediated signaling pathway; multicellular organismal development; positive regulation of JAK-STAT; anterior/posterior pattern specification; positive regulation of cell proliferation | DNA binding; cytokine activity; growth factor activity; sequence-specific DNA binding |
| Mps1      | −18.5       | Hydrogen sulfide biosynthetic process | Transferase activity; thiosulfate sulfurtransferase activity; 3-mercaptopyruvate sulfurtransferase activity |
| Cmtm4     | −12.2       | Chemotaxis | Cytokine activity |
| Mdfi      | −11.8       | Activation of JUN kinase activity; dorsal/ventral axis specification; negative regulation of DNA binding; regulation of Wnt signaling pathway | Protein binding; transcription factor binding |
| Aph1a     | −11.4       | Proteolysis; protein processing; metanephros development; Notch signaling pathway; Notch receptor processing; integral component of plasma membrane | Protein binding; peptidase activity; endopeptidase activity |
| Mdfi      | −9.9        | Activation of JUN kinase activity; dorsal/ventral axis specification; negative regulation of DNA binding; regulation of Wnt signaling pathway | Transcription factor binding; protein binding |
| Atg4b     | −9.68753    | Transport; autophagy; proteolysis; protein transport; autophagosome assembly; positive regulation of autophagy; positive regulation of protein catabolic process | Protein binding; peptidase activity; hydrolase activity; endopeptidase activity; cysteine-type peptidase activity; cysteine-type endopeptidase activity |
| Atp1a1    | −8.80377    | Transport; ion transport; cation transport; dephosphorylation | ATP binding; ADP binding; protein binding; ATPase activity; ankyrin binding; metal ion binding |
| Stk32a    | −8.79321    | Phosphorylation; protein phosphorylation | ATP binding; kinase activity; metal ion binding; nucleotide binding; transferase activity; protein kinase activity; protein serine/threonine kinase activity; transferase activity, transferring phosphorus-containing groups |
| Gpc2      | −8.67856    | Smoothened signaling pathway; neuron differentiation | Heparan sulfate proteoglycan binding |

regulation of transcription, cytoskeleton organization, cell cycle, cell proliferation categories, consistent with the role for the NPC PRR in nephrogenesis.

The Physiological Society and the American Physiological Society.
in the metanephric mesenchyme was reported to induce β-catenin activity in the UB via upregulation of glial cell-derived neurotrophic factor (Gdnf), secreted signaling molecule expressed throughout the CM and necessary for kidney development, (Sarin et al. 2014) we tested whether reduced β-catenin activity in the UB of Mut kidneys is due to decrease in Gdnf expression in the mesenchyme. ISH showed apparent reduction in Gdnf mRNA expression in the mesenchyme of Mut compared with WT kidney (Fig. 4). Inability to detect LacZ staining in the CM using whole mount kidney may result from reduced ability to visualize staining due to high tissue thickness or density, or to relatively lower levels of β-catenin activity in the CM versus the UB epithelia. To determine the effect of PRR signaling on β-catenin transcriptional activity in NPCs in vivo with greater precision, we examined LacZ staining in WT and Mut E14.5 kidney sections. The number of X-gal-positive cells in the cortex, where active nephrogenesis occurs, was reduced in Mut compared to WT kidneys (3.1 ± 0.7 vs. 15.2 ± 1.6, P < 0.01) (Fig. 3). These findings are consistent with decreased β-catenin activity in the CM of Mut/BatGal−/− mice. Mechanistically, decreased β-catenin activity in NPCs in PRR Mut mice may reduce UB β-catenin signaling in a paracrine-dependent manner through downregulation of Gdnf levels in the CM.

**Table 2. Top 10 upregulated genes in Six2+ cells FACS-isolated from Six2PRR−/− kidneys.**

| Gene name | Fold change | GO biological process                                                                 | GO molecular function                        |
|-----------|-------------|--------------------------------------------------------------------------------------|----------------------------------------------|
| Ahdc1     | 7.6         | Any process specifically pertinent to the functioning of integrated living units     | Elemental activities, such as catalysis or binding |
| Ebf1      | 6.2         | Multicellular organismal development regulation of transcription, positive regulation of transcription | DNA binding protein dimerization activity, C2H2 zinc finger domain binding protein binding, metal ion binding |
| Tmem74    | 6.2         | Autophagy                                                                             | Elemental activities, such as catalysis or binding |
| 2010106E10Rik | 5.7 | Any process specifically pertinent to the functioning of integrated living units     | Elemental activities, such as catalysis or binding, describing the actions of a gene product at the molecular level. |
| Cd247     | 5.5         | Cell surface receptor signaling pathway                                               | Protein binding; transmembrane signaling receptor activity |
| Hbb-y     | 5.5         | Transport; protein heterooligomerization; negative regulation of transcription from RNA polymerase II promoter | Hemoglobin alpha binding; oxygen transporter activity; heme binding; metal ion binding; oxygen binding; iron ion binding |
| Cma1      | 5.5         | Proteolysis; positive regulation of angiogenesis; interleukin-1 beta biosynthetic process | Peptide binding; catalytic activity; peptidase activity; hydrolase activity |
| Kirrel3   | 5.4         | Glomerulus morphogenesis; neuron projection morphogenesis; hemopoiesis; neuron migration | Protein binding; PDZ domain binding |
| 4930592O3Rik | 5.4 | Any process specifically pertinent to the functioning of integrated living units     | Elemental activities, such as catalysis or binding |
| IIS       | 5.4         | Immune response; cytokine-mediated signaling pathway positive regulation of JAK-STAT cascade; positive regulation of cell proliferation | Growth factor activity; cytokine activity interleukin-5 receptor binding |

**Novel PRR-dependent genes and control of nephrogenesis**

Notch signaling in NPCs promotes initiation of nephrogenesis and the formation of proximal tubules (Cheng et al. 2007; Chung et al. 2016). Previously, we demonstrated reduced expression of Jagged1 (Jag1), the major Notch ligand in the process of nephrogenesis, in PRR Mut kidneys (Song et al. 2016). Since our array identified downregulation of Notch1 and of Notch-target gene Hes5 in Mut NPCs, we investigated the formation of proximal tubules in Mut kidneys. In WT kidneys, NPCs formed LTL-positive proximal tubules (Fig. 4). In contrast, Mut kidneys had paucity and marked dilation of LTL-positive proximal tubules. Proximal tubular defects observed in Mut mice are most likely due to premature exhaustion of NPCs (Song et al. 2016). Our findings of reduced Notch1 and Hes5 expression in Mut NPCs suggest that additional mechanism may involve aberrant Notch signaling downstream of NPC PRR in a subset of NPCs that were able to differentiation into the proximal tubule segment of the nephron. Expression of Jag1, the major Notch ligand in the process of nephrogenesis and a marker for renal vesicle (RV) nephron stage, was also reduced in Mut NPCs (Fig. 1, Table 4). p53, a tumor suppressor that regulates cell-cycle, differentiation, and apoptosis pathways, is also
important for normal embryonic kidney development. Global p53 deletion in mice results in UB ectopia, reduced UB branching, and hypoplastic metanephroi (Sai-fudeen et al. 2009). Reduced p53 mRNA levels in Mut NPCs suggest that p53 functions downstream of the NPC PRR to promote nephrogenesis.

**Reduced PRR gene dosage in nephron progenitors results in development of hypertension during later life**

We previously demonstrated that Mut (Six2PRR+/−) mice exhibit reduced nephron number at birth due to precocious depletion of NPC pool (Song et al. 2016). Low nephron number has been recognized as a determinant of susceptibility to hypertension both in animal models (Woods et al. 2004) and in humans (Brenner et al. 1988; Hughson et al. 2006). In humans, nephrogenesis is completed by ~36 weeks of gestation and in rodents- by ~3 days after birth (Hinchcliffe et al. 1991; Reidy and Rosenblum 2009). Because Mut mice did not survive beyond the first 48 h of life, we evaluated the effect of reduced PRR gene dosage in Six2PRR+/− (Het) mice on nephron number, glomerular basement membrane (GBM) ultrastructure, and blood pressure at 2 months of age. While the number of glomeruli per kidney section was reduced in Het compared with control mice (69 ± 4.0 vs. 178 ± 4.9, P < 0.001), conscious tail-cuff mean (95.5 ± 2.8 vs. 70.4 ± 3.8, p < 0.01), systolic (143 ± 5.3 vs. 113 ± 6.5, P < 0.01), and diastolic (67 ± 4.5 vs. 51 ± 4.0, P < 0.05) arterial blood pressure was increased in Het mice (Fig. 5). Electron microscopy (EM) of P60 Het kidney section showed segmental GBM thickening with focal microvillus changes and focal podocyte foot process effacement, a hallmark of glomerular injury leading to proteinuria (Fig. 5). Thus, reduced PRR gene dosage in nephron progenitors results in decreased glomerular number, abnormal GBM, and increased blood pressure later in life.

**Reduced PRR gene dosage in nephron progenitors results in increased urinary soluble PRR (sPRR) levels**

The full-length PRR protein (39 kDa) is cleaved by enzyme furin to generate sPRR (28 kDa) which is secreted into the extracellular space and is ultimately found in the blood and urine (Cousin et al. 2009; Gonzalez et al. 2011). Given that elevated plasma sPRR levels correlate positively with histological evidence of renal tissue damage in humans (Ohashi et al. 2016) and that sPRR is functionally active in the urine,(Gonzalez et al. 2011) we next measured plasma and urine sPRR levels in Het and control mice at 2 months of age. While plasma sPRR levels did not differ (60,045 ± 9070 vs. 56,166 ± 4380 pg/mL, P = 0.72), urinary sPRR levels were increased in Het compared with control mice (263 ± 30 vs. 146 ± 14 pg/mL, P < 0.05) (Fig. 5). Because it is unknown whether sPRR (28 kDa) is filtered from plasma to the urine and in view of abundant PRR expression in the collecting duct (CD), we speculate that urinary sPRR excretion likely reflects cleavage of full-length PRR in the CD and intratubular sPRR secretion.

**Discussion**

Previously, we demonstrated that conditional deletion of PRR in Six2+ NPCs results in early neonatal death, a marked decrease in the number of developing nephrons at birth and small cystic kidneys (Song et al. 2016). Yet, the transcriptome downstream of the nephron progenitor PRR that underlies its actions has not been previously defined in vivo. In this study, we first identified genes

---

**Table 3. Top diseases and biological functions altered in Six2+ cells FACS-isolated from Six2PRR−/− kidneys.**

| Category                     | P-value  | Number of molecules |
|------------------------------|----------|---------------------|
| Diseases and disorder        |          |                     |
| Organismal injury and abnormalities | 1.27E-04-2.66E-34 | 2415 |
| Gastrointestinal disease     | 1.27E-04-2.66E-34 | 2424 |
| Hepatic system disease       | 1.28E-04-2.49E-30 | 1782 |
| Reproductive system disease  | 1.06E-04-2.57E-18 | 1076 |
| Cancer                       | 7.04E-05-3.88E-17 | 964 |
| Molecular and cellular functions |        |                     |
| Cellular assembly and organization | 1.11E-04-2.15E-23 | 552 |
| Cellular function and maintenance | 7.14E-05-2.15E-23 | 709 |
| Cell morphology              | 1.21E-04-2.74E-21 | 720 |
| Cellular development         | 1.02E-04-4.60E-16 | 823 |
| Cell death and survival      | 1.27E-04-8.11E-16 | 858 |
| Physiological System Development and Function |          |                     |
| Organismal survival          | 1.50E-05-6.52E-29 | 654 |
| Embryonic development        | 1.21E-04-1.22E-20 | 691 |
| Organismal development       | 1.21E-04-1.22E-20 | 939 |
| Nervous system development   | 1.21E-04-2.25E-17 | 541 |
| Tissue morphology            | 1.21E-04-3.34E-17 | 704 |
| Nephrotoxicity               |          |                     |
| Renal necrosis/cell          | 1.00E00-7.18E-06 | 105 |
| Death nephrosis renal        | 5.86E-01-6.33E-03 | 16 |
| Inflammation renal           | 1.00E00-1.85E-02 | 52 |
| Regeneration                 | 1.00E00-1.85E-02 | 52 |
that are regulated by PRR in Six2+ NPCs using a whole-genome expression analysis of RNA in mice with PRR deficiency targeted to Six2+ NPCs (Mut) and conducted gene ontology analysis to identify functional groups of differentially expressed genes. The expression of seven genes, β-catenin, Notch1, Leif, Lhx1, Jag1, Fgf8, and p53, with expression in NPCs for which deficient state is associated with aberrant nephrogenesis was downregulated in PRR Mut NPCs. Bioinformatic analyses of our data demonstrated that NPC PRR performs essential functions
during nephrogenesis via control of hierarchy of genes that regulate embryonic and cellular development, renal regeneration, cellular assembly and organization, cell morphology, cell death and survival. Next, we demonstrated that reduced PRR gene dosage in NPCs in Six2^{PRR+/-} (Het) mice is associated with reduced number of glomeruli, ultrastructural changes in the GBM, and development of hypertension at 2 months of age. We then showed that levels of soluble PRR (sPRR), PRR cleavage product generated subcellularly and secreted extracellularly, are increased in the urine of Het mice compared with control WT mice at 2 month of age. Our data show that: (1) NPC PRR performs essential functions during nephrogenesis via control of hierarchy of genes that regulate critical cellular processes; and (2) Both reduced nephron endowment and augmented urine sPRR likely contribute to programming of hypertension in Het mice. Our results support a model in which lack of PRR in NPCs results in aberrant expression of genes critical for nephrogenesis in NPC lineage (Fig. 6). Reduced PRR gene dosage in NPCs results in reduced glomerular number, abnormal GBM ultrastructure, and elevated blood pressure at 2 months of age. We propose that reduced PRR gene dosage in NPCs results in compensatory increase in sPRR levels in the CD which may lead to elevated blood pressure by stimulation of ENaC channel activity and increased Na\(^+\) reabsorption in the CD (Fig. 6).

**NPC PRR-dependent genes and control of nephrogenesis**

The balance of NPC self-renewal and differentiation into nephrons ultimately determines nephron endowment (final nephron number) (Little and McMahon 2012).
Because nephrogenesis ends by 36 weeks of gestation in humans, nephron regeneration postnatally is not possible. Reduced nephron endowment is associated with renal hypoplasia, susceptibility to subsequent hypertension and chronic kidney disease (CKD) (Brenner et al. 1988; Bertram et al. 2011). Several key signaling pathways, including Wnt/β-catenin, Six2, Eya1, Notch, Osr1, Pax2, Sall1, Bmp7, Fgf2, Fgf9, and Fgf20, are required in NPCs for

---

**Figure 4.** (A–D) Section in situ hybridization of E14.5 kidneys shows apparent decrease in Gdnf mRNA expression in the mesenchyme of mutants (Mut) compared to controls (WT, dark blue staining). (E–H): Kidney sections of E17.5 Mut and WT mice stained with anti-Lotus Tetragonolobus Lectin (LTL, green) antibody show paucity and dilation (N/P, arrows) of tubules of proximal tubule origin in Mut kidneys.

---

**Figure 5.** Reduced PRR gene dosage in Six2+ NPCs results in decreased glomerular number, altered glomerular basement membranes (GBM) ultrastructure, elevated blood pressure and increased soluble PRR (sPRR) levels in the urine at 2 months of age. (A): Het mice have significantly increased mean (MAP), systolic (SBP), and diastolic (DBP) blood pressure at 2 months of age. *P < 0.01. (B) Het mice have significantly increased sPRR levels in the urine. *P < 0.05. (C) Glomerular number is significantly decreased in Het mice. (D, E) Electron micrograph of WT kidney section (D) shows normally appearing GBM with intact podocyte foot processes (white arrows). Het kidney section (E) shows focal podocyte foot process effacement (black arrows) with focal microvillus change and irregularities in the GBM with segmental thickening (white arrows).
nephrogenesis (Self et al. 2006; Blank et al. 2009; Brown et al. 2011, 2013; Barak et al. 2012; Kanda et al. 2014; Chung et al. 2016). We observed downregulation of several of these genes for which deficient state is associated with aberrant nephrogenesis (Table 4). Wnt/β-catenin is required for both the self-renewal and differentiation of NPCs (Carroll et al. 2005; Park et al. 2007; Karner et al. 2011). Both conditional inactivation or stabilization of β-catenin in the metanephric mesenchyme in mice disrupts nephrogenesis (Park et al. 2007; Sarin et al. 2014). Thus, a finely tuned level of Wnt/β-catenin signaling in the mesenchyme is essential for proper nephrogenesis. Wnt/β-catenin pathway activity was reduced in both NPCs and in the UB of PRR Mut mice, indicating a critical role for β-catenin downstream of NPC PRR in metanephric kidney development. In addition, β-catenin transcriptional activity in vivo was decreased in the UB of Mut mice. Our data suggest that decreased β-catenin activity in NPCs of Mut mice might reduce UB β-catenin signaling in a paracrine-dependent manner through downregulation of Gdnf levels in the mesenchyme. Thus, reduced β-catenin activity in the UB of Mut mice may represent an additional pathological hit that, together with decreased β-catenin signaling in NPCs, contributes to reduced UB branching, decreased number of nephrons and small cystic kidneys observed at birth in Mut mice (Song et al. 2016). We also observed decreased expression of Notch 1, p53, and Fgf8 in Mut mice. Notch signaling in NPCs is crucial for nephrogenesis through downregulation of Six2, a transcription factor required for NPC maintenance (Chung et al. 2016). It is possible that reduced Notch expression in Mut mice might inhibit
nephron induction via disinhibition of Six2. Murine double minute 2 (Mdm2)-p53 pathway is essential to the maintenance of the NPC niche (Hilliard et al. 2014). Reduced p53 mRNA levels in Mut NPCs suggest that p53 functions downstream of the NPC PRR to promote nephrogenesis. Reduced p53 expression may be due to increased levels of Mdm2, a p53 ubiquitin ligase, in Mut NPCs. Fgf8 is required for nephron development (Grieshammer et al. 2005; Perantoni et al. 2005). Mice with pan-mesodermal loss of Fgf8 show lack of CM formation by E16.5 and block in vesicle progression to comma- and S-shaped nephrons (Perantoni et al. 2005). Because Fgf8 is one of the earliest markers that demarcate the transition of NPCs to pre-tubular aggregates and RVs, decreased nephron number in Mut kidneys at birth may be due to block in MET from reduced Fgf8 expression (Song et al. 2016).

NPC PRR and developmental programming of hypertension

Previously, we identified NPC PRR as critical regulator of congenital nephron endowment (Song et al. 2016). Here, we demonstrate that even reduced PRR gene dosage in NPCs in Het mice is associated with reduced glomerular number, aberrant GBM ultrastructure with focal podocyte foot process effacement and increased blood pressure at 2 months of age. It is now well established that adverse events in utero can affect nephrogenesis and result in low nephron endowment (Bertram et al. 2011). Brenner et al. (1988) hypothesized that low congenital nephron number might explain why some individuals are more susceptible to hypertension and renal disease than others later in life (developmental programming). The major factors influencing in utero environment that are associated with a low final nephron number include uteroplacental insufficiency, maternal low protein diet, hyperglycemia, vitamin A deficiency, exposure to or interruption of endogenous glucocorticoids (Chong and Yosipiv 2012). Reduced nephron number may be associated with reduced filtration surface area, thus limiting sodium excretion and leading to higher blood pressure or inappropriate activation of other vasoactive systems (e.g., renin-angiotensin system, RAS). Even though Brenner’s hypothesis offers an explanation for the association of a reduced nephron number with hypertension and renal disease, no definitive proof has been found that low nephron endowment per se causes increased risk for hypertension or renal injury. For example, hypertension is not associated with glomerular number in humans (Hughson et al. 2006). What is the relevance of our study to kidney development and pathogenesis of hypertension in humans? In human neonates, PRR immunoreactivity is present in the glomeruli, proximal tubules, collecting ducts, and arteries (Terada et al. 2017). In addition, the levels of PRR protein expression in neonatal kidney correlate inversely with gestational age and are higher in premature compared with full-term neonates (Terada et al. 2017). These findings suggest that PRR may play an important role in kidney development in humans.

Increased urinary sPRR and developmental programming of hypertension

We demonstrate that urinary levels of sPRR are increased in hypertensive Het mice at 2 months of age. Elevated sPRR levels in the urine of Het mice may reflect kidney tissue damage, the status of the kidney tissue RAS activity or act independently of the RAS and contribute to programming of increased blood pressure in these mice. Mechanistically, increased urinary sPRR levels can enhance activity of renin/prorenin in the CD leading to generation of angiotensin II (Ang II), the major vasoactive peptide of the RAS (Gonzalez et al. 2011). In addition, sPRR may act independent of Ang II/Ang II receptors to induce expression and activity of the epithelial sodium channel (ENaC) in the CD, thus contributing to an increase in blood pressure via enhanced Na+ reabsorption in the CD (Ramkumar et al. 2016).

In summary, NPC PRR performs essential functions during nephrogenesis via control of hierarchy of genes that regulate critical cellular processes in NPC lineage. Reduced PRR gene dosage in NPCs is associated with reduced number of glomeruli, ultrastructural changes in the GBM, and development of hypertension at 2 months of age. We propose that both reduced nephron endowment and augmented urine sPRR may contribute to programming of hypertension in mice with reduced PRR gene dosage in NPCs. This analysis provides important clues on the molecular etiology of reduced nephron endowment that will assist in the development of novel approaches aimed at early diagnosis and counseling of patients with renal hypoplasia/hypoplasia and identification of targets for treatment or prevention.

Acknowledgments

We thank Drs. Atsuhiro Ichihara, Andrew McMahon, and Yiping Chen for providing PRR<sup>flox/flox</sup>, Six2<sup>Cre<sup>+</sup></sup>, and Bat-Gal<sup>+</sup> mice, respectively. We thank Dr. Yuwen Li (Tulane University) for assistance with IPA data analysis.

Conflict of Interest

Nothing to disclose.
References

Barak, H., S. H. Huh, S. Chen, C. Jeanpierre, J. Martinovic, M. Parisot, et al. 2012. FGFR9 and FGFR20 maintain the stemness of nephron progenitors in mice and man. Dev. Cell 22:1191–1207.

Barker, D. J., C. Osmond, J. Golding, D. Kuh, and M. E. Wadsworth. 1989. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ 298:864.

Bertram, J. F., R. N. Douglas-Denton, B. Diouf, M. D. Hughson, and W. E. Hoy. 2011. Human nephron number: implications for health and disease. Pediatr. Nephrol. 26:1529–1533.

Blank, U., A. Brown, D. C. Adams, M. J. Karolak, and L. Oxburgh. 2009. BMP7 promotes proliferation of nephron progenitor cells via a JNK-dependent mechanism. Development 136:3557–3566.

Brenner, B. M., D. L. Garcia, and S. Anderson. 1988. Glomeruli and blood pressure. Less of one, more the other? Am. J. Hypertens. 1:335–347.

Bridgewater, D., B. Cox, J. Cain, A. Lau, V. Athaide, P. S. Gill, et al. 2008. Canonical WNT/beta-catenin signaling is required for ureteric branching. Dev. Biol. 317:83–94.

Brown, A. C., D. Adams, M. de Caestecker, X. Yang, R. Friesel, and L. Oxburgh. 2011. FGF/EGF signaling regulates the renewal of early nephron progenitors during embryonic development. Development 138:5099–5112.

Brown, A. C., S. D. Muthukrishnan, J. A. Guay, D. C. Adams, D. A. Schafer, J. L. Fetting, et al. 2013. Role for compartmentalization in nephron progenitor differentiation. Proc. Natl Acad. Sci. USA 110:4640–4645.

Carroll, T. J., J. S. Park, S. Hayashi, A. Majumdar, and A. P. McMahon. 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Dev. Cell 9:283–292.

Cheng, H. T., M. Kim, M. T. Valerius, K. Surendran, K. Schuster-Gossler, A. Gossler, et al. 2007. Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. Development 134:801–811.

Chong, E., and I. V. Yosypiv. 2012. Developmental programming of hypertension and kidney disease. Int. J. Nephrol. 1–15:2012.

Chung, E., P. Deacon, S. Marable, J. Shin, and J. S. Park. 2016. Notch signaling promotes nephrogenesis by downregulating SIX2. Development 143:3907–3913.

Cousin, C., D. Bracquart, A. Contrepas, P. Corvol, L. Muller, and G. Nguyen. 2009. Soluble form of the (pro)renin receptor generated by intracellular cleavage by furin is secreted in plasma. Hypertension 53:1077–1082.

Gonzalez, A. A., L. S. Lara, C. Luffman, D. M. Seth, and M. C. Prieto. 2011. Soluble form of the (pro)renin receptor is augmented in the collecting duct and urine of chronic angiotensin II-dependent hypertensive rats. Hypertension 57:859–864.

Griesshammer, U., C. Cebraini, R. Ilagan, E. Meyers, D. Herzlinger, and G. R. Martin. 2005. FGFR8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. Development 132:3847–3857.

Harding, S. D., C. Armit, J. Armstrong, J. Brennan, Y. Cheng, B. Haggarty, et al. 2011. The GUDMAP database—an online resource for genitourinary research. Development 138:2845–2853.

Hilliard, S. A., X. Yao, and S. S. El-Dahr. 2014. Mdm2 is required for maintenance of the nephrogenic niche. Dev. Biol. 387:1–14.

Hinchliffe, S. A., P. H. Sargent, C. V. Howard, Y. F. Chan, and D. van Velzen. 1991. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. Lab. Invest. 64:777–784.

Hirose, T., M. Hashimoto, K. Totsune, H. Metoki, K. Asayama, M. Kikuya, et al. 2009. Association of (pro)renin receptor gene polymorphism with blood pressure in Japanese men: the Ohasama study. Am. J. Hypertens. 22:294–299.

Hirose, T., M. Hashimoto, K. Totsune, H. Metoki, A. Hara, M. Satoh, et al. 2011. Association of (pro)renin receptor gene polymorphisms with lacunar infarction and left ventricular hypertrophy in Japanese women: the Ohasama study. Hypertens. Res. 34:530–535.

Hughson, M. D., R. Douglas-Denton, J. F. Bertram, and W. E. Hoy. 2006. Hypertension, glomerular number, and birth weight in African Americans and white subjects in the southeastern United States. Kidney Int. 69:671–678.

Kanda, S., S. Tanigawa, T. Ohmori, A. Taguchi, K. Kudo, Y. Suzuki, et al. 2014. Sall1 maintains nephron progenitors and nascent nephrons by acting as both an activator and a repressor. J. Am. Soc. Nephrol. 25:2584–2595.

Karner, C. M., A. Das, Z. Ma, M. Self, C. Chen, L. Lum, et al. 2011. Canonical Wnt9b signaling balances progenitor cell expansion and differentiation during kidney development. Development 138:1247–1257.

Kobayashi, A., M. T. Valerius, J. W. Mugford, T. J. Carroll, M. Self, G. Oliver, et al. 2008. SIX2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. Cell Stem Cell 3:169–181.

Little, M. H., and A. P. McMahon. 2012. Mammalian kidney development: principles, progress, and projections. Cold Spring Harb. Perspect. Biol. 4:1–18.

Ludwig, J., S. Kerscher, U. Brandt, K. Pfieffer, F. Getlawi, D. K. Apps, et al. 1998. Identification and characterization of a novel 9.2-kDa membrane sector-associated protein of vacuolar proton-ATPase from chromaffin granules. J. Biol. Chem. 273:10939–10947.
Maretto, S., M. Cordenonsi, S. Dupont, P. Braghetta, V. Broccoli, A. B. Hassan, et al. 2003. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc. Natl Acad. Sci. USA 100:32993–304.

Marose, T. D., C. E. Merkel, A. P. McMahon, and T. J. Carroll. 2008. Beta-catenin is necessary to keep cells of ureteric bud/Wolffian duct epithelium in a precursor state. Dev. Biol. 314:112–126.

Nguyen, G., F. Delarue, C. Burcklé, L. Bouzhir, T. Giller, and J. D. Sraer. 2002. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. J. Clin. Invest. 109:1417–1427.

Ohashi, N., S. Isobe, S. Ishigaki, T. Suzuki, T. Iwakura, M. Ono, et al. 2016. Plasma Soluble (Pro)renin Receptor Reflects Renal Damage. PLoS ONE 11:e0156165.

Oshima, Y., K. Kinouchi, A. Ichihara, M. Sakoda, A. Kurauchi-Mito, K. Bokuda, et al. 2011. Prorenin receptor is essential for normal podocyte structure and function. J. Am. Soc. Nephrol. 22:2203–2212.

Park, J. S., M. T. Valerius, and A. P. McMahon. 2007. Wnt/beta-catenin signaling regulates nephron induction during mouse kidney development. Development 134:2533–2539.

Perantoni, A. O., O. Timofeeva, F. Naillat, C. Richman, S. Pajni-Underwood, C. Wilson, et al. 2005. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. Development 132:3859–3871.

Ramkumar, N., D. Stuart, E. Mironova, V. Bugay, S. Wang, N. Abraham, et al. 2016. Renal tubular epithelial cell prorenin receptor regulates blood pressure and sodium transport. Am. J. Physiol. Renal Physiol. 311:F186–F194.

Ramser, J., F. E. Abidi, C. A. Burckle, C. Lenski, H. Toriello, G. Wen, et al. 2005. A unique exonic splice enhancer mutation in a family with X-linked mental retardation and epilepsy points to a novel role of the renin receptor. Hum. Mol. Genet. 14:019–1027.

Reidy, K. J., and N. D. Rosenblum. 2009. Cell and molecular biology of kidney development. Semin. Nephrol. 29:321–337.

Saifudeen, Z., S. Dipp, J. Stefkova, X. Yao, S. Lookabaugh, and S. S. El-Dahr. 2009. p53 regulates metanephric development. J. Am. Soc. Nephrol. 11:2328–2337.

Sarin, S., F. Boivin, A. Li, J. Lim, B. Svaiger, N. D. Rosenblum, et al. 2014. beta-Catenin overexpression in the metanephric mesenchyme leads to renal dysplasia genesis via cell-autonomous and non-cell-autonomous mechanisms. Am. J. Pathol. 184:1395–1410.

Self, M., O. V. Lagutin, B. Bowling, J. Hendrix, Y. Cai, G. R. Dressler, et al. 2006. Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. EMBO J. 25:5214–5228.

Sihn, G., C. Burckle, A. Rousselle, T. Reimer, and M. Bader. 2013. (Pro)renin receptor: subcellular localizations and functions. Front Biosci. 5:500–508.

Song, R., G. Preston, L. Kidd, D. Bushnell, S. Sims-Lucas, C. M. Bates, et al. 2016. Prorenin receptor is critical for nephron progenitors. Dev. Biol. 409:382–391.

Terada, T., M. Urushihara, T. Saijo, R. Nakagawa, and S. Kagami. 2017. (Pro)renin and (pro)renin receptor expression during kidney development in neonates. Eur. J. Pediatr. 176:183–189.

Woods, L. L., D. A. Weeks, and R. Rasch. 2004. Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. Kidney Int. 65:1339–1348.