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Prorenin receptor is critical for nephron progenitors

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ABSTRACT

Deficient nephrogenesis is the major factor contributing to renal hypoplasia defined as abnormally small kidneys. Nephron induction during kidney development is driven by reciprocal interactions between progenitor cells 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 H^+-ATPase. Global loss of *PRR* is lethal in mice and *PRR* mutations are associated with a high blood pressure, left ventricular hypertrophy and X-linked mental retardation in humans. To circumvent lethality of the ubiquitous *PRR* mutation in mice and to determine the potential role of the PRR in nephrogenesis, we generated a mouse model with a conditional deletion of the *PRR* in Six2$^+$ nephron progenitors and their epithelial derivatives (*Six2*$^{PRR-/-}$). Targeted ablation of *PRR* in Six2$^+$ nephron progenitors caused a marked decrease in the number of developing nephrons, small cystic kidneys and podocyte foot process effacement at birth, and early postnatal death. Reduced congenital nephron endowment resulted from premature depletion of nephron progenitor cell population due to impaired progenitor cell proliferation and loss of normal molecular inductive response to canonical Wnt/β-catenin signaling within the metanephric mesenchyme. At 2 months of age, heterozygous *Six2*$^{PRR+/-}$ mice exhibited focal glomerulosclerosis, decreased kidney function and massive proteinuria. Collectively, these findings demonstrate a cell-autonomous requirement for the PRR within nephron progenitors for progenitor maintenance, progression of nephrogenesis, normal kidney development and function.

1. Introduction

Kidney development is driven by reciprocal inductive interactions between a multipotent, self-renewing Six2$^+$;Cited1$^+$ progenitor cells of the cap mesenchyme (CM) and the ureteric bud (UB) (Kobayashi et al., 2008). CM cells are continuously induced during metanephric organogenesis to condense around the surface of the UB tips and form pre-tubular aggregates (PTAs). PTAs undergo a mesenchyme to epithelial transition (MET) to sequentially form renal vesicles (RVs), comma- and S-shaped bodies (SBs), and, ultimately, most components of the mature nephrons, including the glomerulus, proximal tubule, loop of Henle and distal tubule (Little and McMahon, 2012). Although reduced nephron endowment has significant clinical implications such as renal hypoplasia, proteinuria, a limited capacity for kidney repair after injury, susceptibility to subsequent hypertension and chronic kidney disease (CKD), the mechanisms that determine final nephron number in health and disease are not well understood (Bertram et al., 2011).

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 H^+-ATPase (Ludwig et al., 1998). H^+-ATPases are expressed in intracellular compartments of virtually all cell types and play important roles in protein trafficking and degradation *via* acidification of intracellular organelles (Forgac, 2007). In the intercalated cells of the collecting duct, H^+-ATPase is present at the plasma membrane where it is important for urine acidification (Wagner et al., 2004). Global *PRR* knockout is lethal in mice, indicating an essential role of the PRR in embryonic development (Sihn et al., 2013). In humans, *PRR* mutations are associated with a high blood pressure, left ventricular hypertrophy and X-linked mental retardation (Hirose et al., 2009, 2011, Ramser et al., 2005). Recent data demonstrate that PRR is critical for normal kidney development and function. In this regard, *PRR* deletion in mice podocytes using the *Nphs2* promoter results in massive foot process effacement, proteinuria and nephrotic syndrome (Oshima et al., 2011). *PRR* ablation in the UB using the *Hoxb7* promoter leads to kidney hypoplasia, polyuria and

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a reduced capacity to acidify the urine (Song et al., 2013). However, the role of PRR in nephron progenitors remains unknown.

Here, we demonstrate that conditional inactivation of the *PRR* in Six2^{+} nephron progenitors in mice results in small cystic kidneys at birth. We show that nephron progenitor population is depleted due to reduced cell proliferation and enhanced apoptosis, leading to fewer nephrons caused by impaired MET. Subsequently, *Six2*$^{PRR+/-}$ mice develop evidence of glomerular kidney disease with focal glomerulosclerosis, proteinuria and decreased kidney function. Collectively, these findings demonstrate a cell-autonomous requirement for the PRR within nephron progenitors for maintenance of progenitor cell pool, progression of nephrogenesis, normal kidney development and function.

2. Materials and methods

2.1. 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 *Six2*$^{Cre+}$/*PRR*$^{flox/flox}$ mice represent nephron progenitor-specific *PRR*-knockout mice (*Six2*$^{PRR-/-}$). Control mice consisted of *PRR*$^{flox/flox}$ littermates (*Six2*$^{PRR+/+}$). All experiments involving mice were approved by Tulane Institutional Animal Care and Use Committee.

2.2. Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed in the Mx3000P equipment (Stratagene, La Jolla, CA) using MxPro QPCR software (Stratagene) as previously described (Song et al., 2013). mRNA was extracted from snap-frozen E12.5 and E18.5 *Six2*$^{PRR-/-}$ and control kidneys (E15.5 kidneys were pooled, E18.5 – $n=3$ mice per group). The quantity of each target mRNA was normalized by that of GAPDH mRNA expression. RNA samples were analyzed in triplicates in each run. PCR reaction was performed twice.

2.3. Immunohistochemistry and histopathology

Kidneys were fixed in 4% PFA at 4 °C and paraffin embedded. Immunostaining was performed by the immunoperoxidase technique using 4-μm sections with Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Primary antibodies included anti-PRR (1:200, Santa Cruz), anti-Lhx1 (1:10, developmental studies hybridoma bank), anti-Lef1 (1:100, cell signaling), anti-Jagged1 (1:100, Santa Cruz), anti-Sall1 (1:100, Abcam), anti-Pax2 (1:100, Invitrogen), anti-WT1 (1:100, Abcam), anti-active β-catenin (ABC, Millipore, 1:400), anti-Six2 (1:100, Proteintech) and Lotus Tetragonolobus Lectin (LTL) (1:400, Vector Laboratories). Whole intact E13.5 metanephroi from *PRR*$^{Six2-/-}$ and control mice ($n=10$ kidneys per genotype) were processed for the whole mount immunofluorescence using anti-cytokeratin antibody (1:200, Sigma) and the number of UB tips was counted. For immunofluorescence studies, secondary antibodies were detected with Alexa Fluor dyes (Invitrogen). Specificity of immunostaining was documented by the omission of the primary antibody. Kidneys from P0 *Six2*$^{PRR-/-}$ and control mice ($n=3$ mice per group) were cut in the longitudinal midplane, processed through the paraffin, and embedded on the cut surface. Kidneys were sectioned at 4-μm and stained with hematoxylin and eosin. The number of nephrons in each of 3 consecutive sections adjacent to the longitudinal midplane was counted and the mean number of nephrons per section per kidney was calculated. To determine the number of Six2-positive structures, we examined the intensity of Six2 immunofluorescence in P0 kidney sections ($n=3$ mice per group) using Slide book 4.0 software (Intelligent Imaging Innovations, Denver, CO). All counts were performed in a blinded fashion. For electron microscopy, P0 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.

2.4. In situ hybridization (ISH)

Section ISH was performed on E14.5 and P0 *Six2*$^{PRR-/-}$ and control kidneys as previously described (Song et al., 2013). Mouse full length probes for *Cited1*, *Wnt4*, *Pax8, Wnt9b, Bmp7, Etv4* were a kind gift from Drs. Alan Perantoni, Leif Oxburgh and Carl Bates. 3 embryonic kidneys per group per probe were examined.

2.5. Cell proliferation and apoptosis assays

Cell proliferation and apoptosis was examined in E13.5 and P0 kidney sections from *Six2*$^{PRR-/-}$ and control mice ($n=3$ mice per genotype, 3 sections per kidney) as previously described (Song et al., 2013). Anti-phospho-histone H3 (pH3) and anti-cleaved caspase-3 antibodies were used (Cell Signaling, Danvers, MA; 1:50). Nephron progenitors were visualized with anti-Six2 antibody and UBs/UB-derived epithelia with anti-cytokeratin antibody. The number of proliferating and apoptotic cells in nephron progenitors was normalized to the total number of Six2-positive cells in each kidney section. The number of Six2-positive cells was determined by ImageJ software (NIH).

2.6. Measurement of creatinine and albumin

Blood and urine was obtained from P60 *Six2*$^{PRR+/-}$ and control mice ($n=4$ mice per genotype). All urine specimens were collected at 8 am. Each mouse was placed in an individual clean dry cage with no bedding and a sheet of plastic wrap placed on the bottom until urine was produced (approximately 30 min). Urine that was not contaminated with fecal waste was collected into autoclaved Eppendorf tubes and frozen at −20 °C. Plasma and urine creatinine was measured by HPLC with picric acid (Jaffe method). Urinary albumin excretion was determined using the Albuwell ELISA kit (Exocell, Philadelphia, PA).

2.7. Fluorescence-Activated Cell Sorting (FACS)

E15.5 kidneys from *Six2*$^{PRR-/-}$ (*Six2*$^{Cre+}$/*PRR*$^{flox/flox}$) and control (*Six2*$^{Cre+}$/*PRR*$^{+/+}$) mice were digested in 0.25% Trypsin for 1 min at 37 °C, dissociated by repetitive pipetting and resuspended in PBS containing 2% FBS and 10 mM EDTA. The resuspended cells were filtered through 40 μm nylon cell membrane (BD Falcon) and kept on ice until 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 confirm elimination of *PRR* in FACS-isolated Six2^{+} cells from pooled *Six2*$^{PRR-/-}$ kidneys. For ABC immunofluorescence and CM differentiation studies of FACS-isolated CM cells, E15.5 kidneys were digested with collagenase A (25 mg/10 ml PBS) and pancreatin (100 mg/10 ml PBS) at 37 °C for 15 min.

2.8. ABC immunofluorescence and CM differentiation assay

FACS-isolated *Six2*$^{Cre+}$/*PRR*$^{flox/flox}$ and *Six2*$^{Cre+}$/*PRR*$^{+/+}$ cells from E15.5 kidneys were grown on Matrigel-coated 8 chamber slides (20,000 cells/chamber) in DMEM/F12 medium with 10% FBS.

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