Nephron Deficiency and Predisposition to Renal Injury in a Novel One-Kidney Genetic Model


Departments of *Pharmacology and Toxicology, †Medicine (Nephrology), ‡Physiology and Biophysics, §Pathology, and ¶Radiology, University of Mississippi Medical Center, Jackson, Mississippi; and ♦Department of Pediatrics, Medical College of Wisconsin, Madison, Wisconsin

ABSTRACT

Some studies have reported up to 40% of patients born with a single kidney develop hypertension, proteinuria, and in some cases renal failure. The increased susceptibility to renal injury may be due, in part, to reduced nephron numbers. Notably, children who undergo nephrectomy or adults who serve as kidney donors exhibit little difference in renal function compared with persons who have two kidneys. However, the difference in risk between being born with a single kidney versus being born with two kidneys and then undergoing nephrectomy are unclear. Animal models used previously to investigate this question are not ideal because they require invasive methods to model congenital solitary kidney. In this study, we describe a new genetic animal model, the heterogeneous stock-derived model of unilateral renal agenesis (HSRA) rat, which demonstrates 50%–75% spontaneous incidence of a single kidney. The HSRA model is characterized by reduced nephron number (more than would be expected by loss of one kidney), early kidney/glomerular hypertrophy, and progressive renal injury, which culminates in reduced renal function. Long-term studies of temporal relationships among BP, renal hemodynamics, and renal function demonstrate that spontaneous single-kidney HSRA rats are more likely than uninephrectomized normal littermates to exhibit renal impairment because of the combination of reduced nephron numbers and prolonged exposure to renal compensatory mechanisms (i.e., hyperfiltration). Future studies with this novel animal model may provide additional insight into the genetic contributions to kidney development and agenesis and the factors influencing susceptibility to renal injury in individuals with congenital solitary kidney.


A wide variety of disorders and congenital diseases are associated with incompletely developed or absent kidneys (renal agenesis).1 Unilateral renal agenesis is a relatively common developmental defect in both male and female children that occurs in 1:500–1:1000 births.1–3 It is frequently associated with defects in reproductive organs.4–6 In most cases, children born with one kidney appear to have no overt clinical symptoms and develop normally. However, other urinary tract or multiorgan defects may require intervention.7 The long-term outlook for the development of hypertension, proteinuria, and renal failure as an adult is controversial because of the limited number of human studies, inadequate long-term follow-up, and the large number of non–population-based studies.8 Thus, a better understanding of the risk of cardiovascular and kidney disease in the context of developing with a single kidney is needed.

The utility of an animal model that exhibits congenital solitary kidney could be extremely beneficial to...
help address inherit risk (long-term study) as well as the influence of confounding factors (e.g., hypertension, diabetes) on future development of cardiovascular and kidney disease. Many experimental animal models have investigated the consequences of having a single kidney, but most have used invasive procedures, such as early nephrectomy or renal ablation. These studies suggest that compensatory renal changes (due to reduced nephron number) lead to progressive damage and loss of renal function in the remaining kidney and hypertension over time. Studies involving uninephrectomy in sheep performed in utero have observed compensatory nephrogenesis as well as nephron hypertrophy. These animal models, while providing important insight into physiologic mechanisms of renal compensation, may or may not accurately recapitulate circumstances of individuals who develop and are born with a single kidney.

We recently developed a new model of renal agenesis (the heterogeneous stock derived model of unilateral renal agenesis [HSRA]). The HSRA model originates from the genetically and phenotypically diverse heterogeneous stock (HS) rats, which were initially established at the National Institutes of Health by combining eight inbred strains and maintaining animals in a way that minimize inbreeding. Although most HS rats have two kidneys, a small percentage are born with a single kidney (<1%). Subsequent, brother-sister inbreeding of one-kidney HS rats animals was conducted to establish the HSRA model. The HSRA model consistently exhibits unilateral renal agenesis ranging from 50% to 75% in each generation and is characterized by low nephron number, early kidney hypertrophy, and an inherent susceptibility to develop clinically significant kidney injury and decline in renal function with age. The HSRA also exhibits other urogenital abnormalities, including the absence of ipsilateral ureter, seminal vesicles, or uterus horn and ovaries—features commonly seen in humans with unilateral renal agenesis. Thus, this model provides the unique opportunity to assess the effect of developing with only a single kidney on long-term changes of renal and cardiovascular parameters.

The focus of the present study was to establish detailed baseline cardiovascular and renal parameters in the HSRA model as well as address the hypothesis that early development (in utero) with a solitary kidney increases susceptibility to renal injury and hypertension more than what is observed in either control adult animals (two-kidney littermates) or two-kidney controls subjected to nephrectomy at various ages.

RESULTS

General Characteristics of the HSRA Model
The HSRA model exhibits unilateral renal agenesis, with variable occurrence of other urogenital abnormalities, including the ipsilateral absence of sex organs (e.g., testis, epididymis, and seminal vesicles in males) (Supplemental Figure 1A–C). On average, 57% of offspring (ranging from 50% to 75%) are born with a single kidney (HSRA-S) and 43% are born with two kidneys (HSRA-C) with no obvious urogenital abnormalities (Supplemental Figure 1D). Renal agenesis occurs similarly in both sexes, with absence of kidney occurring more on the right, than on the left side (Supplemental Figure 1D). The occurrence of bilateral renal agenesis is estimated at 12% by evaluation of kidney status in embryos (Supplemental Figure 1E). Reproduction is slightly impaired (67% success rate), probably as the result of urogenital abnormalities, but breeding could be maximized by pairing female HSRA-C with male-S to generate 4–10 offspring (average, 6.5) per breeding (Supplemental Figure 2).

Proteinuria, Kidney Hypertrophy, and Nephron Number in HSRA Model
Animals born with a single kidney are more prone to develop proteinuria compared with two-kidney littermate controls. Proteinuria gradually increased from months 1 to 5 in male HSRA–S rats, with a sharp rise after month 5 compared with two-kidney controls (HSRA–C) (Figure 1A). Female HSRA–S rats demonstrated a slower progression to develop proteinuria, which was not significantly different from control rats until month 10 (Figure 1A).

At birth (day 1), kidneys from HSRA–S animals were modestly larger (19%; $p<0.05$) than those from HSRA–C rats; however, within 1 month there was substantial growth in HSRA–S kidneys (51% larger compared with HSRA–C kidneys; $p<0.05$) (Figure 1B). Glomerular hypertrophy did not significantly differ between HSRA–S and HSRA–C rats at birth (Figure 1C). Additionally, there were no obvious differences in other kidney structures between groups (i.e., nephrons demonstrated a similar gradient in development with mature nephrons located in the juxtamedullary area and immature nephrons toward the outer cortex [data not shown]). By month 1, there was a significant difference in glomerular size, with HSRA–S glomeruli becoming substantially larger compared with HSRA–C glomeruli (Figure 1C). Kidneys from HSRA–S rats contained 19% less nephron versus the comparable individual kidney (left) in HSRA–C rats (Figure 1D). The total nephron endowment in HSRA–S kidneys was 60.2% less than the total number of nephrons from both HSRA–C kidneys.

By month 5, glomeruli isolated from HSRA–S kidneys were more permeable to albumin and had a reduced glomerular reflection coefficient, which correlated with a slight but significant increase in proteinuria exhibited by HSRA–S compared with HSRA–C (Figure 2A). The HSRA–S rats demonstrated a 46% increase in single nephron GFR (snGFR) compared with HSRA–C rats (Figure 2B). Micro–computed tomography (CT) analysis of kidneys from HSRA–S rats demonstrated an altered distribution of small and large microvessels compared with HSRA–C (Figure 2, C and D). The HSRA–S kidneys had fewer small microvessels (diameter, 0–80, 80–120, and 120–200 μm) and higher density of larger microvessels in the 200– to 300-μm and 300– to 500-μm range.

By month 10, HSRA–S animals demonstrated elevated BUN (22 ± 2.7 mg/dl) and a decrease in creatinine clearance.
nephron number in left kidney from each strain is calculated as HSRA-C (25093) compared with kidneys from the HSRA-C rats; left kidney, and both left and right (for HSRA-C). Differences in nephron numbers were similar in female HSRA-S and HSRA-C (data not shown). There was no significant difference between male and females. The percentage change in total nephron is calculated as HSRA-C (51187)/HSRA-S (20373)/HSRA-C (25093)=18.8%. Total nephron endowment is based on sum of both kidneys in HSRA-C compared with single kidney in HSRA-S rats. The percentage change in total nephron was calculated as follows: percentage change in total nephron = [(nephron number in left kidney from each strain is calculated as HSRA-C (25093) compared with kidneys from the HSRA-C rats) - (nephron number in left kidney from each strain is calculated as HSRA-C (25093))] / (nephron number in left kidney from each strain is calculated as HSRA-C (25093)).

Temporal Changes in BP, Proteinuria, and Renal Hemodynamics in the HSRA-S rats Compared with Uninephrectomized Rats

An overview of the experimental design and time course measurements of proteinuria and renal hemodynamic parameters from 3 to 20 months is shown in Figure 4, A and B. Four groups of animals were studied: (1) HSRA-S, (2) HSRA-C, (3) HSRA-UNX3 (uninephrectomy at 3 weeks of age), and (4) HSRA-UNX8 (uninephrectomy at 8 weeks of age). From months 3 to 5, there was no significant difference in proteinuria, other measures of renal function, or mean arterial pressure (MAP) among all four groups (Figure 4, C and D, Table 1). By month 9, proteinuria was significantly elevated in HSRA-S, HSRA-UNX3, and HSRA-UNX8 compared with HSRA-C rats. After month 15, the HSRA-S rats demonstrated a steep increase in the level of proteinuria compared with the previous time points. By month 20, HSRA-S animals exhibited the highest proteinuria (126±15.2 mg/24 hours), followed by HSRA-UNX3 (89±14.8 mg/24 hours), HSRA-UNX8 (92±15.6 mg/24 hours), while proteinuria in HSRA-C (19±5.5 mg/24 hours) rats was essentially unchanged during the entire period of study. The majority of proteinuria in the HSRA-S was composed of albumin, with most other protein within the 10–37 kD range, but by month 20, higher-molecular-mass protein was observed (>75 kD) (Supplemental Figure 3A, A and B). MAP did not significantly differ between groups at months 5 and 15 (Figure 4D). However, by month 20, HSRA-S and uninephrectomized animals (HSRA-UNX3 and HSRA-UNX8) exhibited significantly higher MAP (by approximately 10 mmHg) than did the two-kidney control (HSRA-C). MAP measured on conscious animals (by indwelling catheter) demonstrated a similar significant difference in BP among groups, which was supported by differences in cardiac hypertrophy (Supplemental Figure 3B, Table 1).

Renal hemodynamics were evaluated at months 5 (baseline), 15, and 20 in all four groups (Figure 5). Renal blood flow (RBF), not corrected for differences in kidney weight at months 5 and 15, was increased by 50% in nephrectomized rats (HSRA-UNX3 and HSRA-UNX8) compared with HSRA-C (Figure 5A). HSRA-S rats exhibited elevated RBF as well, but this was not significant compared with values in HSRA-C. In contrast, RBF corrected for differences in kidney weight at months 5, 15, and 20 demonstrated the opposite trend because no difference was seen among any of the groups until months 15–20 (Figure 5B). At this time, RBF was significantly decreased in all groups compared with HSRA-C rats. Overall, HSRA-S and both HSRA-UNX groups demonstrated a gradual reduction in RBF from months 5 to 20 compared with HSRA-C animals. RBF autoregulation was normal (at month 5) in the HSRA-S and -C rats as renal perfusion pressure varied from 50 to 150 mmHg (Supplemental Figure 4A). Renal vascular resistance (RVR) became significantly elevated only in
the HSRA-S by month 15 (compared with HSRA-C), but by month 20, RVR in both HSRA-S and HSRA-UNX groups became significantly elevated versus the control (Supplemental Figure 4B).

Both groups of nephrectomized rats demonstrated the highest GFR at month 5 compared with HSRA-C rats, irrespective of kidney weight (Figure 5, C and D). GFR was also elevated in HSRA-S rats, but only when not corrected for kidney weight and not as high as observed in HSRA-UNX groups. By month 20, HSRA-UNX-3, -UNX-8 and -C rats had similar GFRs (uncorrected), whereas HSRA-S rats demonstrated significantly lower GFR compared with all groups, independent of correction for kidney weight (Figure 5C). GFRs in the HSRA-C rats remained essentially unchanged across all time points. From months 15 to 20, GFR dramatically decreased in the HSRA-S (721 ± 676 ml/min per g kidney weight), which was significantly lower than values in nephrectomized rats and HSRA-C rats (1714 ± 680 ml/min per g kidney weight). GFRs in both nephrectomized groups (HSRA-UNX3 and -UNX8) were significantly higher than those in HSRA-S rats but lower than those in HSRA-C rats (1083 ± 676 ml/min per g kidney weight and 1055 ± 680 ml/min per g kidney weight, respectively). Estimated snGFR from months 5 to 20 was consistent with total GFR as HSRA-S and HSRA-UNX groups exhibited hyperfiltration early, but demonstrated a decline in GFR with age compared with HSRA-C rats (Supplemental Figure 5). Several other metabolic parameters, including elevated cholesterol and triglycerides, were elevated in the HSRA-S compared with the HSRA-C and HSRA-UNX groups (Table 1).

Glomerulosclerosis, Tubulointerstitial Injury, and Inflammation in Kidney from HSRA-S Compared with Uninephrectomy

There was significantly more glomerular injury, including glomerulosclerosis and mesangial expansion, in the HSRA-S than in the HSRA-C and HSRA-UNX groups by month 20 (Figure 6A). The HSRA-UNX groups also demonstrated significantly more glomerular injury compared with the HSRA-C animals. Kidneys from HSRA-S

Figure 2. Glomerular permeability, snGFR, and kidney vasculature structure using micro-CT in HSRA-S and HSRA-C rats at month 5. (A) Glomerular permeability. The reflection coefficient (\( r_{alb} \)) was significantly decreased in HSRA-S (more permeability to albumin) compared with HSRA-C rats, which is set as 1. These experiments were performed with a minimum of 20 glomeruli per rat \((n=80–100\) total glomeruli per group). (B) snGFR. (C) Representative three-dimensional micro-CT reconstruction of the renal vasculature structure. (D) Quantification of the cortical microvascular density and vascular volume fraction of microvessels with diameters from small \((0–80\ \mu m)\) to large \((300–500\ \mu m)\). \(n=5\) per group; *\( P<0.05\) compared with HSRA-C rats.

Figure 3. Measurement of kidney hypertrophy, renal function, and BP in HSRA-S and HSRA-C rats at month 10. (A) Renal function measures (BUN and creatinine clearance) in HSRA-S and HSRA-C. (B) MAP. (C) Left kidney weight. (D) Glomerular area. (E) Representative hematoxylin and eosin glomerular images from HSRA-S and HSRA-C rats at 40X. \(n=6\) per group; *\( P<0.05\) compared with HSRA-C rats.
compared with those from HSRA-UNX3, -UNX8, and -C rats demonstrated increased tubular injury, neutrophil gelatinase-associated lipocalin expression (tubular injury marker), and renal interstitial fibrosis (Supplemental Figure 6A and Figure 6B). Both HSRA-UNX groups demonstrated a similar degree of interstitial fibrosis and tubular injury but increased compared with HSRA-C, which remained relatively constant from months 5 to 20. Tubulointerstitial macrophage infiltration (CD68-positive cells) was significantly increased, by approximately 5-fold, in kidneys from HSRA-S compared with HSRA-C rats and by about 2.5-fold compared with HSRA-UNX groups (Supplemental Figure 6B). Kidneys from HSRA-S also demonstrated a significant increase in T cell and B cell infiltration and proinflammatory factors (TNF-α, TGF-β1, and NFκB) compared with HSRA-C and HSRA-UNX groups (Supplemental Figure 6, C–E).

Transcriptome Analysis of Kidney from HSRA-S Compared with HSRA-C

Whole transcriptome analysis was evaluated at day 1 and month 1 to identify early changes in genes/networks that may be involved in increased susceptibility of HSRA-S to develop kidney injury in the long term. At day 1, transcriptome changes were small and marginally significant (data not shown). However, by month 1, a total of 267 genes ($P<0.02$ and fold-change $\pm 20\%$) were differentially expressed between kidney isolated from HSRA-S and HSRA-C (left kidney) (Supplemental Table 1). Expression differences in these genes were modest ($<2$ up- or downregulated). An unbiased pathway analysis on the entire dataset using Ingenuity Pathway Analysis identified two relevant networks (Supplemental Figure 7) linked to organ injury and reproductive system disease (network 1) and cardiovascular system and tissue development.
Summary of body weight, organ weights, and blood and kidney function parameters in HSRA model versus uninephrectomy model

<table>
<thead>
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<th>Trait</th>
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<th>Month 20</th>
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<td>2.06</td>
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<td>0.03</td>
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<td>10.0</td>
</tr>
<tr>
<td>SCr (mg/dl)</td>
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<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>18.7</td>
<td>15.6</td>
<td>15.4</td>
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Values are expressed as mean ± SEM. BW, body weight; KW, kidney weight; HW, heart weight; DV, drinking volume; UV, urine volume; SCr, serum creatinine; BUN, blood urea nitrogen.

Several epidemiologic/clinical studies have examined the likelihood that being born with a solitary kidney increases risk for hypertension, proteinuria, and renal failure in childhood and adulthood. In particular, some studies have found that up to 40% of people born with a single kidney develop proteinuria and hypertension and 10%–15% advance to renal insufficiency. Despite these studies, the long-term outlook for developing hypertension, proteinuria, and renal failure still remains somewhat controversial. Studies that have evaluated children who have had nephrectomy or adults that served as a kidney donor have found little or no difference in renal function and/or injury compared with control subjects. However, several recent studies have found kidney donors are at an increased risk (up to 8-fold) to develop renal failure. Taken together, these studies suggest that those born with two kidneys and either lose or donate a kidney later in life is not the same as developing with a single kidney. Unfortunately, the ability to directly compare long-term effect of being born with a single kidney versus two kidneys or nephrectomy in the same model has been hampered by lack of relevant models.

In the present study, we addressed the hypothesis that animals born with a single kidney are at increased risk for proteinuria, hypertension, and renal function decline compared with those born with two kidneys or uninephrectomized animals in the same model. The HSRA model offers a distinct advantage to investigate the consequences of having a single kidney as other studies have done so through invasive procedures, such as nephrectomy and renal ablation using two-kidney rats. The major findings of this study are as follows. First, kidneys from HSRA-S rats demonstrate fewer nephrons and early kidney and glomerular hypertrophy between postnatal day 1 and month 1 compared with HSRA-C rats, which demonstrated no renal injury or impaired renal...
The lower nephron number exhibited by HSRA-S animals (compared with HSRA-C rats) suggests that these kidneys exhibit less nephrogenesis. Second, single-nephron GFR was significantly elevated (i.e., hyperfiltration) in kidneys from HSRA-S versus HSRA-C, and glomeruli were more permeable to albumin. Third, kidneys from HSRA-S demonstrate distinct morphologic differences in microvascular density compared with HSRA-C. Forth, HSRA-S rats demonstrated a greater degree of proteinuria, histologic kidney injury (including degree of infiltrating immune cells [macrophages, T cells, and B cells]), and lowest GFR compared with the HSRA-C and nephrectomized groups (HSRA-UNX3 and HSRA-UNX8). Finally, molecular pathways related to kidney development and vasculogenesis were dysregulated in the HSRA-S rats compared with the HSRA-C rats, predominantly between postnatal day 1 and month 1.

The predominant mechanism of kidney injury and rise in BP exhibited by HSRA-S animals appears similar to that in uninephrectomized groups (HSRA-UNX3 and HSRA-UNX8) as well as a multitude of rodent studies involving nephrectomy or renal ablation that support the hyperfiltration hypothesis proposed by Brenner and colleagues. Specifically, HSRA-S and HSRA-UNX animals exhibit compensatory glomerular hypertrophy, increased glomerular capillary pressure, and hyperfiltration and with time develop glomerulosclerosis, proteinuria, and tubular injury that lead to decreased renal function and rise in BP (summarized in Supplemental Figure 8). However, the significant susceptibility of the HSRA-S rats to exhibit renal dysfunction is likely due to the reduced nephron numbers and diminished ability of the kidney to compensate for the absence of the second kidney from development to birth compared with that seen HSRA-UNX animals (which occurs after birth).
other words, HSRA-UNX groups do not demonstrate as significant a reduction in renal function as do HSRA-S later in life because of their greater nephron endowment. This is a plausible explanation as reduced nephron endowment has long been postulated to correlate with risk for hypertension as well as renal disease.\footnote{33}

In contrast to the HSRA model, many studies using other models of congenital solitary kidney (rat\footnote{34} or pig\footnote{35}) have observed compensatory nephrogenesis (i.e., increased nephron number). Similarly, studies in a novel sheep fetal nephrectomy model also demonstrated compensatory nephrogenesis.\footnote{16,36}

The predisposition of the HSRA model to exhibit decreased, as opposed to increased, nephron endowment may be attributed to the specific nature of the underlying genetic cause and the role these genes play in kidney development. For example, some gene-specific knockout models (e.g., GDNF) are born with a single kidney that exhibit a significant decrease in nephron number versus similar wild-type kidneys.\footnote{37} With respect to humans, given the significant variability in nephron endowment\footnote{18} and limited work on nephron composition in individuals with a solitary kidney, it remains to be seen whether increased nephrogenesis is the norm or the exception.

Another possibility that could promote or exacerbate renal injury in the HSRA-S model may result from the microvascular changes observed by micro–CT and reflected in the early increase in RVR before the nephrectomized groups. The significant decrease in RBF, combined with significantly fewer small microvessels (0–80 \( \mu \)M), could lead to reduced intrarenal blood flow and hypoxia and promote tubular injury. This is consistent with several studies that suggest reduced blood flow/hypoxia in the kidney stimulates production of collagen I and \( \alpha \)-smooth muscle actin (indicator of fibrosis) \textit{via} epithelial mesenchymal transition, as well as leads to an increase infiltration and maturation of immune cells.\footnote{39} In summary, while the mechanism of injury between the HSRA-S and nephrectomized models appears similar with respect to glomerular injury (hyperfiltration), the reduced nephron composition and vascular changes seen in the HSRA-S rats in the short term (earlier than month 5) have a minimal effect on kidney injury. However, a prolonged state of hyperfiltration, in combination with reduced nephrons,

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Measurement of glomerular and tubulointerstitial injury and representative histologic images. (A) Glomerular injury was scored on a scale from 0 (normal) to 4 (global sclerosis) for each group (HSRA-S, HSRA-C, HSRA-UNX3, and HSRA-UNX8 rats) at month 20. A minimum of 20 randomly selected glomeruli at 40X were scored for each hematoxylin and eosin stained kidney section. (B) Tubulointerstitial fibrosis in kidney cortex in each group (HSRA-S, HSRA-C, HSRA-UNX3, and HSRA-UNX8) at months 5, 15, and 20 was evaluated by Masson’s trichrome at 10X. (C) Tubulointerstitial fibrosis in kidney medulla. For tubulointerstitial injury (fibrosis), a minimum of 20 random images at 10X per kidney section were studied, \( n=6-10 \) animals per group/time. The specific numbers of animals used for each experiment are shown below the x axis. *\( P<0.05 \) compared with HSRA-C; †\( P<0.05 \) for HSRA-S compared with HSRA-UNX3 and HSRA-UNX8 rats.}
\end{figure}
ultimately leads to more accelerated decline in renal function over and above that seen in UNX models.

The study was designed to account for potential age-related (i.e., children versus adult) renal changes associated with loss of a kidney because nephrectomy was performed in young, sexually immature animals during the period when the kidney is still developing (HSRA-UNX3) and after sexual maturity (HSRA-UNX8). However, there was essentially no difference in proteinuria, hemodynamic variables (RBF, GFR, and RVR), or BP in the HSRA-UNX3 compared with HSRA-UNX8 rats. HSRA-UNX8 animals did demonstrate significantly elevated proteinuria over most of the period (months 5–15), but proteinuria increased sharply in the HSRA-UNX3 rats at month 15. There was also a slight increase in immune cell infiltration (T cell and B cell) as well as proinflammatory factors in kidneys from HSRA-UNX3 compared with HSRA-UNX8 rats. This trend suggests that in the long term (after month 20), HSRA-UNX3 animals may ultimately demonstrate significantly more renal injury and decline in renal function than do HSRA-UNX8 rats. Unfortunately, clinical data comparing children and adults who have undergone nephrectomy are sparse because children seldom serve as living kidney donors and nephrectomy is usually performed for other disease conditions (e.g., cancer). 

The transcriptome analysis demonstrated significant alterations in genes/pathways involved in kidney development and vasculogenesis, but only at month 1 as opposed to postnatal day 1. This suggests that the gene expression changes that occur between day 1 and month 1 are likely the result of compensatory changes and/or the combination of compensatory and developmental changes, rather than purely developmental in origin (i.e., linked to underlying genetic cause). At least two genes are worthy of discussion: Hoxb5 and Smoc2 (based on fold-change and significance level). First, homeobox (Hox) genes encode a family of evolutionarily highly conserved transcription factors that are important in determining the basic structure and orientation of an organism. These genes are expressed in embryonic kidney, but the specific role in kidney development is not known. HoxB5 binds to the Hox consensus sequence upstream of transcription start of Ret gene (glial cell line–derived neurotrophic factor [GDNF]-RET pathway) and that deletion of the binding sequence of Hoxb5 abolishes its trans-activation of RET gene in studies related to enteric nervous system.

Therefore, in the context of kidney development, expression differences in Hoxb5 and regulation of Ret (by Hoxb5) could lead to dysregulation of GDNF-RET pathway and alter kidney development. Second, Smoc2 (SPARC-related modular calcium binding 2) proteins are thought to influence growth factor signaling, migration, proliferation, and angiogenesis. Recently, the expression and regulation of Smoc2 were investigated in gonad/mesonephros complexes. Smoc2 expression is associated with Hedgehog signaling, which plays a role regulating branching morphogenesis and is responsible for maintenance and proliferation of metanephric mesenchyme stem cells in the developing kidney. Several mouse models exhibit various degrees of renal agenesis, including the Danforth, urogenital syndrome, limb deformity, and FUBI (failure of ureteric bud invasion) mouse, and those induced by gene manipulation (e.g., knockout of Wt1, Gdnf, and Ret). Abnormalities of the urogenital system and/or skeletal system are common in these models, and the incidence of uni- or bilateral agenesis varies;
however, on average the incidence is far below the 50%–75% observed in the HSRA model. There are two reported models of uni- or bilateral agenesis in the rat: the ACI (5%–15%) and UUA rat (<50%).34,56 The ACI rat is of particular interest because it is one of the progenitor strains used to derive the National Institutes of Health HS rat from which the HSRA model originated. Thus, it is likely that the HSRA and ACI rats share a common genetic cause that leads to renal agenesis, while there are probably other genetic factors in the HSRA (modifier genes) that explain the large disparity in the penetrance of unilateral agenesis observed between the two strains (75% versus 15%, respectively). Further studies will be required to elucidate the exact genetic cause that leads to the solitary kidney in the HSRA model.

The HSRA model has some limitations, such as occurrence of the solitary kidney in the context of other urogenital abnormalities that may also contribute to reduced renal function (compared with nephrectomy). However, there are several important points regarding future uses of the HSRA model that are beyond the scope of the current study but important to consider, including the following: (1) the identification of the genetic factors causative to kidney agenesis, which may lead to important insights into common causes of renal agenesis in humans; (2) the identification of genetic factors that modify the penetrance of renal agenesis, which could identify new genes that play a role in kidney development; (3) how confounding factors, such as predisposition to hypertension and/or diabetes in the presence of a congenital single kidney, affect long-term risk for impaired kidney function; and (4) the ability to test dosing and timing of therapies that could improve or otherwise affect hyperfiltration associated with CKD.

In summary, future genetic, embryologic, and physiologic studies with the HSRA model will likely help improve understanding of the genetic basis of nephrogenesis/kidney development, as well as confounding factors (hypertension and diabetes) that may affect or modulate susceptibility toward kidney injury in patients with a single kidney.

**CONCISE METHODS**

**Animals and Study Design**

All experiments procedures were approved by the Institutional Animal Care and Use Committee of University of Mississippi Medical Center (UMMC). The HSRA strain is maintained at UMMC. The HSRA strain was developed from a single breeding pair of HS rats whose offspring demonstrated a high degree (>60%) of unilateral renal agenesis.18 Brother-sister mating was initiated to establish an inbred strain that demonstrates consistent unilateral renal agenesis in 50%–75% of offspring (calculated on the basis of those that are born and survive to the point when assessed for kidney status). At 6 weeks of age, kidney status is determined by palpitation (previously validated by ultrasonography) and confirmed in experimental animals after euthanasia. Offspring that exhibit a single kidney are denoted as HSRA-S and those born with two kidneys as HSRA-C. All studies were performed using HSRA-S animals that exhibited renal agenesis of the right kidney (i.e., left kidney present). Animals used for this study were caged under controlled temperature, humidity, and 12-hour light/12-hour dark conditions.

**Protocol 1: Kidney-Specific Phenotypes and Other Urogenital Abnormalities**

At 4 weeks of age, groups of sibling-matched HSRA-S and HSRA-C animals (n=6 per group) for each sex were weaned onto a low-salt diet (0.3% NaCl; TD7034; Harlan Teklad, Madison, WI). Twenty-four-hour urine collections were performed to determine proteinuria, as done previously.57,58 At 10 months, MAP was measured by indwelling catheter, and animals were euthanized for collection of serum, organ weights, and histologic analysis.57,58 Careful animal dissections were performed to investigate the nature and type of other urogenital abnormalities that occurred along with unilateral renal agenesis. Animals from three generations (n=487 animals) were analyzed to determine the frequency of unilateral renal agenesis in both female and male animals. Mated females were euthanized (n=29), and embryos (n=130, E15.5) were carefully dissected to determine all possible kidney statuses (bilateral, unilateral, or two-kidney).

**Protocol 2: Nephron Number, Glomerular Permeability, and snGFR**

For nephron number, at 4 weeks of age (1 month), HSRA-S and HSRA-C rats were euthanized (n=5 per group) and kidneys removed and weighted. Whole kidneys were finely diced and incubated with 6 M HCl in a 37°C water bath for 2 hours and processed as described previously.57 The reflection coefficient of albumin (erb) was determined in isolated glomeruli from HSRA-S and HSRA-C at month 5 using a fluorescent-based method on freshly isolated glomeruli at month 5.57 The snGFRs were measured (in anesthetized animals) at month 5 by infusion of isotonic saline containing 1 g/100 ml BSA and 50 μg/ml 3H-inulin (PerkinElmer) at a rate of 2–3 ml/hr. Timed collections of fluid from random proximal or late-proximal tubule surface segments were performed, each lasting 2–4 minutes, with use of glass micropipettes sharpened to a diameter of 8 μm. Blood and tubular fluid samples were counted using LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter). The snGFRs (ml/min) were calculated as ratio of tubular fluid/plasma 3H counts × tubular flow rate.

**Protocol 3: Micro-CT of Kidney Vasculature**

At 4 weeks of age, groups of sibling-matched HSRA-S and HSRA-C animals (n=5 per group) were weaned and aged to month 5. Animals were anesthetized under 2%–3% isoflurane/O2, and, under physiologic pressure (1–2 ml/min), the left kidney was perfused with approximately 10 ml of saline (0.9% NaCl), followed by perfusion with a contrast agent (radio-opaque silicone polymer containing lead chromate, Microfil MV122; Flow Tech, Inc., Carver, MA). The polymer-filled kidneys were left at 4°C overnight and then immersed in 10% buffered formalin for 72 hours before scanning. The kidneys were scanned at 0.3-degree increments using a micro-CT scanner (SkyScan 1076 system; Micro Photonics Inc.), and renal microvessels were analyzed using the Analyze (Biomedical Imaging
Resource, Mayo Clinic, Rochester, MN) software package as described previously.\textsuperscript{59}

Protocol 4: Autoregulation of RBF
At 4 weeks of age, groups of sibling-matched HSRA-S and HSRA-C animals (n=5–6 per group) were weaned and aged to month 5. At month 5, MAP and RBF were measured as previously described.\textsuperscript{57,58}

Protocol 5: Temporal Study of Renal Injury in HSRA-S, HSRA-C, and Uninephrectomized HSRA-C Rats
Four groups of male animals were established to study the time course development of proteinuria: (1) HSRA-S, (2) HSRA-C, (3) HSRA-UNX3 (uninephrectomized at 3 weeks of age), and (4) HSRA-UNX8 (uninephrectomized at 8 weeks of age). Uninephrectomy was performed using two-kidney (HSRA-C) animals, and sham operations were performed on littermate HSRA-S animals. Animals were anesthetized using two-kidney (HSRA-C) animals, and sham operations (uninephrectomized at 8 weeks of age). Uninephrectomy was performed using two-kidney (HSRA-C) animals, and sham operations were performed on littermate HSRA-S animals. Animals were anesthetized with 2%–3% isoflurane/O\(_2\) on a heating pad at 37°C. Under asceptic conditions, an incision on the right flank was made and the right kidney was gently lifted. A single ligature was placed around the renal vessels and ureter and tied tightly. The distal portions of the renal vessels and ureter were then cut, and the kidney was removed. The incision was closed by continuous subcutaneous stitch, and additional closure of the skin was done using independent sutures. The animals was subcutaneously given Baytril (10 mg/kg) to prevent infection and long-acting analgesic Rimadyl (5 mg/kg) to control operative pain.

All 4 groups of age-matched HSRA animals were studied for proteinuria at months 3, 5, 7, 9, 11, 13, 15, 17, and 20. Rats were kept in metabolic cages for 24 hours with free access to water, and proteinuria was determined as previously described.\textsuperscript{57,58} Upon euthanasia, kidney and heart samples were processed for histologic examination, and serum samples were obtained from cardiac puncture to measure blood parameters as done previously.\textsuperscript{57,58}

Protocol 6: BP and Renal Hemodynamic Parameters
At 5, 15, and 20 months of age (n=6–13 rats per group/time), MAP, RBF, and FITC-inulin GFR were measured as previously described.\textsuperscript{57,58} At the end of each experiment, the kidneys were removed and weighed, along with the heart. Tissues were processed for histologic examination. The concentration of FITC-inulin was determined in Fluorescent Bio-Tek plate reader, and GFR was calculated.

Protocol 7: Whole Transcriptome Analysis and Western Blot Analysis
At postnatal day 1 and 1 month of age, groups of sibling-matched HSRA-S and HSRA-C animals (n=4 per group) were euthanized and kidneys isolated. RNA was isolated using TRIzol and Invitrogen PureLink Kit (Life Technologies) and was evaluated for quality and integrity (Experion System; Bio-Rad) as described previously.\textsuperscript{58} Whole genome transcriptome analysis was performed using Affymetrix GeneAtlas platform as previously described through the UMMC Institutional Molecular and Genomics.\textsuperscript{58} Data obtained from these gene expression studies are deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with the GEO accession number GSE62092. Microarray analysis was performed using software provided by Affymetrix and Array Star 5 (DNASTAR Inc.). A P value<0.02 and a difference >±1.2-fold were set as the statistical threshold and fold-change, respectively. No genes demonstrated differential expression using the more stringent Benjamini and Hochberg false-discovery rate. Gene networks and functional analysis were evaluated through the use of Ingenuity Pathways Analysis (Ingenuity Systems). Gene expression differences were confirmed using SYBR-green dye chemistry on Bio-Rad CFX96 (n=4–5 per group) on RNA isolated from kidney on day 1, month 1, and month 20. RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit, and real-time PCR (Bio-Rad) was performed using SsoFast EvaGreen Supermix (Bio-Rad). Western blot analysis was performed using antibodies for tubular injury (neutrophil gelatinase-associated lipocalin, α-actin) and proinflammatory factors (TNF-α, TGF-β1, IL-6) purchased from Santa Cruz Biotechnology as previously described.\textsuperscript{58}

Histologic Analysis
Tissue was fixed in 10% buffered formalin and embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin and Masson’s trichrome. Two central longitudinal sections from each kidney were examined under light microscopy. Glomerular injury was assessed using a semi-quantitative scoring as previously described.\textsuperscript{57,58} Tubulointerstitial injury (fibrosis) by thresholding and tubular injury using semi-quantitative scoring was assessed as previously described.\textsuperscript{60} Macrophage, T-cell, and B-cell infiltration was assessed by immunohistochemistry on unstained sections using primary antibodies directed at CD-68/ED-1, CD-22, and CD43 (Santa Cruz Biotechnology) and detected by DAB (Ultravision LPValue Detection System; Thermo Fisher Scientific).\textsuperscript{58} Slides were counterstained with methyl green (n=4 section per group; 15–20 images). Images were captured using Nikon 55i microscope with DS-Fi1 5-Meg Color C digital camera (Nikon, Melville, NY) and analyzed using Nis-Elements image analysis software (version 3.03; Nikon Instruments Inc., Melville, NY).

Statistical Analyses
HSRA-S and HSRA-C phenotypic data (e.g., nephron numbers, glomerular area) were analyzed by t test. For comparison of four groups of HSRA animals (HSRA-S, HSRA-C, HSRA-UNX3, and HSRA-UNX8) experimental data (e.g., proteinuria, BP) were evaluated by one-way or two-way ANOVA using the Dunnett or Bonferroni test. A P value<0.05 was considered to indicate statistically significant differences. All data are presented as mean±SEM (GraphPad Prism 6, La Jolla, CA).

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DISCLOSURES

None.

REFERENCES


