Osmoregulatory defect in adult mice associated with deficient prenatal expression of six2

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Somponpun SJ, Wong B, Hynd TE, Fogelgren B, Lozanoff S. Osmoregulatory defect in adult mice associated with deficient prenatal expression of six2. Am J Physiol Regul Integr Comp Physiol 301: R682–R689, 2011. First published June 8, 2011; doi:10.1152/ajpregu.00187.2011.—Suboptimal kidney development resulting from a genetic deficit in nephron number can have lifelong consequences that may lead to cardiorenal complications upon exposure to secondary insults in later life. To determine whether the inherited reduced renal reserve compromises the ability to handle osmotic stress in the adult animal, we challenged the heterozygous 3H1 Brachyrhynh (Br/+) mouse, which displays heritable renal hypoplasia associated with reduced embryonic six2 expression, to a solution of 2% NaCl for 5 days or to fluid restriction for 48 h. Blood chemistry, fluid intake, and physiological parameters, including renal measurements, were determined. Systemic hypertonicity by prolonged salt loading led to significant increases in plasma osmolality and plasma Na⁺, along with polydipsia and polyuria, with a significant urine-concentrating defect that was resistant to DDAVP treatment in the adult Br/+ mouse compared with wild-type littermates. The Br/+ mouse also developed a significant increase in blood urea nitrogen at baseline that was further elevated when 2% NaCl was given. Fluid restriction for 48 h further enhanced plasma osmolality and plasma Na⁺ responses, although the Br/+ mouse was evidently able to produce a small amount of concentrated urine at this time. Hypothalamic c-Fos expression was appropriately activated in the Br/+ mouse in response to both osmotic challenges, indicating an intact central neuroendocrine pathway that was not affected by the lack of congenital six2 expression. Collectively, our results demonstrate impaired osmoregulatory mechanisms consistent with chronic renal failure in the Br/+ mouse and indicate that six2 haploinsufficiency has a direct effect on postnatal fluid and electrolyte handling associated with fluid imbalance.

nephrogenesis; glomeruli; nephron endowment; renal hypoplasia; vasopressin

THE KIDNEY IS A VITAL ORGAN THAT SERVES AS A HOMEOSTATIC REGULATOR OF FLUIDS AND ELECTROLYTES. THIS REGULATION IS ACHIEVED PRIMARILY BY THE NEPHRON, THE FUNCTIONAL SUBUNIT OF THE KIDNEY. CHRONIC RENAL FAILURE (CRF), OR THE PROGRESSIVE LOSS OF KIDNEY FUNCTION, OCCURS WHEN THE KIDNEY NO LONGER MAINTAINS A CRITICAL NUMBER OF FUNCTIONAL NEPHRONS. CRF MAY ARISE DURING THE COURSE OF NUMEROUS DISEASES, BUT IT MIGHT ALSO OCCUR AS A RESULT OF IN SUFFICIENT NEPHRON NUMBER AT BIRTH (1, 4, 21). WHILE KIDNEY DISEASE AFFECTS POPULATIONS WORLDWIDE, ITS INCIDENCE IS DISPROPORTIONATELY HIGH AMONG CERTAIN POPULATIONS, SUCH AS AFRICAN-AMERICANS (14) AND PACIFIC ISLANDERS, FOR REASONS THAT ARE NOT ENTIRELY CLEAR BUT MAY BE RELATED TO REDUCED RENAL RESERVE ASSOCIATED WITH SUBOPTIMAL NEPHRON DIFFERENTIATION DURING DEVELOPMENT.

Several morphogenetic factors interact specifically during the critical period of kidney organogenesis to determine and give rise to final nephron formation (for review see Refs. 2, 3, 22). In an effort to understand how the kidney develops nephrons and achieves a critical number that adequately regulates and maintains fluid balance in the organism, we are investigating the role of an embryonic transcription factor, sine oculis 2 (six2), in the establishment of nephron number. Six2 belongs to the homeodomain-containing transcription factors of the Six family that are expressed and function to regulate a variety of developmental processes, principally during embryonic induction (9). Its expression pattern in embryonic tissues includes the frontal region of the head mesenchyme, the cells in the hindbrain, and the developing pituitary arising from Rathke’s pouch (13). In the developing kidney, six2 expression is restricted primarily to the mesenchymal populations in the nephrogenic zone, where loss of six2 expression is known to reduce the number of progenitor cells (17).

Previous work in our laboratory established a colony of adult heterozygous 3H1 Brachyrhynh (Br/+) mice that display heritable renal hypoplasia (RH) associated with diminished expression of six2 during renal development as a result of a radiation-induced mutation (5, 11). The homozygous Br/Br mutants die neonatally because of the severity of their genetic defect, but the heterozygous Br/+ mutants survive into adulthood. As a consequence, the Br/+ mice demonstrate reduced nephron number and develop CRF and a hypertensive phenotype compared with their wild-type littermates (6). Because RH is inherited in a haploinsufficient manner in the adult Br/+ mouse, this animal is uniquely valuable for identification of genes that are associated with this renal disease, as well as for complications arising from CRF. Interestingly, while the Br/+ mice survive into adulthood and inherit renal insufficiency with reduced nephron number, no obvious abnormalities were reported in the six2-heterozygous transgenic mice, and the six2-nullizygous mice are embryonic-lethal (17). This suggests that additional factors interact specifically with six2 to contribute to reduced nephron number but does not eliminate renal formation altogether. Thus we have a valuable model of adult RH of embryonic origin that can be used to address a number of important biological questions, including the role of suboptimal kidney development in long-term blood pressure regulation. Additionally, this strain is particularly useful, since it can be subjected to experimental alterations to determine the consequence of reduced nephron number without surgical intervention, such as those associated with nephrectomy models, thus reducing variables inherent to surgery.

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In the current study, we characterize and investigate the functional role of reduced embryonic six2 expression in adult renal function and body fluid homeostasis. We demonstrate a significant loss of fluid homeostatic control in the adult Brl/+ mouse exposed to prolonged high-salt loading or fluid restriction. We also determined whether the defect in osmoregulation inherent to the Brl/+ mouse arises exclusively from renal inadequacies associated with underexpression of six2 or whether a neural component is also involved. The results demonstrate that brain osmoregulatory mechanisms are not affected by the lack of six2 expression and suggest that the impaired fluid-regulating ability observed in the adult Brl/+ mouse lies in the structural abnormality associated with reduced nephron number as a consequence of diminished nephrogenesis.

MATERIALS AND METHODS

Animals

Adult male and female mice (20–30 g body wt, 12–16 wk old) of 3H1 background and Brl/+ were allowed to acclimatize and maintained on a 12:12-h light-dark cycle, with lights on at 6 AM and ambient temperature at 20–23°C for 5 days prior to experimentation. All female mice were examined for vaginal plugs. The date when the plugs were first identified is designated embryonic day 0.5 (E0.5). At the appropriate embryonic stage, the gestational female was anesthetized with isoflurane inhalant, cervical dislocation was performed, and the embryos were removed. All animals were used in compliance with federal, state, and local laws and institutional regulations. The experimental protocol was approved by the University of Hawaii Institutional Animal Care and Use Committee. Adequate measures were taken to minimize pain and discomfort in the animals throughout the study.

Kidney Dissection, mRNA Collection, and Quantitative RT-PCR for six2

At embryonic days 13.5 and 17.5 (E13.5 and E17.5), the uterus was removed and placed in 1 X PBS. Under an Olympus SZ-CTV dissecting microscope, each embryo was dissected from the uterus and placed in 1X PBS in its own 35-mm tissue culture dish. Microforceps were used to remove the viscera of the abdomen, with care taken not to damage the posterior abdominal wall. Once the nephric duct was identified, it was resected laterally to expose the kidney, gonad, and adrenal gland. With the microforceps, the kidney was gently loosened to damage the posterior abdominal wall and the embryos were removed. All animals were used in compliance with federal, state, and local laws and institutional regulations. The experimental protocol was approved by the University of Hawaii Institutional Animal Care and Use Committee. Adequate measures were taken to minimize pain and discomfort in the animals throughout the study.

Water loading and DDAVP experiment. Adult 3H1 wild-type and Brl/+ mice were loaded with sterile irrigation water at 5% of body weight intraperitoneally and concomitantly injected with vehicle (100 μl of 0.9% saline sc) or DDAVP (100 μl of 1.0 μg/kg sc). Spot urine samples were collected at 0, 6, and 24 h following water loading for subsequent urinalyses.

Hypothalamic c-Fos Expression

The expression of brain c-Fos was determined in the hypothalamic supraoptic nuclei (SON) and in the forebrain circumventricular organ neurons. Experiments were performed as previously described (18). Briefly, at the end of salt-loading and fluid deprivation experiments, mice were anesthetized with pentobarbital sodium and perfused transcardially with physiological saline and then with phosphate-buffered 4% paraformaldehyde, pH 6.7 (Sigma). After fixation, brains and kidneys were removed, and cryostat sections (30 μm thick) were obtained in a one-in-four series and stored in cryoprotectant solution until processing. For brain tissue, a single set of sections was used to localize Fos immunoreactivity in SON nuclei. Quantification of Fos expression in the forebrain nuclei surrounding the anterior third ventricle included cells of the organum vasculosum of the lamina terminalis (OVLT) and the subformical organ (SFO) nuclei. Cryoprotectant was removed from freely floating tissue sections with multiple rinses of K+ -containing PBS (0.05 M KBPS, pH 7.4). The tissues were subsequently incubated with a rabbit polyclonal antiserum raised against amino acids 4–17 of human c-Fos (Ab5, Calbiochem) at a 1:35,000 dilution made up in KPBS with 0.4% Triton X-100 for 60 min at room temperature and then for 72 h at 4°C. After multiple rinses in KBPS, the sections were incubated with a biotinylated donkey anti-rabbit serum (Jackson ImmunoResearch) at a 1:600 dilution in KPBS with 0.4% Triton X-100 for 1 h at room temperature. After several more rinses, the sections were incubated in avidin-biotin complex solution for 1 h at room temperature. Primary antibody was
localized using a conventional immunoperoxidase method with a 15-min exposure to nickel sulfate plus diaminobenzidine HCl in sodium acetate solution in the presence of H2O2. This yielded a blue-black reaction product in the nuclear compartment. Sections were mounted on poly-L-lysine-treated slides and air-dried overnight, and coverslips were applied. Cells that expressed nuclear blue-black immunoprecipitants after the diaminobenzidine-nickel reaction were considered positive. c-Fos expression was quantified in each nucleus by counting the number of Fos-positive nuclei in one or more sections from each animal. Only intensely stained nuclei were counted to avoid interference with background staining, and the same threshold was applied to count all sections of Fos immunoreactivity. For each variable, the mean value from multiple measurements from each animal was calculated, and these values were used to calculate group means.

Statistical Analysis

Student’s t-test (or rank sum test), one-way ANOVA (or Kruskal-Wallis analysis on rank), and two-way ANOVA with repeated measures were used as appropriate to determine the statistical significance between groups. The α-value was set at P < 0.05. Results are expressed as group means ± SE.

RESULTS

Haploinsufficient Expression of Renal six2 Is Nonlinear and Contributes to a Large Degree of Variation in Nephron Number in the Adult Br Mouse

We previously reported a haploinsufficient expression of six2 in the E13.5 Br kidneys compared with the wild-type littermates (5). Given the large variation in the glomerular number seen in the colony of adult Br/+ mice, we reevaluated and further examined the expression of six2 at an additional stage (E17.5) from the Br kidneys and standardized it to the level expressed in E13.5 kidneys. As shown in Fig. 1, six2 expression in the wild-type embryo decreased as development proceeded and became undetectable by postnatal day 2. Similarly, the expression of six2 in Br/+ kidneys decreased over the course of development and was gone by postnatal day 2. While comparison of relative expression of six2 transcripts demonstrates a haploinsufficient expression (1.0:0.5:0.0) for Br at E13.5 as initially reported, this relationship was not maintained in E17.5 kidneys. Instead, the expression of six2 in the Br mouse declined at a more rapid pace in E17.5 than E13.5 kidneys. This demonstrates that although expression of six2 is haploinsufficient initially, the decrease in six2 expression over the course of development in the Br kidney is not linear. Thus we do not necessarily expect a 50% decrease in the glomerular number in the Br/+ mouse postnatally.

Consequences of Reduced Embryonic six2 Expression and Impaired Nephrogenesis: Abnormal Handling of Fluid and Electrolyte in Postnatal Life

To demonstrate the functional consequence of suboptimal metanephric development associated with reduced embryonic six2 expression on body fluid regulation, we challenged adult heterozygous Br/+ mice with 2% NaCl solution as a source of their fluid intake for 5 days or total fluid deprivation for 48 h with ad libitum food intake. While 3H1 wild-type animals were able to maintain their Posmol and PNa against the rising concentration of salt intake associated with 5 days of 2% NaCl loading, their Posmol and PNa were significantly increased after 48 h of fluid deprivation. In the Br/+ animal, Posmol was already elevated while PNa remained relatively unchanged at baseline. In contrast, a significant increase in Posmol and PNa developed in the Br/+ mouse in response to 5 days of 2% NaCl and further increased with 48 h of fluid deprivation. The increases in response to fluid challenges were significantly higher in the Br/+ than the wild-type mice (Table 1).

BUN was determined in a separate experiment to assess kidney function in these animals. In the Br/+ mouse, the significantly elevated BUN at baseline may contribute to the increase in basal Posmol. The increase in BUN was further enhanced when the animals were exposed to 2% NaCl loading for 72 h. In contrast, no change in BUN was observed in the 3H1 wild-type animals in response to 2% NaCl intake. Additionally, while the elevated BUN in the Br/+ mouse slightly decreased after the animals had been replete with water for an additional 2 days after NaCl treatment, BUN never returned to the resting baseline value (Fig. 2). Collectively, the results indicate inadequate osmoregulation and decreased renal function in the adult Br/+ mouse as a result of reduced expression of embryonic six2.

Impaired Urine-Concentrating Ability, Polyuria, and Polydipsia in the Adult Br/+ Mouse Indicative of Chronic Renal Failure

We used the mouse metabolic isolator unit to monitor and record fluid intake and body weight change and performed 24-h urine collection over the course of 5 days of high-salt challenge, as well as during 48 h of fluid deprivation. In response to prolonged salt loading, the Br/+ mouse consumed significantly more 2% NaCl over a 24-h period than the wild-type mouse. Br/+ animals were already consuming more tap water at baseline, suggesting a heightened diuresis response and a partial nephrogenic diabetes insipidus phenotype in the Br/+ colony. The higher consumption of 2% NaCl...
solution may have accounted for the higher $P_{\text{Na}}$ in the $Br^+/+$ than the wild-type mice. The average amount of fluid intake remained unchanged in the 3H1 wild-type animal when challenged with 2% NaCl (Table 1).

The higher fluid intake observed in the $Br^+/+$ mouse is associated with a corresponding output in dilute urine, suggesting that the $Br^+/+$ animals had a reduced urine-concentrating capacity when challenged with systemic hypertonicity (Table 1). Furthermore, to directly test their urine-concentrating ability, animals of both genotypes were water-loaded to suppress the endogenous vasopressin (AVP) production and concurrently given a supraphysiological dose of the vasopressin V2 receptor (V2R) agonist DDAVP. In response to exogenous DDAVP, the wild-type animals increased their $U_{\text{osmol}}$ from 1,500 ± 146 to 2,747 ± 119 mosmol/kgH$_2$O ($P < 0.05$). In contrast, $Br^+/+$ mice only partially increased their $U_{\text{osmol}}$ in response to DDAVP (from 1,422 ± 126 to 1,686 ± 201 mosmol/kgH$_2$O), consistent with partial nephrogenic diabetes insipidus. $U_{\text{osmol}}$ in all animals returned to baseline values after 24 h. Thus, while wild-type animals fully increased urine concentration in response to exogenous DDAVP, $Br^+/+$ mice modestly responded to the treatment and were only able to partially concentrate their urine, suggesting a reduced renal concentrating ability in these animals (Fig. 3). Overall, the results indicate an impaired urine-concentrating ability that is resistant to DDAVP treatment in the $Br^+/+$ animals, consistent with the physiological feature prominent in CRF.

In response to fluid restriction for 48 h, animals of both genotypes were able to produce a concentrated urine, but while the 3H1 wild-type animals were relatively resistant to systemic hyperosmolality, the $Br^+/+$ mice were unable to protect themselves against plasma hypertonicity associated with fluid restriction (Table 1).

Finally, 3H1 wild-type and $Br^+/+$ mice displayed a significant body weight loss when fluid-deprived for 48 h. Both groups of animals also lost weight in response to 5 days of hypertonic saline consumption, although the degree of weight loss observed in the $Br^+/+$ animal was markedly steep and occurred more rapidly than in the wild-type controls. These changes in body weight agree well with the degree of hyperosmolality, the plasma hypertonicity and reduction in body weight water load suppression. In contrast, $Br^+/+$ mice responded modestly to DDAVP treatment and were only able to partially concentrate their urine. Results indicate an impaired urine-concentrating ability that is resistant to DDAVP treatment in the $Br^+/+$ animals, consistent with partial diabetes insipidus. $P < 0.05$. 

### Table 1. Physiological parameters of wild-type and $Br^+/+$ mice exposed to 5 days of 2% NaCl ingestion or 48 h of fluid deprivation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-Type</th>
<th>$Br^+/+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{\text{osmol}}$, mosmol/kgH$_2$O</td>
<td>325.4 ± 3.27</td>
<td>344.8 ± 2.47</td>
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<tr>
<td>$P_{\text{Na}}$, meq/l</td>
<td>131.9 ± 1.48</td>
<td>134.2 ± 1.24</td>
</tr>
<tr>
<td>$U_{\text{osmol}}$, mosmol/kgH$_2$O</td>
<td>1,461 ± 105.70</td>
<td>1,415 ± 71.61</td>
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<tr>
<td>Body weight, g</td>
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<td></td>
</tr>
<tr>
<td>Initial</td>
<td>33.96 ± 2.29</td>
<td>25.19 ± 1.22</td>
</tr>
<tr>
<td>Final</td>
<td>33.45 ± 2.08</td>
<td>25.53 ± 1.13</td>
</tr>
<tr>
<td>%Body weight loss</td>
<td>1.50</td>
<td>+0.34</td>
</tr>
<tr>
<td>Fluid intake, g</td>
<td>0.93 ± 0.03</td>
<td>1.58 ± 0.09</td>
</tr>
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Values are means ± SE. 3H1 (wild-type) and heterozygous Brachyrrhine ($Br^+/+$) mice were subjected to 5 days of 2% NaCl (S) or 48 h of fluid deprivation (DH). Fluid intake and body weight change were measured and urine was collected from animals in the mouse metabolic isolator unit. Plasma osmolality and Na$^+$ ($P_{\text{osmol}}$ and $P_{\text{Na}}$) were determined from blood drawn upon termination of the experiment. Fluid intake was calculated per 10 g of body weight average over 24 h. $U_{\text{osmol}}$, urine osmolality; NS, untreated control; NA, not applicable. *$P < 0.05$ vs. wild-type (NS, S, DH). $^{a}$P < 0.01 vs. wild-type (NS, S, DH) and $Br^+/+$ (NS). $^{b}$P < 0.01 vs. wild-type (NS) and $Br^+/+$ (NS). $^{c}$P < 0.001 vs. wild-type (NS) and $Br^+/+$ (NS). $^{d}$P < 0.001 vs. wild-type (NS, S, DH) and $Br^+/+$ (NS). $^{e}$P < 0.05 vs. wild-type (S) and $Br^+/+$ (NS, S, DH). $^{f}$P < 0.05 vs. wild-type (NS, S, DH). $^{g}$P < 0.001 vs. wild-type (NS, S, DH). $^{h}$P < 0.001 vs. wild-type (NS, S, DH). $^{i}$P < 0.05 vs. wild-type (S, DH). $^{j}$P < 0.05 vs. wild-type (NS, S, DH).
tonic difference between the two groups of animals when challenged with high-salt consumption (Table 1).

**Morphological Defect in the Adult Br/+ Kidney Is Exaggerated With Salt Loading**

The adult Br/+ mouse exhibits renal hypoplasia, characterized by a smaller volume and fewer numbers of nephrons, with compensatory sclerosis and hypertrophy in the remaining glomeruli. Furthermore, treatment with hypertonic saline loading led to a progressive enlargement of the nephron in the adult Br/+ mouse. To quantitatively demonstrate changes in kidney morphology, glomerular surface area was compared between samples using standard stereological analysis (DisectorZ, www.akuaware.com). There was a significant increase in the glomerular surface area of the kidneys from high-salt-treated mice (Fig. 5, A and B) and caused expansion of Bowman’s capsule, with vacuoles forming in mesangial cells in Br/+ nephrons (Fig. 4).

**Increased Hypothalamic c-Fos Expression in Response to Fluid Challenges in the Br/+ Mouse Suggests an Intact Osmoregulatory Brain Center That Is Not Affected by the Lack of Embryonic six2 Expression**

To further establish whether the abnormal handling of fluids and osmoieties inherent to the Br/+ mouse arises exclusively from reduced renal glomerular number or whether such osmoregulatory defects are secondary to malfunction of brain regulatory systems, a quantitative immunohistochemical analysis was undertaken to determine the pattern of neural activity in response to experimental fluid perturbation. A marked increase in Posmol and PNa in response to the prolonged hypertonic saline load resulted in a modest increase in Fos expression in SON neurons of animals of both genotypes. However, this did not achieve statistical significance. Conversely, Fos expression was significantly upregulated following fluid deprivation for 48 h in the SON of wild-type and Br/+ animals, although the degree of Fos activation was much more substantial in the Br/+ animals than nonstimulated and salt-treated mice (Figs. 5A and 6). Therefore, it is evident that the brains of Br/+ animals remained functionally intact and appropriately expressed Fos in the SON nuclei, particularly in response to the combined plasma hypertonicity-volume depletion associated with fluid restriction.

We also determined the expression of Fos in the osmoreceptive neurons of the forebrain circumventricular organs, including the SFO and OVLT nuclei. Similar to the response observed in SON nuclei, immunohistochemistry revealed a significant increase in the number of Fos-expressing cells in both nuclei in response to fluid deprivation and, to a lesser extent, salt loading (Fig. 5, B and C). The results suggest intact osmosensitivity of these forebrain neurons. Furthermore, gross histological analysis of the rest of the brain tissues collaborated and revealed no significant alterations in most of the brain structures (data not shown). Taken together, the results suggest that absence of embryonic six2 expression did not alter the central neuroendocrine pathway controlling osmoregulation and indicate that the impaired fluid-regulating ability in the adult Br/+ mouse derives exclusively from renal inadequacy associated with reduced nephron number.

**DISCUSSION**

Successful regulation of body fluid is essential for proper function of most cells in the body and depends primarily on the normal function of the kidney in establishing extracellular fluid homeostasis. Because renal filtering capacity is determined by the number of nephrons, it is crucial that the embryonic metanephros (the mammalian kidney) develops properly to achieve an optimal number of nephrons. While it is generally assumed that all kidneys possess the same number of nephrons, the number of functional nephrons varies greatly among individuals (8, 12, 15). Since this variability appears to be present early in neonates (23), it has been suggested that final nephron number is developmentally established during the period of embryogenesis.

While progress has been made to uncover factors and mechanisms that regulate nephron differentiation and branching morphogenesis, it remains unknown which genes give rise to the final number of nephrons in the developing kidney. As demonstrated previously in transgenic mice harboring the mutated gene, absence of six2 upset the delicate balance between pools of native mesenchymes and the differentiated epithelial cells, leading to irreversible loss of nephron subunits that results in RH at birth (17), indicating the critical role of six2 in nephron development. Indeed, RH associated with decreased

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Fig. 4. Kidney histology shown in hematoxylin-eosin-stained sections. Note progressive enlargement of the nephron due to prolonged salt loading in 3H1 wild-type (A and B) and Br/+ (C and D) mice. Salt loading had a minimal effect on wild-type nephrons (A) and caused expansion of Bowman’s capsule, with vacuoles forming in mesangial cells in Br/+ nephrons (D). Arrowheads, glomeruli. Scale bar, 300 μm.
nephron number has been linked with chronic hypertension in human newborns (1).

In the current study, we demonstrate a developmental decrease in embryonic renal six2 expression that is absent in adult tissue. This is evident in wild-type and Br/Br+ animals. Furthermore, while Br/Br+ kidneys contain half as much six2 transcript at E13.5 as the wild-type kidneys, indicating haploinsufficiency, the level of six2 was found to decrease more rapidly, such that the haploinsufficient expression is no longer maintained at E17.5. Since the metanephros proceeds through development by continuously expanding itself over successive rounds of repetitive branching, the rapid disappearance of six2-positive progenitors in the Br/Br+ and, almost immediately, in the Br/Br embryos probably accounts for the nonlinear expression of six2 at a later stage as these six2-positive cells continue their rapid proliferation as usual in the wild-type metanephros. In other words, it is the relative difference in the amount of six2-positive cells among the growing mix of others during the exponential growth of the embryonic kidney that results in the nonlinear expression change of six2 over nephrogenesis.

The rapid decline in six2 expression at the later stage of development possibly accounts for the >50% decrease in glomeruli postnatally with relatively high variability in nephron number that is evident among the adult Br/Br+ colony. We previously reported variable nephron numbers of 13,000–24,000 per kidney in 3H1 wild-type mice and ~700–3,200 per kidney in Br/Br+ mice (6). These observations support the contention that nephron number is variable, even in the general population. That such variability is enhanced (>4-fold) among the Br/Br+ colony provides observational evidence that further suggests the possible role of six2 in the determination of final nephron number.

In this study, we challenged the adult Br/Br+ mice to two specific experimental fluid perturbations and provide evidence that indicates an osmoregulatory defect that persists well into adulthood, including hypernatremia, plasma hyperosmoticity, polydipsia, and polyuria with significantly dilute urine output that fails to respond fully to exogenous DDAVP. The attenuated renal response indicates an impaired urine-concentrating ability that, together with a significant increase in BUN at rest and under stimulated conditions, further indicates reduced renal function, reflecting suboptimal nephron number development.

Although the Br/Br+ mouse clearly develops a DDAVP-resistant urine-concentrating defect that typically characterizes the progression of CRF (7, 20), they were nonetheless able to concentrate their urine when fluid-deprived for 48 h. The residual urine-concentrating ability in response to fluid deprivation is probably due to an increased aquaporin 2 signaling in the remaining nephrons (19). Indeed, while the Br/Br+ mice were physically able to withstand and survived sustained high-salt consumption, they were much more vulnerable to fluid restriction and predictably died shortly after 48 h of fluid restriction if water was not returned soon (S. J. Somponpun, personal observation). Overall, changes in physiological parameters observed in the current study are consistent with renal insufficiency associated with CRF in the adult Br/Br+ mice.

Histomorphological defects such as cysts and distended renal tubules were consistently observed in the Br/Br+ mouse. We previously demonstrated the smaller total kidney volume, smaller glomerular density-to-number ratio, and larger glomerular surface area, confirming nephropathy, in the mutant mouse (6). Prolonged exposure to high-salt loading in the current experiment exacerbates the condition and causes a progressive enlargement of the nephron in the Br/Br+ compared with the wild-type mice.

Since six2 demonstrates broad expression during embryogenesis, we also determined whether the diminished six2 expression in the Br/Br+ mouse might, in addition to causing renal dysmorphology, affect the central neuroendocrine pathway critical for osmoregulation. The elevated P_{osmol} and P_{Na} provoked by prolonged salt loading for 5 days led to an appropriate, although modest, increase in Fos expression in SON nuclei in the Br/Br+ and wild-type mice. In contrast, upregulation of

Fig. 5. Cell counts of Fos-immunopositive cell nuclei in supraoptic nucleus (SON, A), organum vasculosum of the lamina terminalis (OVLT, B), and subfornical organ (SFO, C). Fos expression was activated in response to prolonged salt loading and significantly elevated by fluid deprivation in all brain regions examined in both genotypes. *P ≤ 0.05. This indicates an intact central neuroendocrine pathway in response to fluid perturbation that is not affected by lack of congenital six2 expression in Br/Br+ mouse. DH, fluid deprivation; S, salt-loading; NS, no salt (untreated control).
Fos was clearly established in SON neurons in response to 48 h of fluid restriction, indicating that these nuclei remain functionally intact and are capable of responding to signals generated by the combined hyperosmotic and hypovolemic stress associated with fluid deprivation. The SFO and OVLT nuclei in the basal forebrain also expressed Fos appropriately, suggesting that the osmosensitive mechanism residing in the forebrain circumventricular organ neurons remains able to sense the osmotic changes in response to salt loading and fluid deprivation. Since the lack of six2 is known to reduce glomerular number and Br/+ mice display reduced renal nephrons compared with wild-type mice, our data suggest that the osmoregulatory defect in these animals likely resides in the kidney’s inability to reabsorb sufficient fluid to maintain a constant $P_{\text{osmol}}$ when exposed to osmotic challenges, while the hypothalamic magnocellular neurons in the SON and the basal forebrain nuclei are intact and were not developmentally altered by the lack of embryonic six2 expression in the Br/+ animal. This is important, because it isolates the kidney as the primary target of six2 and sets up an opportunity for us to further investigate and attempt to restore six2 expression to the six2-deficient Br/+ mouse in our ongoing gene replacement protocol. We are currently generating a transgenic rescue mouse reexpressing six2 in our Br colony to test the hypothesis that successful reexpression of six2 leads to a restoration of nephron number, thus rescuing the defective renal function in vivo, thereby confirming the role of six2 in nephron endowment.

**Significance and Perspective**

Successful metanephric development is crucial to achieving appropriate postnatal renal function. Results from the current study strongly argue the role of six2 as an important embryonic regulatory gene of nephron development and suggest that six2 may constitute the genetic basis for renal nephron number. We have shown that reduced expression of six2 during embryonic development initiates glomerular dysplastic changes that are associated with the subsequent development of multicystic kidneys, with nephron deficit leading to fluid and electrolyte imbalance in the adult.

The deficit in the number of functioning nephrons marks the 3H1 Br/+ mouse as an experimentally useful model to study the role of nephron numbers in renal diseases and establishes the adult Br/+ colony as one of only a few existing nonsurgical models of CRF. This mouse model allows for an investigation into genetic attributes of initial nephron differentiation in the embryo as well as physiological defects that develop in the affected adult. Since a deficit in nephron endowment leads to increased mean arterial pressure, owing to reduced glomerular filtration rate, the current study also allows us to establish an experimental mouse model for understanding the role of reduced nephron numbers in the genesis of cardiovascular diseases including high blood pressure. As demonstrated in a recent experimental model, difference in nephron endowment has functional consequences in the regulation of blood pressure and renal abnormality (15, 16).

Ongoing experiments are directed at identifying factors that are the targets of six2 as well as genes associated with RH and complications arising from this renal disease. Determining such markers associated with six2 might allow for potential clinical applications for an early detection of renal ailments or identification of a subpopulation that might be more susceptible to renal damage because of the reduced nephron number.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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