Comparative Medicine
Copyright 2016
by the American Association for Laboratory Animal Science

Vol 66, No 1
February 2016
Pages 10–20

*Original Research*

# Renal Function and Hematology in Rats with Congenital Renal Hypoplasia

**Hidenori Yasuda, Kohei Amakasu, Yuki Tochigi, Kentaro Katayama, and Hiroetsu Suzuki***

**Renal hypoplasia due to a congenitally reduced number of nephrons progresses to chronic kidney disease and may cause renal anemia, given that the kidneys are a major source of erythropoietin in adults. Hypoplastic kidney (HPK) rats have only about 20% of the normal number of nephrons and develop CKD. This study assessed the renal function and hematologic changes in HPK rats from 70 to 210 d of age. HPK rats demonstrated deterioration of renal excretory function, slightly macrocytic erythropenia at all days examined, age-related increases in splenic hemosiderosis accompanied by a tendency toward increased hemolysis, normal plasma erythropoietin levels associated with increased hepatic and decreased renal erythropoietin production, and maintenance of the response for erythropoietin production to hypoxic conditions, with increased interstitial fibrosis at 140 d of age. These results indicate that increases in splenic hemosiderosis and the membrane fragility of RBC might be associated with erythropenia and that hepatic production of erythropoietin might contribute to maintaining the blood Hgb concentration in HPK rats.**

**Abbreviations:** CKD, chronic kidney disease; HGN, hypogonadism; HPK, hypoplastic kidney.

Approximately 10% to 13% of the general population has chronic kidney disease (CKD), including an estimated 13.3 million people in Japan.[13,15,20] Moreover, more than 1.1 million patients worldwide require maintenance dialysis, and that number continues to increase.[24] Determining the pathogenesis of CKD, identifying clinical makers of early stages of CKD, and developing effective methods to treat CKD are required, especially given that CKD has been reported to be a risk factor for cardiovascular disease, with high mortality rates.[11,13,15] Patients with CKD are frequently anemic, due to a low level of erythropoietin and inhibition of erythropoiesis.[12,27] The decreased production of erythropoietin may result from the transdifferentiation of interstitial fibroblasts to myofibroblasts, resulting in increased production of extracellular matrix in the kidneys.[4,29] The number of nephrons in the kidneys at birth varies greatly,[5,19] and a congenital reduction in number of nephrons is thought to be related to the occurrence and prognosis of CKD.[9,17,18,21] Therefore, a CKD animal model with a reduced number of nephrons is useful for studying the pathophysiology of and treatments for CKD.

Affected rats in the hypogonadism (HGN) inbred strain are characterized by male sterility due to hypogonadism,[37,41] reduced female fertility due to ovarian hypoplasia,[30,31] and progressive renal dysfunction due to bilateral hypoplastic kidneys (HPK).[32,33] These defects are controlled by a single autosomal recessive gene, *hgn*.[38,40] Linkage analysis and sequencing of candidate genes revealed a 25-bp duplicated insertion mutation in exon 7 of *Astrin/Spag5*, which encodes a microtubule-associated protein.[39] Because this mutation causes a premature terminal codon resulting in a truncated Astrin protein that lacks the primary spindle-targeting domain, the cause of the phenotype is considered to be a loss-of-function type mutation of the *Astrin* gene.[38,39] The recovery of normal fertility and renal function in homozygous mutant rats by a transgene comprising normal *Astrin* cDNA indicates that Astrin is required for normal testicular and renal development.[22]

The HGN strain was isolated from the sixth filial generation of a polygenic hydronephrotic rat strain derived from the original stock of the Wistar–Imamichi rat closed colony.[41] Because the occurrence of hydronephrosis would influence renal development and function, we established another hypogonadism strain (HGN II) that was directly derived from the original closed colony.[36] The HGN II strain has been maintained by inbreeding between carriers, and the mutated gene responsible for the phenotype in the HGN II strain is identical to that in the HGN strain.[39] The affected rats of the HGN II strain show a similar phenotype as that of the HGN strain with regard to hypogonadism and HPK.[35,36]

Although male HPK rats in the HGN and HGN II strains have only about 20% of the nephrons present in normal kidney, the total glomerular filtration rate per kidney is compensated by hyperfiltration of individual glomeruli.[32,36] However, continuous glomerular hyperfiltration and functional overload of individual nephrons can result in a deterioration in renal excretion. Histologically, HPK rats demonstrate glomerular hypertrophy and dilation of the renal tubules.[32,36] As these rats age, cast formation in tubular lumen, glomerular sclerosis, and cellular infiltration into interstitial tissue occur.[33,35] In addition, age-related features of renal deterioration, including polyposia, polyuria, azotemia, albuminuria, and hypertension, follow,[35] and secondary hyperparathyroidism, osteodystrophy, and anemia emerge at advanced age in HPK rats.[33] Therefore HPK rats are a model for studying

*Received: 18 Apr 2015. Revision requested: 14 Jun 2015. Accepted: 10 Aug 2015.*
*Laboratory of Veterinary Physiology, Unit of Functional Morphology, Department of Basic Veterinary Medicine, School of Veterinary Medicine, Faculty of Veterinary Science, Nippon Veterinary and Life Science University, Tokyo, Japan.*
**Corresponding author. Email: hiroetsu@nvlu.ac.jp*

**Table 1.** Plasma concentrations of biochemical markers in normal and HPK at 70, 140, and 210 d of age

| | | 70 d | 140 d | 210 d |
|---|---|---|---|---|
| BUN (mg/dL) | Normal | 8.2 ± 2.0 | 12.7 ± 1.8 | 12.3 ± 0.8 |
| | HPK | 17.0 ± 3.0[b] | 28.8 ± 15.1[a] | 36.9 ± 8.8[c] |
| Creatinine (mg/dL) | Normal | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.0 |
| | HPK | 0.5 ± 0.1[a] | 0.8 ± 0.5[a] | 1.0 ± 0.2[c] |
| Albumin (g/dL) | Normal | 3.2 ± 0.2 | 3.5 ± 0.2 | 3.3 ± 0.2 |
| | HPK | 2.6 ± 0.6 | 2.8 ± 0.3[b] | 2.9 ± 0.3[a] |
| Total cholesterol (mg/dL) | Normal | 62.0 ± 3.4 | 83.6 ± 16.1 | 99.6 ± 13.9 |
| | HPK | 98.5 ± 26.5 | 156.8 ± 46.5[b] | 182.0 ± 49.0[b] |
| $Na^+$ (mEq/L) | Normal | 134.8 ± 0.5 | 133.2 ± 8.9 | 131.6 ± 7.9 |
| | HPK | 136.3 ± 2.5 | 128.7 ± 5.1 | 134.3 ± 7.8 |
| $K^+$ (mEq/L) | Normal | 3.5 ± 1.1 | 3.5 ± 0.6 | 3.0 ± 0.2 |
| | HPK | 3.5 ± 0.7 | 4.1 ± 1.2 | 3.9 ± 0.4[b] |
| $Cl^-$ (mEq/L) | Normal | 95.5 ± 1.0 | 96.0 ± 8.6 | 94.0 ± 6.5 |
| | HPK | 99.0 ± 6.9 | 92.0 ± 4.9 | 94.8 ± 6.2 |
| $Ca^{2+}$ (mg/dL) | Normal | 10.1 ± 0.8 | 10.0 ± 0.8 | 9.1 ± 1.9 |
| | HPK | 9.7 ± 1.2 | 8.9 ± 1.9 | 9.9 ± 1.9 |
| Inorganic P (mg/dL) | Normal | 9.7 ± 0.8 | 7.2 ± 2.3 | 6.3 ± 2.6 |
| | HPK | 8.6 ± 1.1 | 8.3 ± 1.5 | 6.9 ± 3.1 |
| $Mg^{2+}$ (mg/dL) | Normal | 2.1 ± 0.3 | 2.4 ± 0.3 | 2.1 ± 0.2 |
| | HPK | 2.3 ± 0.3 | 3.4 ± 1.1 | 3.2 ± 0.6[b] |
| Total protein (g/dL) | Normal | 5.5 ± 0.7 | 6.3 ± 0.8 | 5.8 ± 0.3 |
| | HPK | 5.0 ± 0.9 | 5.5 ± 0.9 | 6.0 ± 0.6 |
| AST (U/L) | Normal | 80.8 ± 22.3 | 98.2 ± 22.0 | 103.8 ± 20.1 |
| | HPK | 73.5 ± 9.1 | 104.3 ± 45.0 | 85.8 ± 32.9 |
| ALT (U/L) | Normal | 34.0 ± 6.9 | 33.0 ± 8.0 | 29.0 ± 3.9 |
| | HPK | 29.0 ± 4.5 | 26.5 ± 3.7 | 20.0 ± 9.8 |

Data are given as mean ± 1 SD.
Value in HPK rats is significantly ([a], $P < 0.05$; [b]$P < 0.01$, [c]$P < 0.001$) different from that in normal rats.

how a congenitally reduced nephron mass may induce CKD and secondary renal diseases, and HPK rats might be useful for identifying biomarkers related to these diseases. Because our previous studies in HPK rats[33,35] provided only limited information about the progression of CKD and renal anemia, the current study was designed to analyze multiple parameters related to renal function and hematology and to characterize the anemic tendencies in 70- to 210-d-old HPK rats. We found that the hematologic condition of HPK rats is characterized by reduced renal excretive function, erythropenia, increased hemolysis in the spleen, progressive renal fibrosis, and maintenance of normal plasma erythropoietin concentrations.

## Materials and Methods

**Animals.** Rats of the HGN II strain were kept in a conventional environment in our laboratory under a controlled photoperiod, temperature, and relative humidity, and regularly checked for common pathogens as described previously.[7] They were housed in stainless steel cages after weaning and had unlimited access to a standard pellet diet (CR-LPF, Oriental Yeast, Tokyo, Japan) and

**Table 2.** CBC counts in normal and HPK rats at 70, 140, and 210 d of age

| | | 70 d | 140 d | 210 d |
|---|---|---|---|---|
| RBC (×10⁴/μL) | Normal | 817.6 ± 22.0 | 917.8 ± 34.6 | 933.0 ± 39.8 |
| | HPK | 718.8 ± 22.0[c] | 809.8 ± 45.1[b] | 782.5 ± 40.4[c] |
| Hgb (g/dL) | Normal | 15.3 ± 0.2 | 15.9 ± 0.1 | 15.7 ± 0.2 |
| | HPK | 14.7 ± 0.6 | 15.4 ± 0.7 | 15.1 ± 0.8 |
| Hct (%) | Normal | 46.5 ± 1.7 | 47.9 ± 2.0 | 47.9 ± 1.5 |
| | HPK | 42.5 ± 0.7[b] | 46.3 ± 1.1 | 44.6 ± 2.0[a] |
| MCV (fL) | Normal | 56.8 ± 2.3 | 52.0 ± 1.6 | 51.8 ± 1.5 |
| | HPK | 59.4 ± 1.9 | 57.4 ± 3.0[b] | 56.8 ± 0.5[c] |
| MCH (pg) | Normal | 18.8 ± 0.6 | 17.4 ± 0.5 | 16.9 ± 0.7 |
| | HPK | 20.4 ± 0.3[c] | 19.0 ± 0.3[c] | 19.2 ± 0.1[c] |
| MCHC (g/dL) | Normal | 33.0 ± 1.6 | 33.4 ± 1.2 | 32.5 ± 0.8 |
| | HPK | 34.5 ± 1.4 | 33.1 ± 1.5 | 33.8 ± 0.3 |
| Platelets (× 10⁴/μL) | Normal | 76.7 ± 23.5 | 85.4 ± 9.7 | 83.8 ± 10.7 |
| | HPK | 59.2 ± 4.8 | 66.3 ± 30.8 | 73.5 ± 14.2 |
| WBC (× 10²/μL) | Normal | 85.2 ± 7.0 | 79.4 ± 10.6 | 74.5 ± 11.6 |
| | HPK | 98.8 ± 19.9 | 109.4 ± 13.4[b] | 98.5 ± 22.5 |

Data are given as mean ± 1 SD.
Value in HPK rats is significantly ([a], $P < 0.05$; [b]$P < 0.01$, [c]$P < 0.001$) different from that in normal rats.

water.[3,7,34] Genotyping for the *hgn* allele was performed by PCR amplification of the 25-bp insertion into exon 7 of the *Astrin* gene.[39] Male rats derived from HGN II strain were obtained by mating heterozygous (*+/hgn*) male and female rats.[35,36] Because the mutation is an autosomal recessive trait, heterozygous (*+/hgn*) and homozygous mutant (*hgn/hgn*) rats were regarded as normal and affected (HPK), respectively. This study was approved by the Animal Care and Use Committee of Nippon Veterinary and Life Science University. All experimental procedures and animal care were performed in accordance with the guidelines of the committee.

**Preparation of blood and organ samples.** Blood samples from the tail veins of normal and HPK male rats at 70, 140, and 210 d of age ($n$ = 3 to 6) were collected into EDTA under ether anesthesia. Heparinized blood samples were collected from the vena cava, and plasma was stored at –20 °C until assay. After the rats were euthanized by exsanguination under anesthesia, they were necropsied, and their kidneys and spleens were weighed on an electric balance and fixed in 4% neutral formalin.[32,33]

**Hematology and blood biochemistry.** Hematologic parameters including RBC count, Hgb concentration, Hct, MCV, MCH, MCHC, and platelet count were analyzed automatically (Celltac, Nihon Koden, Tokyo, Japan). Reticulocytes were counted by using the Brecher methods,[6] and their percentage in RBC was calculated. Plasma concentrations of sodium, potassium, chlorine, BUN, creatinine, AST, ALT, albumin, total protein, total cholesterol, calcium, inorganic phosphorus, and magnesium were measured automatically (DriChem 3500V, Fujifilm Medical Company, Tokyo, Japan).[2,7,34] Plasma iron, transferrin, and total iron binding capacity were measured by a clinical reference laboratory (Monolis, Cyofu-shi, Tokyo).

**Osmotic resistance of RBC membranes.** The osmotic resistance of RBCs was measured essentially as described.[28] Briefly, 30 μL of heparinized blood was added to 1.5 mL of 0.1% to 0.85% NaCl in PBS in a graded concentration series and centrifuged at 825 × $g$ for 10 min. The absorbance of each supernatant at 540 nm was measured, with the supernatant in the 0.85% salt concentration mixture used as a blank. The percentage absorbance of each supernatant relative to that of the 0.1% salt supernatant was calculated, and the relative degree of hemolysis was determined. The salt concentration at 50% hemolysis was calculated from the approximate hemolysis curve.

**Anemic hypoxia induced by bleeding.** Several 210-d-old HPK male rats died during bleeding-induced hypoxia. Therefore, data under anemic hypoxia was obtained from 140-d-old normal and HPK male rats, as reported previously.[16] Ether was used for terminal blood collection, whereas isoflurane was used for the recovery procedures. Under isoflurane anesthesia controlled by small-animal anesthetizer (TK-7, Biomachinery, Chiba, Japan), blood samples equivalent to 1.5% of body weight were removed from the jugular vein and replaced by the same amount of saline. The serum erythropoietin concentration in these samples was measured. At 12 h after bleeding, the left kidney and quadrate lobe of the liver were ligated and removed under isoflurane anesthesia, and the organs were immersed in RNAlater (Life Technology, CA) at 4 °C for 24 h and stored at –80 °C until total RNA extraction. Blood samples were removed from the vena cava, and rats

were euthanized by exsanguination under anesthesia. The serum samples were stored at –80 °C until erythropoietin was assayed.

**Radioimmunoassay of plasma and serum erythropoietin level.** Plasma and serum erythropoietin levels were measured by radioimmunoassay (Mitsubishi Chemical Medicine, Minato-ku, Tokyo, Japan; Monolis, Cyofu-shi, Tokyo, Japan).

**Total RNA extraction and real-time RT-PCR.** Total RNA was isolated from the liver and left kidney of normal and HPK rats by using TRIzol reagent (Life Technologies, ThermoFisher Scientific, Carlsbad, CA) according to the manufacturer's instructions. Each sample was treated with DNase I (Takara, Shiga, Japan), followed by reverse transcription of 1 to 2 µg total RNA by using ReverTra Ace RTase (Toyobo, Osaka, Japan) and random primers, as described by the manufacturer. The reaction conditions consisted of annealing at 30 °C for 10 min, enzyme reaction at 42 °C for 60 min, and denaturation at 99 °C for 5 min. The resultant cDNA was PCR amplified by using Power SYBR Green Master Mix (Life Technologies), specific primer pairs for erythropoietin (forward, 5′ CAC GAA GCC ATG AAG ACA GA 3′; reverse, 5′ GGC TGT TGC CAG TGG TAT TT 3′) and GAPDH (forward, 5′ AAG ATG GTG AAG GTC GGT GTG 3′; reverse, 5′ AAT GAA GGG GTC GTT GAT GG 3′), and a 7500 Fast Real-Time PCR system (Life Technologies). The reaction conditions included activation of AmpliTaq Gold polymerase at 95 °C for 10 min; followed by 40 cycles of denaturation for 15 s at 95 °C and annealing–extension for 1 min at 60 °C. Amplification was followed by a dissociation stage (95 °C for 15 min, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s) to confirm the presence of a single PCR product. The level of expression of GAPDH mRNA in the same samples was used as an internal control to evaluate the relative expression of erythropoietin mRNA.

**Histopathologic analysis.** Formalin-fixed spleens were embedded in paraffin, cut into sections 2 µm thick, deparaffinized in xylene, hydrated through a graded ethanol series, immersed in water, and stained with Berlin blue. More than 10 areas, each of which was defined as a square field (77126 µm$^2$), in the splenic red pulp were selected randomly in each rat. The area of hemosiderin deposition in each square field was measured by using ImageJ software (imagej.nih.gov). In addition, formalin-fixed kidneys were embedded in paraffin, cut into sections 4 µm thick, and stained with elastic–van Gieson stain. Digital images were obtained under optical and fluorescence microscopy (Biozero, Keyence, Osaka, Japan).[22]

**Statistical analysis.** Results are reported as mean ± 1 SD, with differences evaluated by using unpaired Student *t* tests (Excel for Windows 2011, Microsoft, Redmond, WA). A *P* value less than 0.05 was defined as statistically significant.

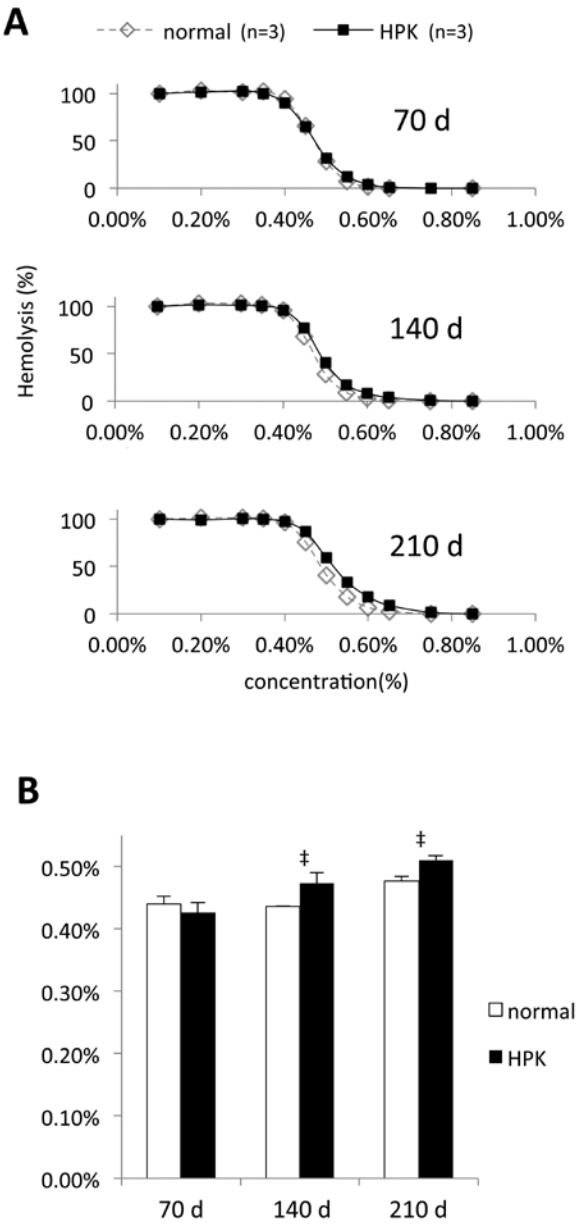


**Figure 1.** (A) Percentage hemolysis curves in normal and HPK rats at 70, 140, and 210 d of age. The *x*-axis represents the NaCl concentration in PBS. (B) Salt concentration at 50% hemolysis in male normal and HPK rats at 70, 140 and 210 d of age. Each error bar represents 1 SD ($n$ = 3 or 4); value significantly (‡, $P < 0.001$) different from that of normal rats.

## Results

**Blood biochemistry.** Plasma concentrations of BUN and creatinine were significantly (70 d, $P < 0.01$ and $P < 0.05$; 140 d, $P < 0.05$; 210 d, $P < 0.001$) higher in HPK than in normal rats at all ages examined. In addition, total cholesterol was higher ($P < 0.01$) and concentrations of albumin lower (140 d, $P < 0.01$; 210 d, $P < 0.05$) at 140 and 210 d of age in HPK rats. Plasma $K^+$ was higher ($P < 0.01$) in HPK than in normal rats at 210 d of age. In addition, plasma $Mg^{2+}$ concentration was significantly ($P < 0.01$) higher in HPK than in normal rats at 210 d of age. The age-related deterioration in these parameters indicates that HPK rats undergo progressive renal dysfunction. In contrast, hepatic enzyme concentrations were similar in normal and HPK rats on all days examined (Table 1).

**Hematology.** RBC counts were significantly (70 d and 210 d, $P < 0.001$; 140 d, $P < 0.01$) lower in HPK than in normal rats at all ages examined, and Hct was lower in HPK than in normal rats at 70 ($P < 0.01$) and 210 d ($P < 0.05$) of age. Hgb values were consistently (albeit nonsignificantly) lower in HPK than in normal rats, with the average from all rats aged 70 to 210 d significantly ($P < 0.005$) lower in HPK than in normal rats. MCH at all ages examined ($P < 0.001$) and MCV at 140 ($P < 0.01$) and 210 d ($P < 0.001$) of age were higher in HPK than in normal rats. In contrast, MCHC did not differ between normal and HPK rats at any ages examined. Total

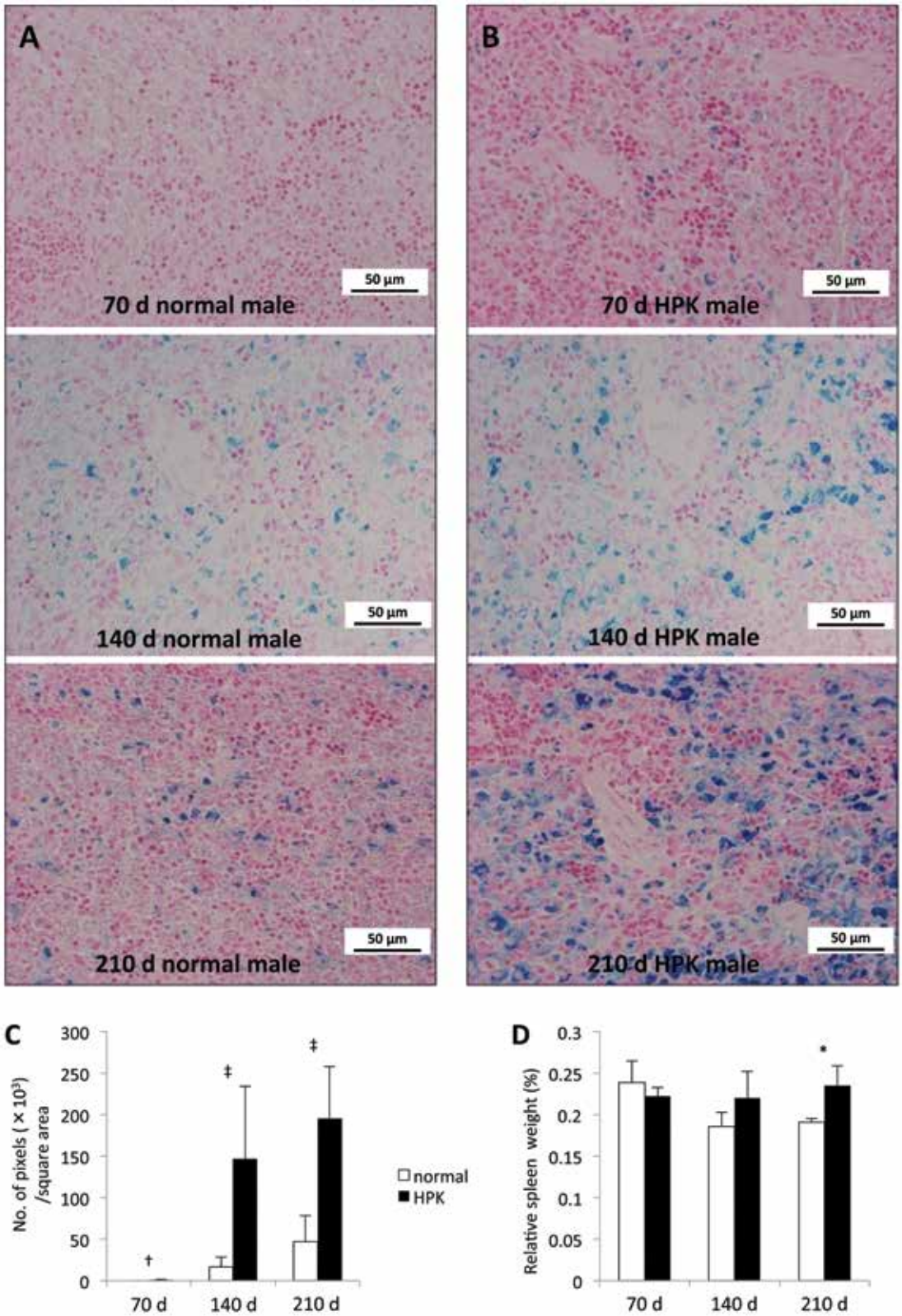


**Figure 2.** Splenic histology of (A) normal and (B) HPK rats at 70, 140, and 210 d of age. Splenic red pulp showed age-associated increases in hemosiderin-containing macrophages (blue), with greater deposition of hemosiderin in the spleens of HPK rat than in normal rats. Berlin blue stain. (C) Hemosiderin deposition and (D) relative spleen weight (%; spleen weight / body weight × 100%) at 70, 140, and 210 d of age (open bars, normal rats; solid bars, HPK rats). Areas of hemosiderin deposition were larger in HPK than in normal rats at all ages examined, and relative spleen weights at 210 d of age were greater in male HPK than in normal rats. Error bar, 1 SD ($n$ = 3 to 6); value is significantly (*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$) from that of normal rats.

**Table 3.** Serum iron and related parameters in normal and HPK rats at 70, 140, and 210 d of age

| | | 70 d | 140 d | 210 d |
|---|---|---|---|---|
| Fe (µg/dL) | Normal | 171.3 ± 46.3 | 211.3 ± 69.8 | 202.3 ± 7.4 |
| | HPK | 216.8 ± 71.2 | 203.7 ± 34.5 | 171.7 ± 65.7 |
| Total iron-binding capacity (µg/dL) | Normal | 613 ± 95.8 | 537.3 ± 230.6 | 626 ± 41.1 |
| | HPK | 414.8 ± 44[b] | 438 ± 97.4 | 361 ± 124.8[a] |
| Transfferin (mg/dL) | Normal | 145.5 ± 21.4 | 160 ± 6 | 160.3 ± 5.1 |
| | HPK | 109.3 ± 8.3[a] | 107.3 ± 11.2[b] | 93.3 ± 24.7[b] |

Data are given as mean ± 1 SD.
Value in HPK rats is significantly ([a], $P < 0.05$; [b]$P < 0.01$, [c]$P < 0.001$) different from that in normal rats.

WBC count and WBC components (data not shown) did not show consistent differences (Table 2). These results indicate that HPK rats show a tendency toward anemia with slightly macrocytic erythropenia. At all ages examined, the percentage of reticulocytes showed no significant difference between normal and HPK rats (about 1%; data not shown).

**Osmotic resistance of RBC membranes.** Although the hemolysis curves were comparable between 70-d-old HPK and normal rats, the curve shifted progressively toward higher osmotic pressure in HPK male rats at 140 and 210 d of age (Figure 1 A). Moreover, the salt concentrations required for 50% hemolysis were significantly ($P < 0.001$) higher in HPK than in normal male rats at 140 and 210 d of age (Figure 1 B). These results indicate that the osmotic resistance of RBC was decreased in HPK rats.

**Histopathologic analysis in spleen and spleen weight.** Macrophages containing hemosiderin deposits, stained blue, were localized in the red pulp area of the spleen. Staining increased with age in both normal and HPK rats but appeared to be greater in HPK than in normal spleens at all ages examined (Figure 2 A and B). The areas of hemosiderin deposition gradually increased with age, and were significantly (70 d, $P < 0.01$; 140 and 210 d, $P < 0.001$) larger in HPK than in normal spleens at all ages examined (Figure 2 C). At age 210 d, the relative spleen weight was significantly ($P < 0.05$) greater in HPK than in normal rats (Figure 2 D).

**Plasma iron and related parameters.** The total iron binding capacity at 70 ($P < 0.01$) and 210 d ($P < 0.05$) of age and the plasma transferrin level at all ages examined were significantly (70 d, $P < 0.05$; 140 and 210 d, $P < 0.01$) lower in HPK than in normal rats, whereas plasma iron levels remained normal in HPK rats at all ages examined (Table 3). Therefore, transferrin saturation (100% × Fe / total iron binding capacity) was significantly higher in HPK than in normal rats at 70 d (51.7% ± 13.5% compared with 28.0% ± 6.5%, $P < 0.05$) and 210 d (47.7% ± 6.5% compared with 32.5% ± 3.2%, $P < 0.05$).

**Histopathology of the kidneys.** The number of glomeruli in HPK rats was reduced at 70 d of age, with glomerular hypertrophy present at 140 d, degenerated glomeruli at 210 d, and mildly and severely dilated tubules at 140 and 210 d, respectively (Figure 3). The number of pink-stained collagen fibers gradually increased with age in the interstitium of HPK rats (Figures 3 and 4).

**Erythropoietin levels and erythropoietin mRNA expression in kidney and liver.** Under normoxic conditions, plasma erythropoietin concentrations were similar in HPK and normal rats at all ages examined (Figure 5 A). However, erythropoietin mRNA expression at 140 d of age was lower ($P < 0.05$) in kidney and higher ($P < 0.01$) in liver in HPK compared with normal rats (Figure 5 B). Hypoxia induced at least a 10-fold increase in serum erythropoietin levels in both normal and HPK rats, with no significant difference between them (Figure 5 C). In addition, hypoxic conditions increased the expression of erythropoietin mRNA in both the kidney and liver, with no significant differences between normal and HPK rats (Figure 5 D).

## Discussion

As previously reported,[33,35] gradually increased levels of BUN and creatinine in HPK rats indicate that their renal function deteriorated with advanced age. The normal levels of AST and ALT in HPK rats at all ages suggest that their low albumin and high total cholesterol concentrations are likely not due to hepatic dysfunction. The hypoalbuminemia is probably caused by the loss of albumin to urine, because proteinuria in male HPK rats gradually progressed with advancing age.[35,36] In nephrotic syndrome, hypoalbuminemia is accompanied by hyperlipidemia, which results primarily from increased apolipoprotein concentrations.[8,25] The gradually increased total cholesterol level suggests that a similar process occurs in HPK rats. In addition, electrolyte imbalances accompany advanced-stage CKD. Although the renal dysfunction in HPK rats was not severe enough to completely disrupt the plasma electrolytic balance, the elevations in the plasma $K^+$ and $Mg^{2+}$ levels at 210 d of age indicate reduced excretion of these electrolytes.

In general, renal anemia is characterized by a decrease in blood Hgb resulting from normocytic, normochromic erythropenia.[1] In the present study, HPK rats had a slight, consistent decrease in Hgb arising from slightly macrocytic erythropenia. Although the number of RBC was significantly lower in HPK than in normal rats at all ages examined, the increased MCV and MCH values of HPK rats partially compensate for their decreased Hgb and Hct. Similar phenomena have been noted in rats with unilateral renal agenesis in the unilateral urogenital anomalies (UUA) strain, which showed slightly macrocytic erythropenia at 70 and 140 d of age.[2] Although HPK and UUA rats both have congenitally reduced nephron mass, the genetic etiologies differ,[3,39] and it is therefore unlikely that the astrin mutation in HPK rats directly causes the macrocytic changes in RBC. In normal rats, the nearly constant levels of Hgb and Hct at the ages examined results from age-related increases in the number of RBC coupled with age-related decreases in MCV and MCH. Similar age-related changes in hematology were reported in F344 rats.[23,42] Postnatal changes in rat hematology include the replacement of larger, fetal-type

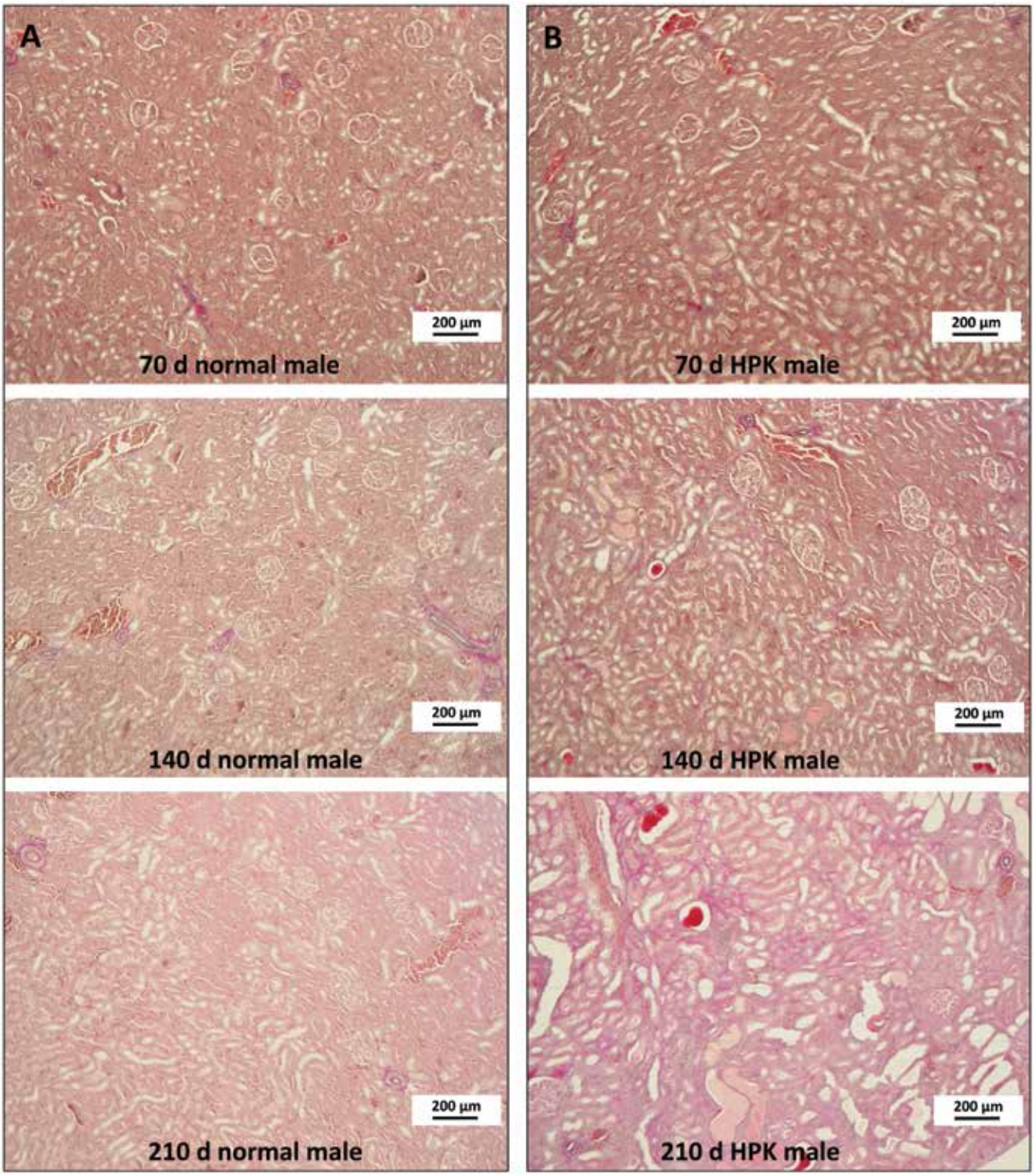


**Figure 3.** Renal histopathology of (A) normal and (B) HPK rats at 70, 140, and 210 d of age. At 70 d, HPK rats appeared to have fewer glomeruli than did normal rats. Glomerular hypertrophy was apparent in HPK at 210 d of age. In addition, collagen fibers (pink) in the interstitial tissue were markedly increased, and dilated tubules were frequent in HPK rats at 210 d of age. Elastic–van Gieson stain.

RBC by smaller adult-type RBC.[23,42] Given that slightly macrocytic erythropenia is already present in HPK (and UUA) rats at 70 d of age, the fetal-type RBC might be retained longer in HPK than in normal rats. A low level of erythropoietin or the possible presence of an inhibitor of erythropoiesis due to congenital reduction of renal mass might impair the erythropoiesis required for rapid body growth during the juvenile period. In a previous study,[33] we found that HPK rats from HGN strain were anemic after 140 d of

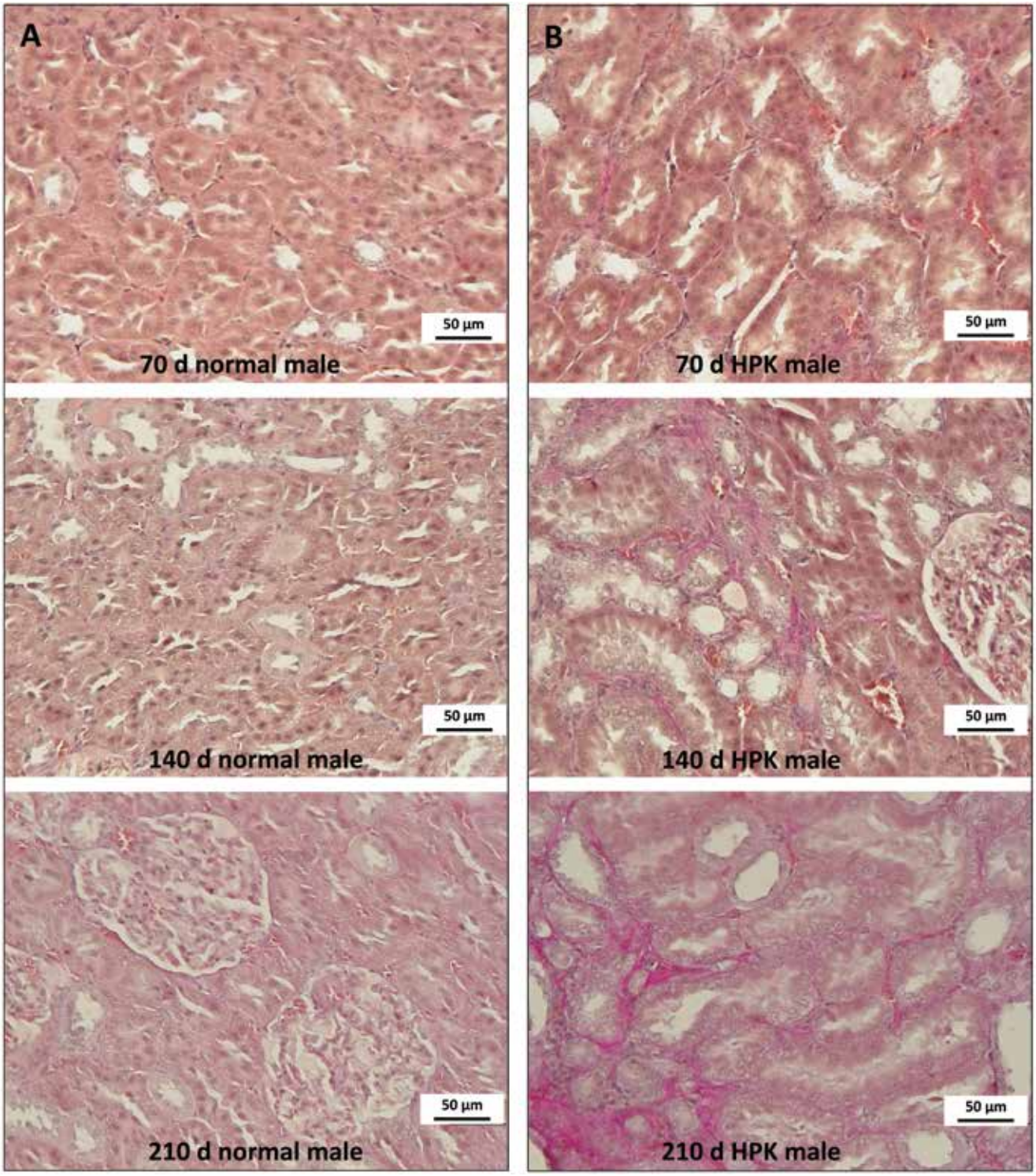


**Figure 4.** Renal histopathology in normal (A) and HPK (B) rats at 70, 140, and 210 d of age. Collagen fibers stained pink gradually increased in interstitial tissue with age in HPK rats. Elastic–van Gieson stain.

age. The original HGN strain had been established from a polygenic hydronephrosis strain that demonstrated various degrees of hydronephrosis,[41] whereas HPK rats of the HGNII strain that we used in the current study were derived from a closed colony and were free from hydronephrosis.[36] This situation might explain why the anemic tendency of HPK rats in the present study was more moderate than that in the previous study, although HPK rats from both strains showed slightly macrocytic erythropenia.

During end-stage CKD, renal damage leads to the urinary excretion of transferrin and iron.[14] The accumulation of iron in the

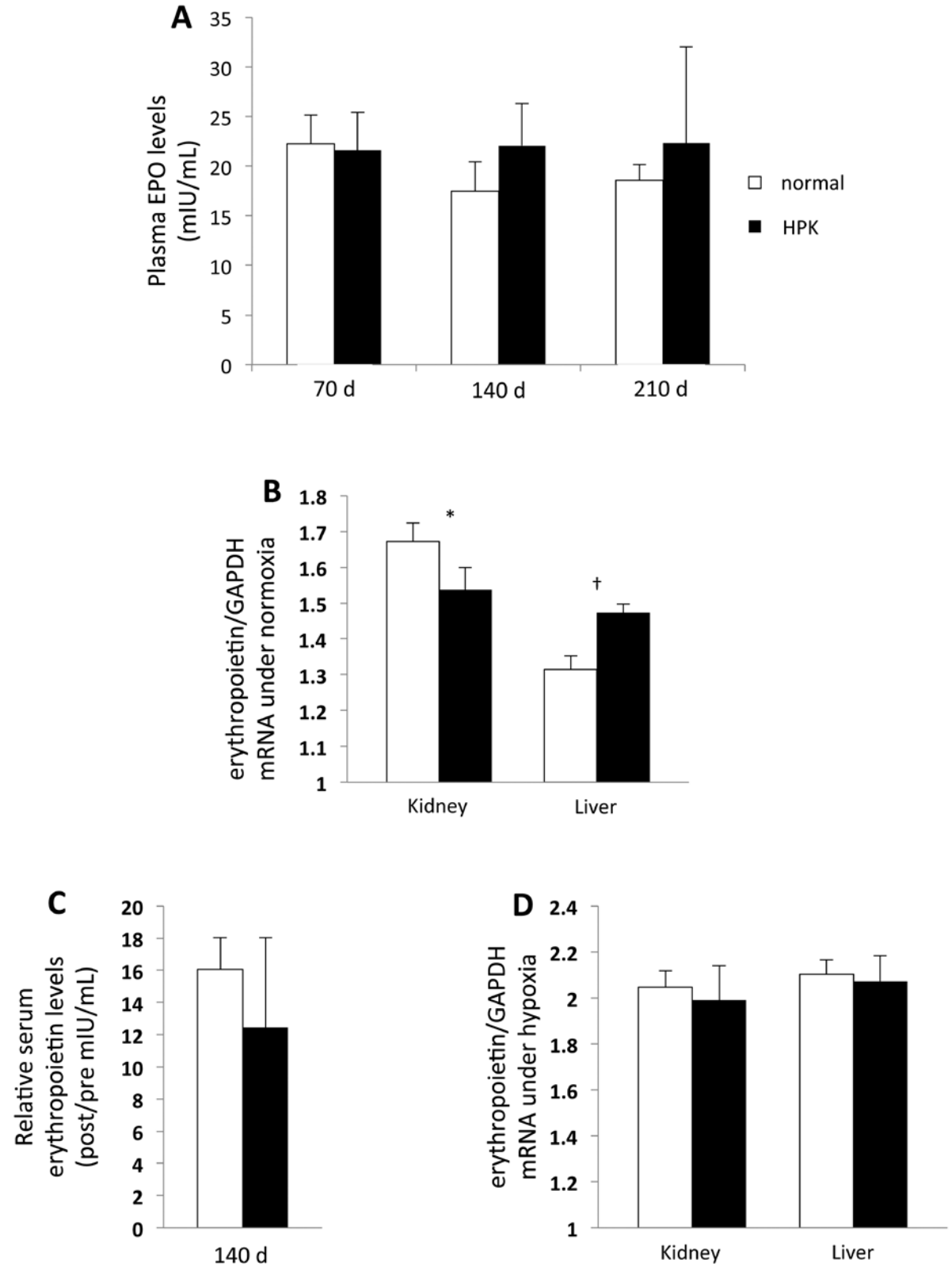


**Figure 5.** (A) Plasma erythropoietin concentrations at ages 70 to 210 d in normal and HPK rats. No differences were observed at any age. (B) erythropoietin mRNA expression at 140 d in the kidney and liver of normal and HPK males. Erythropoietin mRNA expression was lower in HPK than in normal kidneys but higher in HPK than in normal liver. (C) Effect of bleeding-induced hypoxia on relative serum erythropoietin levels at 140 d in normal and HPK rats. No significant difference was observed. (D) Effect of bleeding-induced hypoxia on erythropoietin mRNA expression at 140 d in kidneys and livers of normal and HPK rats. No significant differences were observed. Error bar, 1 SD ($n$ = 3 to 5); value significantly (*, $P < 0.05$; †, $P < 0.01$) different from that in normal rats.

spleen as well as the decreased availability of iron in the bone marrow reduce the blood cell proliferation rate in the bone marrow and cause microcytic changes in RBC.[1,14,43] The plasma total iron binding capacity and transferrin levels were reduced in HPK rats, whereas their plasma iron and transferrin saturation levels were normal or increased, respectively. In addition, the percentage of reticulocytes was within the normal range in HPK rats, and their erythrocytes were macrocytic rather than microcytic. These findings suggest that there is no severe shortage of iron in the bone marrow of HPK rats. Splenic hemosiderin pigmentation was accompanied by increased fragility of RBC membranes, and these conditions gradually deteriorated with age in HPK rats. Similar defects have been reported in mice with ICR-derived glomerulonephritis.[43] The characteristic increases in RBC volume and uremic oxidative stress associated with renal dysfunction may contribute to a hemolytic tendency and splenic hemosiderosis, which may aggravate erythropenia.

In general, the percentage of reticulocytes in the peripheral blood is normal or low during renal anemia, although the RBC lifespan is apparently shortened. This condition has been thought to result from the reduced production of renal erythropoietin. In situ hybridization and transgenic techniques have shown that the erythropoietin-producing cells in the kidney are fibroblasts in the inner layer of the cortex and the outer layer of the medulla.[26] Although the mechanism underlying decreased erythropoietin production associated with renal fibrosis has not been elucidated completely, the decreased erythropoietin production in CKD may result from the transdifferentiation of erythropoietin-producing cells into scar-producing myofibroblasts, which lose their ability to produce erythropoietin.[4,29] In the present study, increased collagen fibers clearly indicate that renal fibrosis progressed in HPK. In addition, preliminary experiments including immunohistochemistry indicated increases in type I collagen and α-SMA-positive myofibroblasts in the interstitial tissue of 140-d-old HPK rats (data not shown). Moreover, renal erythropoietin mRNA expression decreased in HPK rats under normoxic conditions. Therefore, the normal plasma concentration of erythropoietin in HPK rats apparently reflects a compensatory increase in hepatic (and other) erythropoietin production.

During postnatal development, the major site of erythropoietin production switches from the liver to the kidneys.[10,44] In renal hypoplasia, therefore, the erythropoietin production capacity of the liver may be maintained longer. Responsive increase of serum erythropoietin concentrations in HPK rats under conditions of bleeding-induced hypoxia were accompanied by increased erythropoietin mRNA expression in both the kidneys and liver. These results suggest that, at least through 140 d of age, increased renal production of erythropoietin in response to severe hypoxia remains in HPK rats, although the sensitivity of renal erythropoietin-producing cells to the oxygen partial pressure under normoxic conditions might be impaired. Similarly, the induction of anemia reportedly counteracts the decreased expression of erythropoietin mRNA in transdifferentiated myofibroblasts in fibrotic kidneys.[4] Because of limitations in the experimental design and the number of rats used, we were unable to assess the time course of the hypoxic response in renal erythropoietin mRNA expression and the serum erythropoietin level of each rat. The broad standard deviation in the relative serum erythropoietin level in HPK rats at 140 d of age and the deaths of several HPK rats at 210 d of age during bleeding-induced hypoxia suggest that the maximal response might already be blunted at 140 to 210 d of age.

In the present study, we found that HPK rats demonstrated deterioration of renal excretory function, slightly macrocytic erythropenia, normal plasma erythropoietin levels accompanied by increased hepatic erythropoietin production, and splenic hemosiderosis accompanied by a tendency toward increased hemolysis during the progression of CKD.

## Acknowledgments

We thank all the members of our laboratory for their technical support and advice. This work was supported in part by a Grant for Pathophysiological Research Conference of Chronic Kidney Disease (to H Suzuki, JKFB 13-8 and 14-15) from the Kidney Foundation, Japan, and a Grant-in-Aid for Scientific Research (to H Suzuki, no. 26450414) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

## References

1. **Afshar R, Sanavi S, Salimi J, Ahmadzadeh M.** 2010. Hematological profile of chronic kidney disease (CKD) patients in Iran, in predialysis stages and after initiation of hemodialysis. Saudi J Kidney Dis Transpl **21:**368–371.
2. **Amakasu K, Suzuki K, Katayama K, Suzuki H.** 2011. Age-related pathophysiological changes in rats with unilateral renal agenesis. J Vet Med Sci **73:**787–795.
3. **Amakasu K, Suzuki K, Suzuki H.** 2009. The unilateral urogenital anomalies (UUA) rat: a new mutant strain associated with unilateral renal agenesis, cryptorchidism, and malformation of reproductive organs restricted to the left side. Comp Med **59:**249–256.
4. **Asada N, Takase M, Nakamura J, Oquchi A, Asade M, Suzuki N, Yamamura K, Nagoshi N, Shibata S, Rao TN, Fehling HJ, Fukatsu A, Minegishi N, Kita T, Kimura T, Okano H, Yamamoto M, Yanagita M.** 2011. Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. J Clin Invest **121:**3981–3990.
5. **Bertram JF, Douglas-Denton RN, Diouf B, Hughson MD, Hoy WE.** 2011. Human nephron number: implications for health and disease. Pediatr Nephrol **26:**1529–1533.
6. **Brecher G.** 1949. New methylene blue as a reticulocyte stain. Am J Clin Pathol **19:**895.
7. **Chiba J, Suzuki K, Suzuki H.** 2008. The petit rat (*pet/pet*), a new semilethal mutant dwarf rat with thymic and testicular anomalies. Comp Med **58:**551–559.
8. **Demant T, Mathes C, Gutlich K, Bedynek A, Steinhauer HB, Bosch T, Packard CJ, Warwick GL.** 1998. A simultaneous study of the metabolism of apolipoprotein B and albumin in nephrotic patients. Kidney Int **54:**2064–2080.
9. **Drukker A.** 2002. Oligonephropathy: from a rare childhood disorder to a possible health problem in the adult. Isr Med Assoc J **4:**191–195.
10. **Eckardt KU, Ratcliffe PJ, Tan CC, Bauer C, Kurtz A.** 1992. Age-dependent expression of the erythropoietin gene in rat liver and kidneys. J Clin Invest **89:**753–760.
11. **Elewa U, Sanchez-Nino MD, Martin-Cleary C, Fernandez-Fernandez B, Egido J, Ortiz A.** 2012. Cardiovascular risk biomarkers in CKD: the inflammation link and the road less traveled. Int Urol Nephrol **44:**1731–1744.
12. **Fukushima Y, Fukuda M, Yoshida K, Yamaguchi A, Nakamoto Y, Miura AB, Harada T, Tsuchida S.** 1986. Serum erythropoietin levels and inhibitors of erythropoiesis in patients with chronic renal failure. Tohoku J Exp Med **150:**1–15.
13. **Hajhosseiny R, Khavandi K, Goldsmith DJ.** 2012. Cardiovascular disease in chronic kidney disease: untying the Gordian knot. Int J Clin Pract **67:**14–31.

14. **Hancock DE, Onstad JW, Wolf PL.** 1976. Transferrin loss into the urine with hypochromic, microcytic anemia. Am J Clin Pathol **65:**73–78.
15. **Hernandez GT, Sippel M, Mukherjee D.** 2013. Interrelationship between chronic kidney disease and risk of cardiovascular disease. Cardiovasc Hematol Agents Med Chem **11:**38–43.
16. **Horiguchi H, Oguma E, Sakamoto T, Murata K, Kayama F.** 2013. Suppression of erythropoietin induction by diethylstilbestrol in rats. Arch Toxicol **88:**137–144.
17. **Hoy WE, Hughson MD, Singh GR, Douglas-Denton R, Bertram JF.** 2006. Reduced nephron number and glomerulomegaly in Australian Aborigines: a group at high risk for renal disease and hypertension. Kidney Int **70:**104–110.
18. **Hoy WE.** 2006. Lessons in ethnonephrology. Kidney Int **70:**251–257.
19. **Hughson M, Farris AB 3rd, Douglas-Denton R, Hoy WE, Bertram JF.** 2003. Glomerular number and size in autopsy kidney: the relationship to birth weight. Kidney Int **63:**2113–2122.
20. **Imai E, Matsuo S, Makino H, Watanabe T, Akizawa T, Nitta K, Iimuro S, Ohashi Y, Hishida A.** 2010. Chronic kidney disease Japan Cohort study: baseline characteristics and factors associated with causative diseases and renal function. Clin Exp Nephrol **14:**558–570.
21. **Kandasamy Y, Smith R, Wright IM.** 2011. Oligonaphropathy of prematurity. Am J Perinatol **29:**115–120.
22. **Katayama K, Yasuda H, Tochigi Y, Suzuki H.** 2012. The microtubule-associated protein astrin transgenesis rescues spermatogenesis and renal function in hypogonadic (*hgn/hgn*) rats. Andrology **1:**301–307.
23. **Kojima S, Haruta J, Enomoto A, Fujisawa H, Harada T, Maita K.** 1999. Age-related hematological changes in normal F344 rats: during the neonatal period. Exp Anim **48:**153–159.
24. **Lysaght MJ.** 2002. Maintenance dialysis population dynamics: current trends and long-term implications. J Am Soc Nephrol **13 Suppl 1:**S37–S40.
25. **Maugeais C, Braschi S, Ouguerram K, Maugeais P, Mahot P, Jacotot B, Darmaun D, Magot T, Krempf M.** 1997. Lipoprotein kinetics in patients with analbuminemia. Evidence for the role of serum albumin in controlling lipoprotein metabolism. Arterioscler Thromb Vasc Biol **17:**1369–1375.
26. **Obara N, Suzuki N, Kim K, Nagasawa T, Imagawa S, Yamamoto M.** 2008. Repression via the GATA box is essential for tissue-specific erythropoiesis gene expression. Blood **111:**5223–5232.
27. **Parmar MS.** 2002. Chronic renal disease. BMJ.**325:**85–90.
28. **Parpart AK, Lorenz PB, Parpart ER, Gregg JR, Chase AM.** 1947. The osmotic resistance (fragility) of human red cells. J Clin Invest **26:**636–640.
29. **Souma T, Yamazaki S, Moriguchi T, Suzuki N, Hirano I, Pan X, Minegishi N, Abe M, Kiyomoto H, Ito S, Yamamoto M.** 2013. Plasticity of renal erythropoietin-producing cells governs fibrosis. J Am Soc Nephrol **24:**1599–1616.
30. **Suzuki H, Daigo K, Okada M, Yagi M, Nakamiya H, Saito K, Suzuki K.** 2006. Influence of the hypogonadism (*hgn*) locus on female reproduction and ovarian development in an altered genetic background. Reprod Med Biol **5:**227–234.
31. **Suzuki H, Hakamata Y, Kamei T, Kikukawa K, Suzuki K.** 1992. Reduced fertility in female homozygotes for *hgn* (male hypogonadism) selected by *hgn*-associated hypoplastic kidney. Congenit Anom (Kyoto) **32:**167–178.
32. **Suzuki H, Suzuki K.** 1995. Pathophysiology and postnatal pathogenesis of hypoplastic kidney (*hpk/hpk*) in the male hypognadic mutant rat (*hgn/hgn*). J Vet Med Sci **57:**891–897.
33. **Suzuki H, Suzuki K.** 1998. Rat hypoplastic kidney (*hpk/hpk*) induces renal anemia, hyperparathyroidism, and osteodystrophy the end stage of renal failure. J Vet Med Sci **60:**1051–1058.
34. **Suzuki H, Takenaka M, Suzuki K.** 2007. Phenotypic characterization of spontaneously mutated rats showing lethal dwarfism and epilepsy. Comp Med **57:**360–369.
35. **Suzuki H, Tokuriki T, Kamita H, Oota C, Takasu M, Saito K, Suzuki K.** 2006. Age-related pathophysiological changes in rat oligomeganephronic hypoplastic kidney. Pediatr Nephrol **21:**637–642.
36. **Suzuki H, Tokuriki T, Saito K, Hishida A, Suzuki K.** 2005. Glomerular hyperfiltration and hypertrophy in the rat hypoplastic kidney as a model of oligomeganephronic disease. Nephrol Dial Transplant **20:**1362–1369.
37. **Suzuki H, Yagi M, Saito K, Suzuki K.** 2004. Dysplastic development of seminiferous tubules and interstitial tissue in rat hypogonadic (*hgn/hgn*) testes. Biol Reprod **71:**104–116.
38. **Suzuki H, Yagi M, Saito K, Suzuki K.** 2007. Embryonic pathogenesis of hypogonadism and renal hypoplasia in *hgn/hgn* rats characterized by male sterility,reduced female fertility and progressive renal insufficiency. Congenit Anom (Kyoto) **47:**34–44.
39. **Suzuki H, Yagi M, Suzuki K.** 2006. Duplicated insertion mutation in the microtubule-associated protein Spag5 (astrin/MAP126) and defective proliferation of immature Sertoli cells in rat hypogonadic (*hgn/hgn*) testes. Reproduction **132:**79–93.
40. **Suzuki K, Suzuki H, Hakamata Y, Kamei T, Kikukawa K.** 1991. Genetic analysis and histology of hypoplastic kidneys in the male hypogonadic mutant (*hgn/hgn*) rat. Congenit Anom (Kyoto) **31:**305–314.
41. **Suzuki K, Hakamata Y, Hamada A, Kikukawa K, Wada MY,** Imamichi T**.** 1988. Male hypogonadism as a candidate of deficiency of postnatal testicular growth or differentiating factor(s): a new autosomal recessive mutation in the rat. J Hered **79:**54–58.
42. **Turton JA, Hawkey CM, Hart MG, Gwynne J, Hicks RM.** 1989. Age-related changes in the haematology of female F344 rats. Lab Anim **23:**295–301.
43. **Yamaguchi-Yamada M, Manabe N, Uchio-Yamada K, Akashi N, Goto Y, Miyamoto Y, Nagao M, Yamamoto Y, Ogura A, Miyamoto H.** 2004. Anemia with chronic renal disorder and disrupted metabolism of erythropoietin in ICR-derived glomerulonephritis (ICGN) mice. J Vet Med Sci **66:**423–431.
44. **Zanjani ED, Ascensao JL, McGlave PB, Banisadre M, Ash RC.** 1981. Studies on the liver to kidney switch of erythropoietin production. J Clin Invest **67:**1183–1188.