


# The Transcription Factor Sox6 Controls Renin Expression during Renal Artery Stenosis

Mohammad Saleem, Luz Saavedra-Sánchez, Pierina Barturen-Larrea, and Jose A. Gomez 

## Key Points

- Sox6 controls the increased renin expression that is induced during renal artery stenosis, and thus has a novel function in renovascular hypertension.
- Sox6 knockout in Ren1d+ cells inhibited the renovascular hypertension and kidney injury induced by renal artery stenosis.
- The results presented in this manuscript point to a new transcriptional regulatory network in renal artery stenosis which is controlled by Sox6.

## Abstract

**Background** Renal artery stenosis (RAStenosis) or renal artery occlusion is an intractable problem affecting about 6% of people >65 and up to 40% of people with coronary or peripheral vascular disease in the United States. The renal renin-angiotensin-aldosterone system plays a key role in RAStenosis, with renin (which is mainly produced in the kidney) being recognized as the driver of the disease. In this study, we will determine a new function for the transcription factor Sox6 in the control of renal renin during RAStenosis.

**Methods** We hypothesize that knocking out Sox6 in Ren1d-positive cells will protect mice against renovascular hypertension and kidney injury. To test our hypothesis, we used a new transgenic mouse model, Ren1d<sup>cre</sup>/Sox6<sup>fl/fl</sup> (Sox6 KO), in which Sox6 is knocked out in renin-expressing cells. We used a modified two-kidney, one-clip (2K1C) Goldblatt mouse model to induce RAStenosis and renovascular hypertension. BP was measured using the tail-cuff method. Renin, prorenin, Sox6, and NGAL expressions levels were measured with Western blot, *in situ* hybridization, and immunohistochemistry. Creatinine levels were measured using the colorimetric assay.

**Results** Systolic BP was significantly lower in Sox6 KO 2 weeks after RAStenosis compared with Sox6 WT (Ren1d<sup>cre</sup>/Sox6<sup>wt/wt</sup>). Renin, prorenin, and NGAL expression levels in the stenosed kidney were lower in Sox6 KO compared with Sox6 WT mice. Furthermore, creatinine clearance was preserved in Sox6 KO compared with Sox6 WT mice.

**Conclusions** Our data indicate that Sox6 controls renal renin and prorenin expression and, as such, has a function in renovascular hypertension induced by RAStenosis. These results point to a novel transcriptional regulatory network controlled by Sox6.

KIDNEY360 2: 842–856, 2021. doi: <https://doi.org/10.34067/KID.0002792020>

## Introduction

Renal artery stenosis (RAStenosis) is a common condition in patients with atherosclerosis (1) and affects 5% of patients with hypertension (2). Progression to severe stenosis is well documented and leads to hypertension and kidney damage (3,4). RAStenosis is implicated in causing renovascular hypertension. Seminal studies demonstrating the link between vascular perfusion to the kidney and the development of hypertension remain fundamental to the field of BP research (5,6). Goldblatt two-kidney, one-clip (2K1C) animal models facilitated the discovery and

elucidation of the role played by the renin-angiotensin-aldosterone system (RAAS) in renovascular hypertension (6). We used a modified 2K1C mouse model to study renovascular hypertension during RAStenosis (5,7). In this model, the renal artery in one kidney is clipped to reduce blood flow and, as a result, the contralateral (noninjured) kidney compensates to maintain BP homeostasis (5). This phenomenon increases the synthesis and release of renin in the stenosed kidney. Since the discovery of renin about a century ago (8), renin has been implicated in hypertension, cardiac hypertrophy, and related cardiovascular diseases (9). RAAS

Clinical Pharmacology Division, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee

**Correspondence:** Dr. Jose A. Gomez, Clinical Pharmacology Division, Department of Medicine, Vanderbilt University Medical Center, 2220 Pierce Avenue, 502 RRB, Nashville, TN 37232-6602. Email: [jose.a.gomez@vumc.org](mailto:jose.a.gomez@vumc.org)

initiates signals involved in renovascular hypertension, AKI, and CKDs (10,11). Renin is mainly produced in the kidney and renal renin, being the rate-limiting protease in RAAS, is the key driver of renovascular hypertension during RAS stenosis (12). The treatments for renovascular hypertension targeting RAAS are effective in controlling hypertension, but may cause deterioration of kidney function (13). Moreover, the clinical trials targeting renal vascularization to improve disease outcomes failed to show any improvement in renal function, cardiovascular events, or mortality when added to complete multifactorial therapy (14–16). Therefore, it is imperative to find new therapeutic targets to regulate renin expression and renovascular or resistant hypertension during RAS stenosis. The exact molecular and genetic mechanism of renovascular hypertension caused by RAS stenosis has not yet been completely defined and warrants further investigation.

We recently reported that knocking out Sox6 in renin-expressing cells inhibited the increase in renin expression during sodium restriction and dehydration (17). Sox6 is a member of the Sry (sex determining region Y) subfamily of SoxD proteins (18) that activates or represses gene transcription through association with multiple transcription factors (19). It binds with other members of the Sox family, such as Sox5 and Sox9, during chondrogenesis (19). Moreover, Sox6 binds to various cofactors to regulate diverse cellular functions during embryonic development and adulthood (20–22). The renin promoter possesses the binding site for Sox6 (17) within the superenhancer region (23). In addition, Sox6 has been associated with hypertension in humans (24,25); however, no underlying mechanism has been described. We hypothesized that Sox6 controls increases in renin expression in the kidney during RAS stenosis, and that its knockout (KO)—specifically in Ren1d-positive (Ren1d+) cells—will contribute to protecting mice against the renovascular hypertension and kidney injury induced by RAS stenosis.

To define this novel function of Sox6, we used Ren1d<sup>Cre</sup>-Sox6<sup>fl/fl</sup> and Ren1d<sup>Cre</sup>-Sox6<sup>wt/wt</sup> mice. These animals will be used to define Sox6's function in renin regulation in the kidney and measure the effect of Sox6 ablation in Ren1d+ cells on renin expression, renovascular hypertension, and kidney injury during RAS stenosis. The results from this study suggest that knocking out Sox6 in renin-expressing cells contributes to protecting against renovascular hypertension. Moreover, kidney function is preserved and kidney injury markers are attenuated in the Sox6-KO, stenosed mice. Our data indicate that Sox6 is a novel transcription factor controlling prorenin and renin expression in the kidney during renovascular hypertension.

## Materials and Methods

### Animals

Mice were housed and cared for at the Vanderbilt University Medical Center (VUMC) Division of Animal Care, following the National Institutes of Health guidelines and the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the VUMC Institutional Animal Care and Use Committee before starting the experiments.

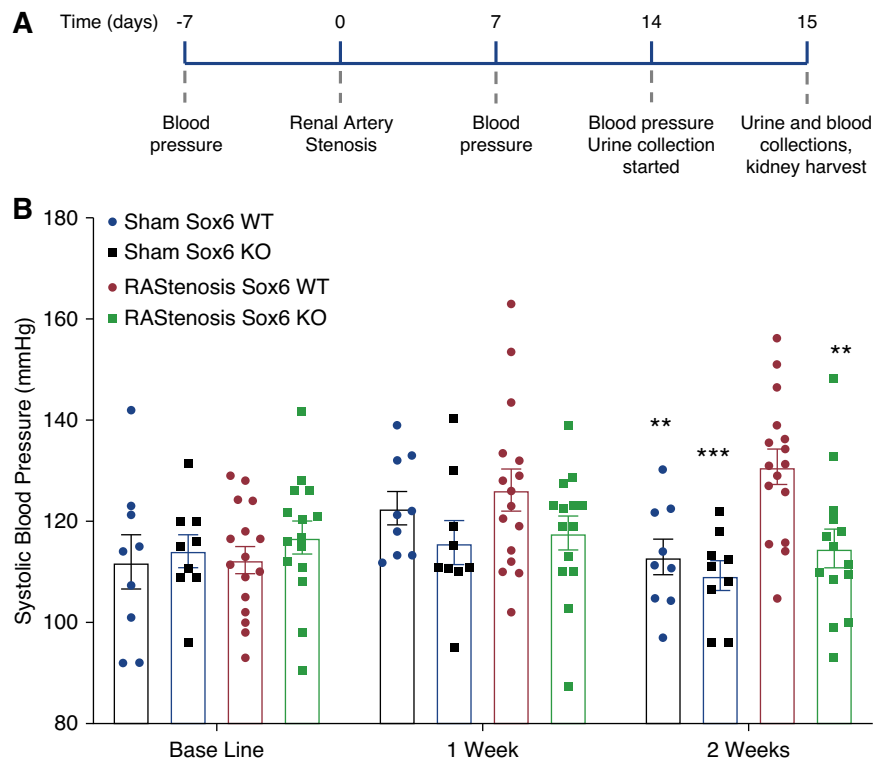
Ren1d<sup>Cre</sup> (26) and Sox6<sup>fl/fl</sup> (27) were crossed to obtain Ren1d<sup>Cre</sup>-Sox6<sup>fl/fl</sup> mice in the C57BL/6 background. The resulting mice, Ren1d<sup>Cre</sup>-Sox6<sup>fl/fl</sup>, do not express Sox6 in Ren1d+ cells. All of the animals used in the *in vivo* studies were maintained on a 12-hour light/dark cycle, at an ambient temperature of 24°C and 60% humidity. Six-week-old Ren1d<sup>Cre</sup>/Sox6<sup>fl/fl</sup> (Sox6-KO) mice and Ren1d<sup>Cre</sup>/Sox6<sup>wt/wt</sup> (Sox6 wild-type [WT]) control littermate mice, weighing 20–24 g, were used in this study. At the physiologic level, the Sox6-KO mice and WT littermates did not show any significant differences in body weight, physical appearance, movement, food consumption, or urine volume. The electrolyte composition in plasma and urine was similar in Sox6-KO mice and WT littermates. The precise numbers of animals used in each experiment are reported in the figure legends. Male and female mice were included in the study to determine sex differences. The sequences of the primers for genotyping are: Ren-Cre, Ren1d Cre primer, 5'-GAA GGA GAG CAA AAG GTA AGA G-3' (located in the renin promoter); Cre primer, 5'-TTG GTG TAC GGT CAG TAA ATT GGA C-3' (located in Cre); Ren1d, Ren1d Cre primer, 5'-GAA GGA GAG CAA AAG GTA AGA G-3' (located in the renin promoter of Ren1d and Ren1c); Ren1d, Ren1c, and Ren2 primer, 5'-GTA GTA GAA GGG GGA GTT GTG-3' (in the first renin intron of Ren1d, Ren1c, and Ren2); Sox6<sup>fl/fl</sup> forward primer, 5'-GTC ACT CAG AGG TTA CTA TGG TG-3'; Sox6<sup>fl/fl</sup> reverse primer, 5'-TTG GAG GCT TTA GCA GCT CTC-3'.

### BP Measurement

BP was measured using the tail-cuff method, following previously reported recommendations for precise measurements (28). BP was measured for two consecutive days 1 week before the surgery (Figure 1A). After surgery, mice were rested for 1 week to recover from the surgery, and BP was measured again for two consecutive days in week 1 and 2 after surgery. The BP measurements from the first and second day were averaged and reported. Three readings were recorded for each mouse each day (*N* stenosed WT=15, *N* stenosed KO=14, *N* sham WT=9, *N* sham KO=9).

### Surgical Procedure

The modified 2K1C murine model of renovascular hypertension was established by placing a polyurethane cuff on the right renal artery, according to a method previously reported with some modifications (29,30). We used this method because, according to Lorenz *et al.* (29), using conventional, U-designed silver clips induces a low success rate of hypertension (40%–60%) because the clip laterally presses the artery, triggering a few constrictions, and placement of a plastic cuff would result in a constriction in two dimensions (constriction) rather than one (flattening), as with a metal clip. Due to the variability in the levels of hypertension obtained with the conventional, U-design silver clip, Lorenz *et al.* (29) successfully used rounded polyurethane tubing to initiate RAS stenosis in mice to avoid these disadvantages. Therefore, we used a small segment of polyurethane tubing (internal diameter, 0.30 mm; outside diameter, 0.63 mm; wall thickness, 0.16 mm; MRE 025, Braintree Scientific), sliced lengthwise, to act as a cuff to produce a constriction around the right renal artery. After



**Figure 1. | Specific knock out of Sox6 in renin-expressing cells protect mice against renovascular hypertension induced by renal artery stenosis.** (A) Schematic representation of experimental design for 2-week study. BP was measured 1 week before and 2 weeks after surgery by tail-cuff method. BP was measured for two consecutive days each week. (B) Systolic BP.  $N$  stenosed wild type (WT)=16,  $N$  stenosed knockout (KO)=14,  $N$  sham WT=9,  $N$  sham KO=9. Data are presented as the mean $\pm$ SEM.  $P$  values calculated with two-way ANOVA followed by Tukey *post hoc* test. \*\* $P$ <0.01, \*\*\* $P$ <0.001, comparing all samples with Sox6-WT mice that underwent renal artery stenosis (RAS stenosis).

a subcutaneous injection of ketoprofen (dose, 5 mg/kg body wt), mice were anesthetized with intraperitoneal injections of ketamine (dose, 100 mg/kg body wt) and xylazine (dose, 10 mg/kg body wt). The tube was cut open lengthwise and placed around the right main renal artery, approximately equidistant between the aorta and renal bifurcation. The cuff was closed and held in place with two sutures. Sham mice underwent the same procedure, but the cuff was not placed. A subcutaneous injection of ketoprofen (dose, 5 mg/kg body wt) was given after 24 hours as postoperative analgesia.

#### Urine and Blood Collection

Individual metabolic cages (MMC100; Hatteras Instruments) were used for each mouse urine collection. To collect urine, metabolic cages were placed in a separate surgery room with a 12-hour light/dark cycle. Each mouse was placed in an individual cage at 9 AM for 24 hours of urine collection without food supply. Water was provided *ad libitum*. On the next day at 9 AM, mice were placed back into their respective, original cages and urine was collected and put on ice. Urine was centrifuged at  $1000 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes. Urine was aliquoted in Eppendorf tubes, flash frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for the biochemical analysis. For blood collection, mice were euthanized and blood was collected, by puncturing the heart with a syringe, and then processed with the anticoagulant EDTA. Plasma was obtained by centrifugation at  $2100 \times g$

for 10 minutes at room temperature (RT). Plasma was aliquoted in Eppendorf tubes, flash frozen in liquid nitrogen, and then stored at  $-80^{\circ}\text{C}$  for further analysis.

#### Creatinine Measurements

Urinary creatinine was measured using the colorimetric assay kit (item number 500701; Cayman Chemical). Urine was collected as mentioned above. Creatinine is a breakdown product of creatine phosphate, and urine creatinine clearance is an index of impairment and deterioration of kidney function (31). Urine was diluted  $10\times$ . Prepared standards and diluted urine samples ( $15 \mu\text{l}$  each) were added to designated wells and the reaction was initiated by adding  $150 \mu\text{l}$  alkaline picrate solution, which develops as a yellow/orange color. The covered plate was incubated on a shaker for 10 minutes at RT. The plate cover was then removed and initial absorbance was measured at 490 nm. The developed color was destroyed using an acidic solution, and the plate was again incubated for 20 minutes on a shaker at RT. The final absorbance was then measured at 490 nm. The difference in the color intensity before and after acidification is proportional to the creatinine concentration (31,32), so the initial absorbance was subtracted from the final absorbance to obtain the corrected absorbance. The adjusted absorbance of the standards was then plotted as a function of the final concentration of creatinine from table in the booklet from manufacturer. The creatinine concentration of the samples was calculated by using the following equation obtained

from the linear regression of the standard curve, substituting adjusted absorbance values for each sample: creatinine (mg/dl) = (sample absorbance – [y intercept]) / slope. The obtained values were then multiplied by the sample dilution.

### Western Blotting

Western blotting was performed as previously described (17). Briefly, kidney tissues were minced with a razor and homogenized with a Tissue-Tearor (model number 985370; BioSpec Products) following the manufacturer's instructions. Homogenates were lysed using lysis buffer (radioimmunoprecipitation assay (RIPA) buffer [catalog number R0278; Sigma], 1× protease inhibitor cocktail [catalog number P2714; Sigma], 1 mM PMSF [catalog number P20270; RPI]), sonicated for 30 seconds (three times for 10 seconds, with one 10-second interval between each sonication), and then centrifuged at  $16,000 \times g$  for 20 minutes at 4°C. After supernatant collection, samples were prepared in RIPA buffer, 1× Laemmli buffer (catalog number 1610747; Bio-Rad), and 10%  $\beta$ -mercaptoethanol (catalog number 1610710; Bio-Rad). Samples containing 30  $\mu$ g of protein were loaded into the gels. Tissue lysates were resolved on 8%–16% Tris-glycine gels (catalog number 456-1063; Bio-Rad) by SDS/PAGE. Gels were transblotted onto a polyvinylidene fluoride membrane for 2 hours at 4°C. Thereafter, membranes were blocked with 5% milk in Tris-buffered saline/Tween 20 (TBST) at RT for 1 hour, and then probed with the respective primary antibodies overnight at 4°C. After three washes with TBST (three times for 10 minutes), membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP; anti-mouse reference number, W4028; anti-rabbit reference number, W4018) followed by TBST washes (three times for 10 minutes). The chemiluminescent reagent Clarity Western ECL Substrate was used (catalog number 1705062; Bio-Rad) to visualize protein bands using the Bio-Rad image system. Protein bands were quantified and normalized using the housekeeping gene  $\beta$ -actin (catalog number A1978; Sigma-Aldrich), using software integrated within the image system. Primary antibody dilutions used were as follows: 1:100 for renin (catalog number sc-137252; Santa Cruz Biotechnology), 1:1000 for neutrophil gelatinase-associated lipocalin (NGAL; catalog number ab63929; Abcam), and 1:5000 for  $\beta$ -actin (catalog number A1978; Sigma-Aldrich). Secondary antibody (Promega) dilutions were used at 1:500. The renin band position and specificity was determined by using a renin peptide from Santa Cruz (catalog number sc-137252p) in a competition assay with the renin antibody (catalog number sc-137252). In addition, renin antibody specificity and distinction between prorenin and renin bands in Western blot were determined using commercial recombinant renin (catalog number 4277-AS; R&D Systems) and prorenin (catalog number AS-72174; AnaSpec) Western blot analysis (Supplemental Figure 1). The monoclonal primary antibody used for the detection of renin (from Santa Cruz Biotechnology) recognizes prorenin and mature renin. Our study and previous studies used commercially available recombinant prorenin and renin to confirm that the upper band (approximately 50 kD) and lower band (approximately 40 kD) represent prorenin and renin, respectively (17,33,34). We found these two distinct bands (corresponding to prorenin and

renin) in the Western blots of mice kidneys after low-sodium and furosemide treatment (17). To determine which protein was represented by each band, we used a competition assay and commercially available recombinant prorenin and renin in Western blot experiments as described in the *Materials and Methods* and Saleem et al. (17). Similarly, we detected two distinct bands of prorenin and renin in Western blots during RASenosis, and, using the same approach, we determined these two bands correspond to prorenin and renin.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed by following previously published protocols (17,35). Briefly, kidneys were perfusion fixed with 10% neutral buffered formalin solution, dehydrated in a graduated ethanol series, and embedded in paraffin. Kidney sections were cut at a thickness of 10  $\mu$ m. Histo-Clear solution (catalog number HS-202; National Diagnostics) was used to deparaffinize the sections, and they were then permeabilized with 0.2% Triton X-100 at RT. Thereafter, sections were blocked with 5% BSA-PBS at RT and incubated with primary antibodies prepared in 1% BSA-PBS overnight at 4°C. The next morning, sections were washed with PBS (three times for 5 minutes). After the three washes, sections were incubated with fluorochrome-conjugated secondary antibodies for 1 hour at RT. Anti-renin (10  $\mu$ g/ml, AF4277; R&D systems), anti-Sox6 (1:1000, ab30455; Abcam), and anti-aquaporin 2 (anti-Aq2; 1:1000, ab15116; Abcam) primary antibodies were used. The specificity of the Sox6 antibody was determined using tissues from Sox6-KO mice. In addition, a Sox6 peptide competition assay was performed using Sox6 peptide (ab30530; Abcam) to determine the specificity of the Sox6 antibody. The secondary antibodies were prepared in 1% BSA-PBS (1:500) and were chosen on the basis of the primary antibodies and Alexa Fluor fluorophores (Thermo Fisher). 4',6-Diamidino-2-phenylindole was used to counterstain the nuclei.

### Fluorescent *In Situ* Hybridization

Expression of Sox6, renin, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) mRNA was studied using the RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics [ACD] Bio-Techne, Newark, CA) for fluorescent *in situ* hybridization (ISH), following the manufacturer's protocols (17). Briefly, kidneys were perfusion fixed with 10% neutral buffered formalin solution, dehydrated in a graduated ethanol series, and embedded in paraffin. Kidney sections were cut at a thickness of 5  $\mu$ m. Sections were deparaffinized using Histo-Clear Solution, dehydrated in absolute ethanol at RT, and then the endogenous peroxidase was blocked using RNAscope Hydrogen Peroxide. The tissue was retrieved by boiling in target retrieval solution (ACD Bio-Techne) at 100°C–104°C for 15 minutes, and then treated with Protease Plus at 40°C for 30 minutes. Target probes (Mm-Ren1-O1, reference number 558571; Mm-Sox6-C2, reference number 472061-C3; Mm- $\alpha$ -SMA; designed by ACD Bio-Techne) were hybridized for 2 hours at 40°C, followed by a series of signal amplifications (amplifications 1–3), and, in between, washing with RNAscope Wash buffer (twice for 2 minutes). Renin, Sox6, and  $\alpha$ -SMA mRNA

**Table 1. Results in stenosed mice at a glance**

Parameters	Sox6-WT Mice	Sox6-KO Mice
BP	Increased BP	Protected against high BP
Prorenin and renin expression	Increased in stenosed kidney Repressed in contralateral kidney	Inhibited in both stenosed and contralateral kidneys
JG cell recruitment	Increased in stenosed kidney No JG cell recruitment in contralateral kidneys	Inhibited in both stenosed and contralateral kidneys
Colocalization of renin and Sox6 expression	Increased in stenosed kidney No colocalization in contralateral kidneys	Inhibited in both stenosed and contralateral kidneys
NGAL expression	Increased in stenosed kidney Decreased expression in contralateral kidney	Inhibited in stenosed kidney Decreased expression in contralateral kidney
Creatinine clearance	Diminished	Preserved

WT, wild type; KO, knockout; JG, juxtaglomerular; NGAL, neutrophil gelatinase-associated lipocalin.

probes were assigned to channels HRP-channel 1 (HRP-C1), HRP-C2, and HRP-C3, respectively. The HRP-C1 signal was developed using RNAscope Multiplex FL v2 HRP-C1 with Opal 520 fluorophore (reference number FP1487001KT; PerkinElmer), followed by blocking with RNAscope Multiplex FL v2 HRP blocker, and washing with RNAscope Wash buffer in between. Similarly, HRP-C2 and HRP-C3 signals were developed using Opal 650 (reference number FP1496001KT), and Opal 570 (reference number FP14001KT) fluorophores, respectively. HRP-C1, HRP-C2, and HRP-C3 were assigned to renin, Sox6, and  $\alpha$ -SMA probes, respectively. All hybridization steps at 40°C were performed in a HybEZ Hybridization System (ACD Bio-Techne). After the completion of the RNAscope assay, tissue sections were counterstained with 4',6-diamidino-2-phenylindole; incubated for 30 seconds at RT; mounted onto slides using VectaMount mounting medium (H-500; Vector Laboratories, Burlingame, CA); and the slides were then dried, overnight, in the dark, at RT. The tissue sections were viewed using a Nikon Eclipse Ti (software NIS-Elements AR 4.40.00, 64-bit).

### Randomization and Blinding

Two separate, blinded investigators counted the glomeruli containing renin, Sox6 double-positive cells, and renin and Aq2 colocalization in connective tubules (CNTs) and collecting ducts (CDs). Investigators were blinded with respect to animal identifiers and group assignments. Values were averaged between the two investigators.

### Statistical Analyses

Two-way ANOVA was used for experiments with three or more conditions, followed by Tukey tests for comparisons between individual groups. A *P* value of  $\leq 0.05$  was considered significant. All statistical analyses were performed using GraphPad Prism 8.2.

### Results

For a summary of all of the results from stenosed and sham mice, see Tables 1 and 2, respectively.

#### Effect of Sox6 KO on BP Control during RAStenosis-Induced Hypertension

To determine the role of the transcription factor Sox6 in BP control, a mouse model with Sox6 KO in Ren1d+ cells was used, the Ren1d<sup>cre</sup>/Sox6<sup>fl/fl</sup> (Sox6-KO) mouse (17). Two weeks after surgery, Sox6-WT mice exhibit significantly higher systolic BP (Figure 1, A and B) when compared with sham Sox6-WT and sham-KO mice. Sox6-KO mice did not develop high BP after 2 weeks of RAStenosis. These mice exhibit systolic BPs (Figure 1B) similar to sham Sox6-WT and sham-KO mice. Analysis of BP in male and female mice showed no differences due to sex (Supplemental Figure 2).

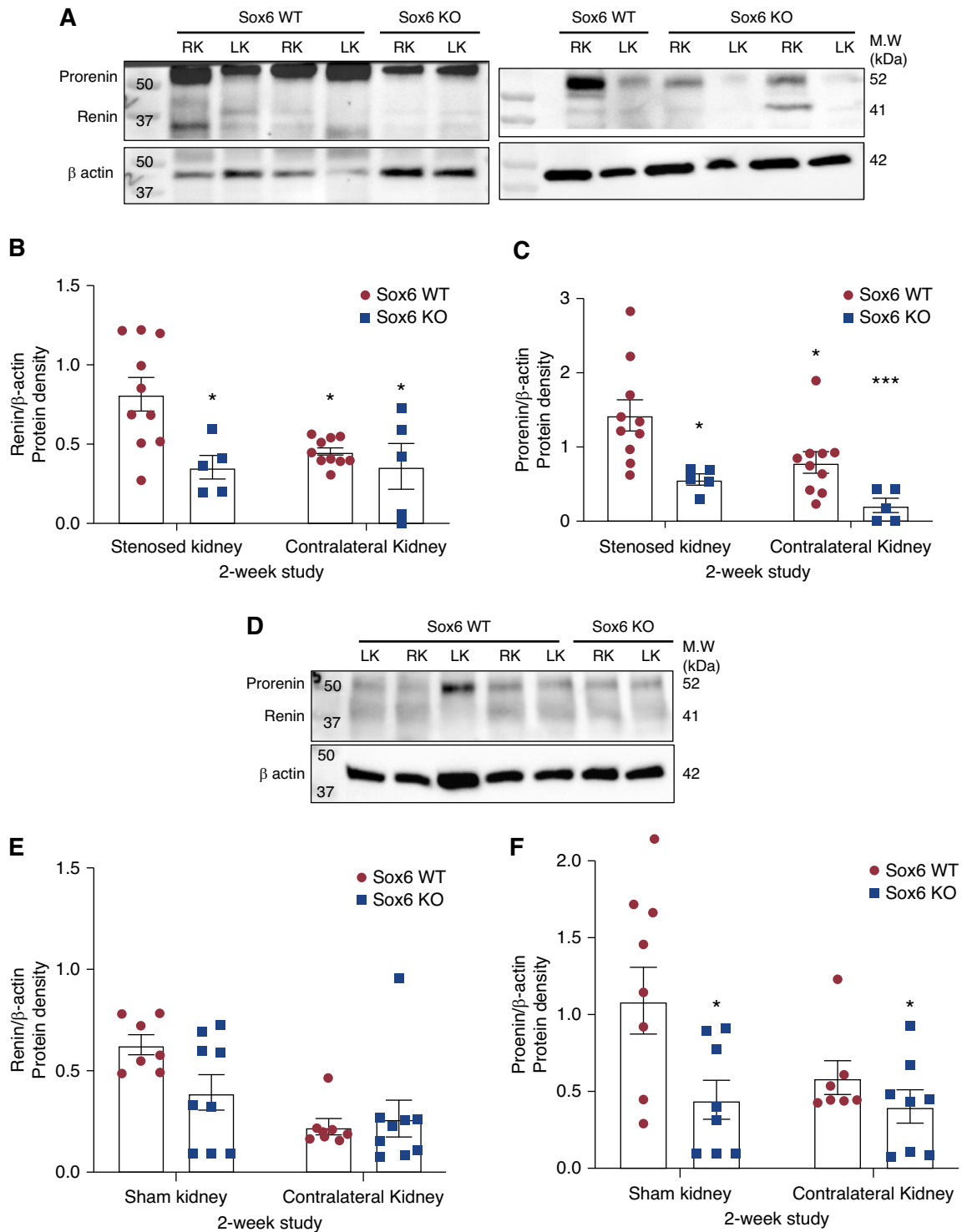
#### Effect of Knocking Out Sox6 on Renin-Expression Control during Renovascular Hypertension Induced by RAStenosis

Renin is mainly produced in the kidney and catalyzes the production of angiotensin I (Ang I) from angiotensinogen, which is the rate-limiting step in the production of the vasoconstrictor Ang II. To determine the effect of Sox6 on

**Table 2. Results in sham mice at a glance**

Parameters	Sox6-WT Mice	Sox6-KO Mice
BP	Normal	Normal
Prorenin and renin expression	Did not increase in both sham and in contralateral kidneys, especially with IHC and ISH	Hard to detect in both sham and in contralateral kidneys, especially with IHC and ISH
JG cell recruitment	No JG cell recruitment in both sham and in contralateral kidneys	No JG cell recruitment in both sham and in contralateral kidneys
NGAL expression	Decreased expression in both sham and in contralateral kidneys	Decreased expression in both sham and in contralateral kidneys
Creatinine clearance	Preserved	Preserved

WT, wild type; KO, knockout; IHC, immunohistochemistry; ISH, *in situ* hybridization; JG, juxtaglomerular; NGAL, neutrophil gelatinase-associated lipocalin.



**Figure 2. | Specific KO of Sox6 in Ren1d+ cells inhibits renin expression in the stenosed kidney during RASstenosis.** Two weeks after RASstenosis surgery, kidneys were harvested and Western blot was performed. (A) Representative Western blots images showing levels of prorenin/renin expression in stenosed mice.  $\beta$ -Actin was used as a loading control. (B) Densitometric analysis of renin protein bands. (C) Densitometric analysis of prorenin protein bands. (D) Representative Western blot images showing levels of prorenin/renin expression in sham animals. (E) Densitometric analysis of renin protein bands. (F) Densitometric analysis of prorenin protein bands. To show the specificity of the antibody, we performed the experiments using commercially available recombinant prorenin and renin proteins and renin peptide. Results are shown in Supplemental Figure 1. *N* stenosed WT=10, *N* stenosed KO=5, *N* sham WT=9, *N* sham KO=9. Data are presented as the mean  $\pm$  SEM. *P* values calculated with two-way ANOVA followed by Tukey *post hoc* test. \**P*<0.05, \*\*\**P*<0.01, comparing all samples to Sox6-WT mice that underwent RASstenosis. LK, left kidney; RK, right kidney.

renin expression in the kidney during RAS stenosis, we measured the levels of renin and prorenin in the kidney cortices (using Western blot) 2 weeks after surgery, as described in the *Materials and Methods* section. When stenosed kidneys were compared, the expression levels of prorenin and renin were significantly higher in Sox6-WT compared with Sox6-KO mice (Figure 2, A–C). The increase in renal renin expression caused an increase in systolic BP in Sox6-WT mice (Figure 1B). When sham kidneys were compared, the expression of prorenin was significantly higher in Sox6-WT compared with Sox6-KO mice (Figure 2, D–F). However, in sham kidneys, the expression of renin was not significantly higher in Sox6-WT compared with Sox6-KO mice (Figure 2D). When prorenin and renin expression was analyzed by sex, there were no significant differences detected (Supplemental Figure 3).

#### **Initial Effect of Knocking Out Sox6 in Ren1d+ Cells on Renin Expression Control during Renovascular Hypertension Induced by RAS stenosis**

To determine the initial effects of Sox6 on renin gene expression during RAS stenosis (Figure 3A), renin and prorenin expression levels were measured in kidney cortices after 3 days of RAS stenosis, using Western blot. When stenosed kidneys were compared, the expression levels of prorenin and renin were significantly higher in Sox6-WT compared with Sox6-KO mice (Figure 3, B–D). When sham kidneys were compared, the expression levels of renin and prorenin were not different between Sox6-WT and Sox6-KO mice (Figure 3, B, E, and F). The levels of expression of renin and prorenin in the contralateral kidneys in sham animals were similar in both Sox6-KO and Sox6-WT mice (Figure 3, B, E, and F). We did not find differences in prorenin and renin expression due to sex (Supplemental Figure 4).

#### **Effect of Knocking Out Sox6 in Ren1d+ Cells on Renin Expression and Recruitment of Juxtaglomerular Cells during Renovascular Hypertension Induced by RAS stenosis**

To establish the expression of renin and Sox6, and their colocalization in the kidney, we performed IHC. Renin expression was higher in stenosed kidneys from Sox6-WT mice and they exhibited juxtaglomerular (JG) cell recruitment along the afferent arteriole (Figure 4A, upper panel). The increased renin expression and JG cell recruitment was inhibited in stenosed kidney from Sox6-KO mice (Figure 4A, lower panel). The number of glomeruli showing renin and Sox6 colocalization and JG cell recruitment was significantly higher in Sox6-WT than in Sox6-KO mice when stenosed kidneys were compared (Figure 4, A and B). Moreover, the number of glomeruli with JG cell recruitment was significantly higher in stenosed kidneys from Sox6-WT and Sox6-KO mice compared with the contralateral kidneys from Sox6-WT and Sox6-KO mice (Figure 4B). Except in a few glomeruli, renin expression was not detected in the contralateral kidneys from the stenosed Sox6-WT or Sox6-KO mice (Figure 4B, Supplemental Figure 5, upper and lower panels). In sham mice, expression of renin was detected in both kidneys (sham and contralateral) from Sox6-WT mice; however, there were no differences in renin expression between the kidneys (Supplemental Figure 6, A and B, upper panels). A total of 300 glomeruli per kidney were counted in sham mice. The number of

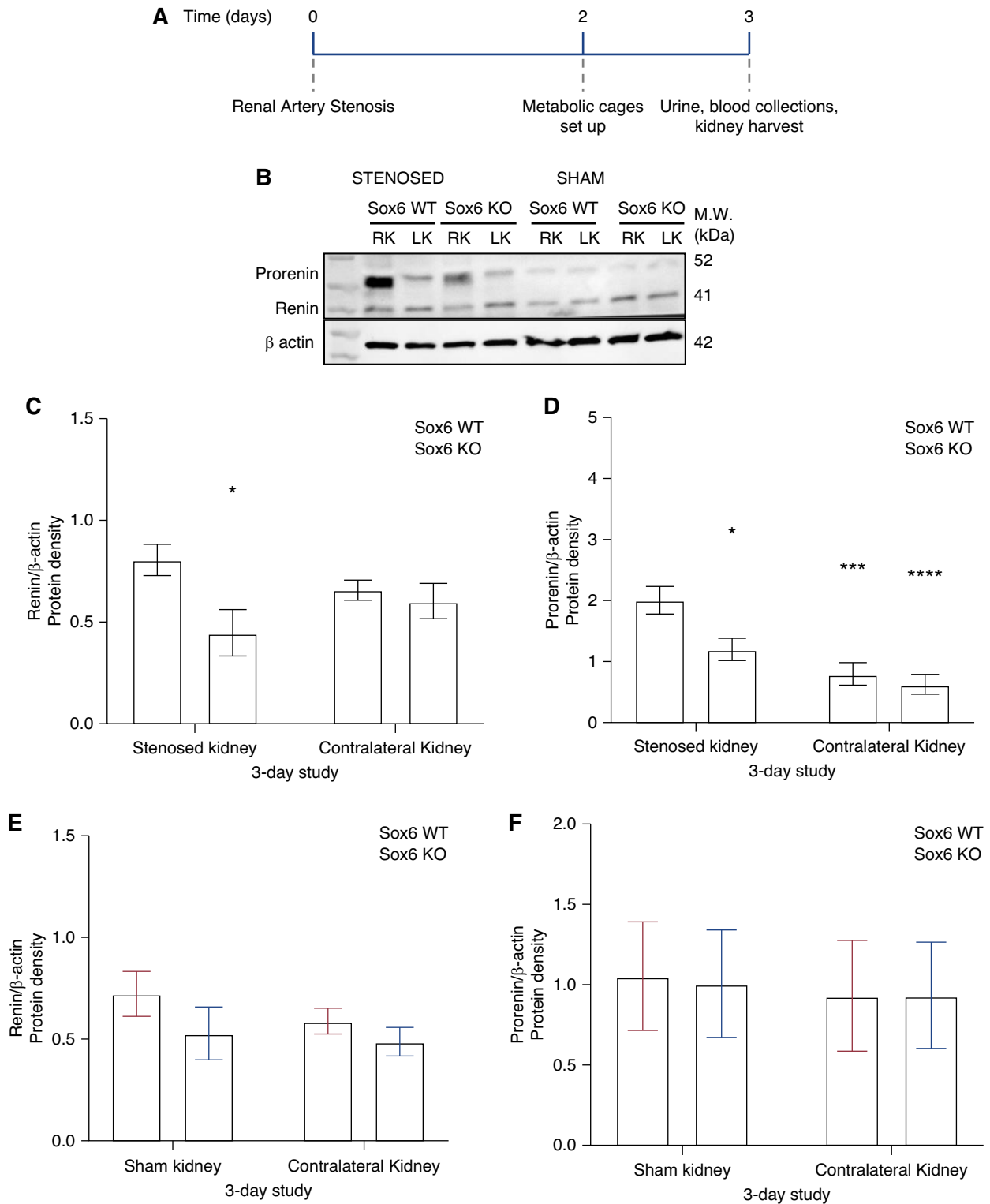
glomeruli expressing renin was significantly higher in both sham and the contralateral kidneys compared to the respective kidneys from Sox-KO mice (Supplemental Figure 6C). Next, we measured renin expression in the CNTs and CDs using Aq2 as a marker for the CNT and CD regions of nephron (36). We found that renin expression colocalized with Aq2 both in CNTs and CDs in stenosed kidneys from Sox6-WT mice (Figure 4C, upper panel). Renin expression was significantly lower in the stenosed kidney from Sox6-KO mice (Figure 4C, lower panel). For the quantification, about 100 cells in both CNTs and CDs were counted, per kidney, for the visualization of renin and Aq2 colocalization in both stenosed and sham mice. When stenosed kidneys were compared, we found a significant increase in colocalization in Sox6-WT compared with Sox6-KO mice (Supplemental Figure 6D). In sham kidneys, we did not detect any colocalization of renin and Aq2 in Sox6-WT and Sox6-KO mice. Similarly, CNT and CD renin and Aq2 colocalization was not detected in the contralateral kidneys; colocalization was also not found in Sox6-WT or Sox6-KO stenosed or sham mice (data not shown for sham mice). There were no differences in the parameters analyzed above due to sex (Supplemental Figure 7).

#### **Effect of Knocking Out Sox6 on Renin mRNA Expression and Recruitment of JG Cells during Renovascular Hypertension Induced by RAS stenosis**

To determine the expression of renin, Sox6, and  $\alpha$ -SMA mRNA, fluorescent ISH was performed in kidneys after 3 days of RAS stenosis. The number of glomeruli showing recruitment of JG cells was significantly higher in Sox6-WT than Sox6-KO mice when stenosed kidneys were compared (Figure 5, A–C). The expression of renin mRNA or recruitment of JG cells was not detected in contralateral kidneys from stenosed Sox6-WT or Sox6-KO mice (Supplemental Figure 8, upper and lower panels). In sham mice, expression of renin mRNA was detected in both sham and contralateral kidneys from Sox6-WT mice, but expression was absent in both kidneys from Sox6-KO mice (Supplemental Figure 9, A and B, upper and lower panels). When 300 glomeruli were counted per kidney in sham mice, the number of glomeruli expressing renin mRNA was significantly higher in both sham and contralateral kidneys compared with the respective kidneys from Sox-KO mice (Supplemental Figure 9C). We did not find any differences in renin mRNA expression due to sex in the stenosed animals (Supplemental Figure 10).

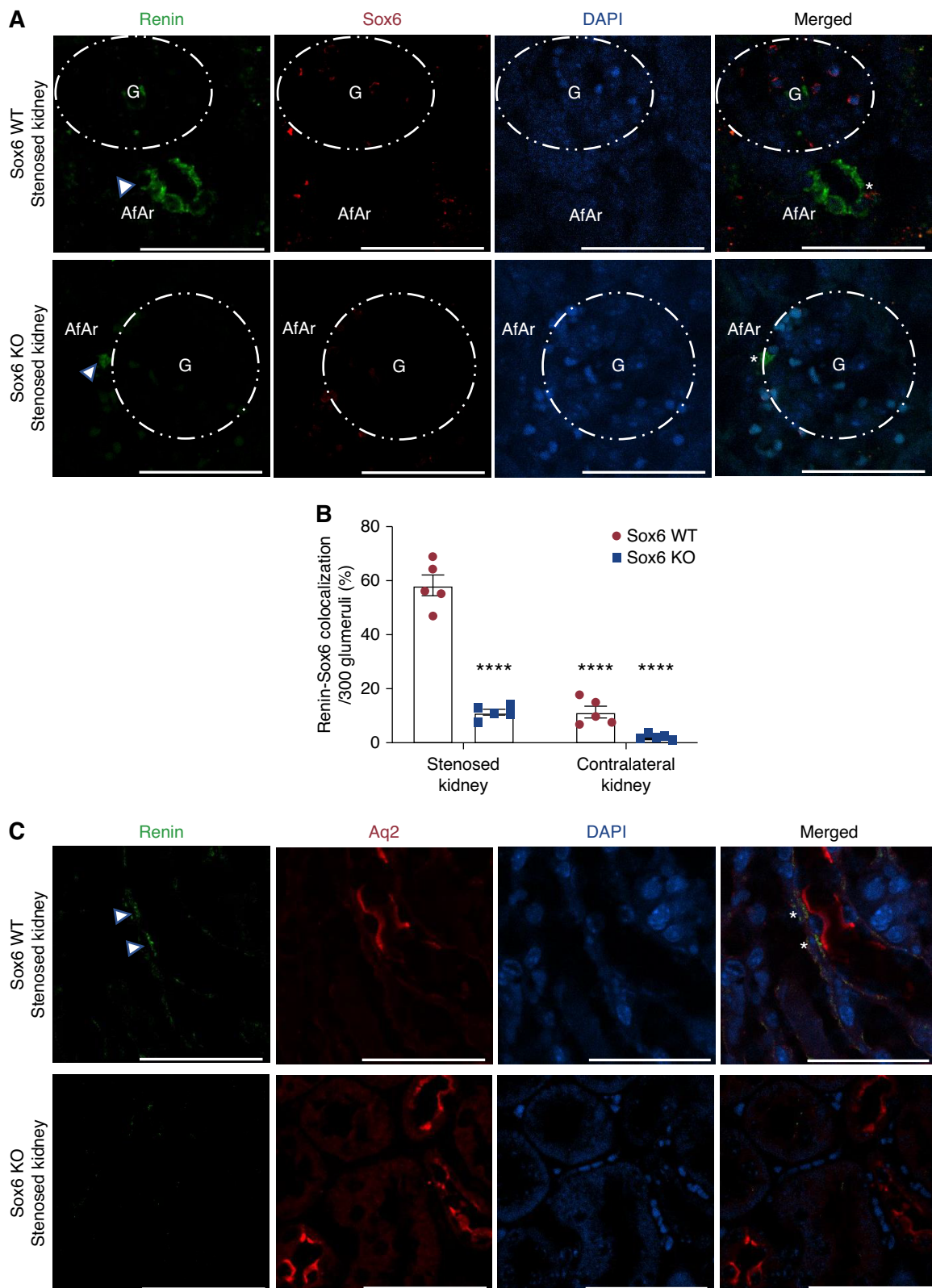
#### **Effect of Knocking Out Sox6 on AKI during Renovascular Hypertension Induced by RAS stenosis**

Next, we evaluated the effects of knocking out Sox6 in renin-expressing cells in RAS stenosis-induced kidney injury. We measured the AKI marker NGAL using Western blot. When stenosed kidneys were compared, the expression level of NGAL was significantly higher in Sox6-WT compared with Sox6-KO mice (Figure 6, A and B). Also, the levels of NGAL expression in stenosed kidneys from Sox6-WT mice was significantly higher than that in the contralateral kidneys of both Sox6-WT and Sox6-KO mice (Figure 6, A and B), and these differences were not attributable to sex (Supplemental Figure 11).

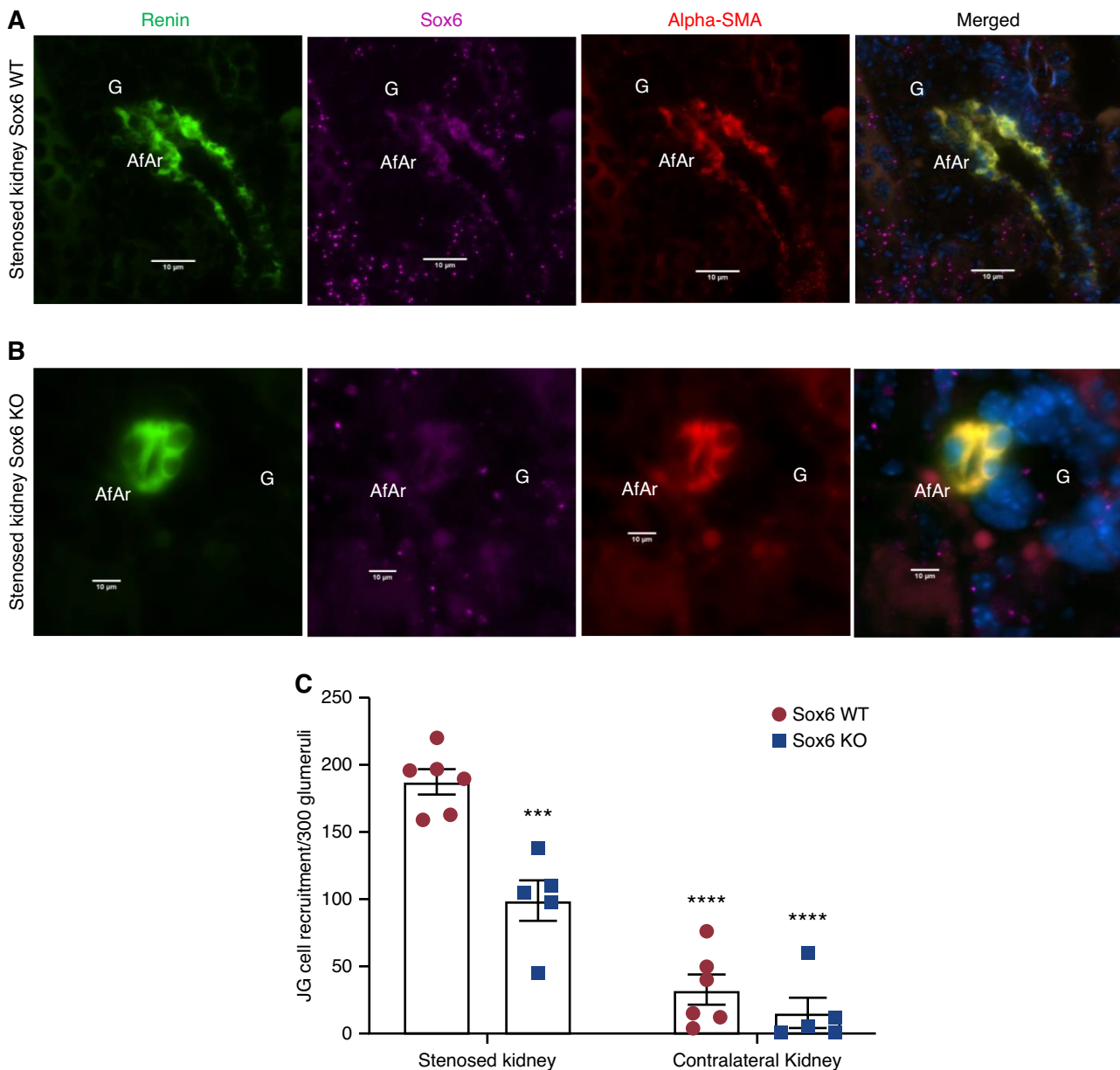


**Figure 3. | Specific KO of Sox6 in Ren1d+ cells inhibits renin expression in the stenosed kidney acutely.** (A) Schematic representation of experimental design for 3-day study. Three days after surgery, kidneys were harvested and Western blot was performed. (B) Representative Western blots images showing levels of prorenin/renin expression in stenosed and sham mice.  $\beta$ -Actin was used as a loading control. Densitometric analysis of (C) renin protein bands in stenosed mice, (D) prorenin protein bands in stenosed mice, (E) renin protein bands in sham-operated mice, and (F) prorenin protein bands in sham-operated mice. *N* stenosed WT=16, *N* stenosed KO=11, *N* sham WT=4, *N* sham KO=4. Data are presented as the mean $\pm$ SEM. *P* values calculated with two-way ANOVA followed by Tukey *post hoc* test. \**P*<0.05, \*\*\**P*<0.001, \*\*\*\**P*<0.0001, comparing all samples with Sox6-WT stenosed kidney.





**Figure 4. | Specific KO of Sox6 in Ren1d+ cells inhibits renin expression in the stenosed kidney during RAS stenosis.** Three days after surgery, kidneys were harvested, fixed, and immunohistochemistry was performed. Representative fluorescence microscopy images are shown. (A) Upper panel shows expression of renin (green), Sox6 (red) and 4',6-diamidino-2-phenylindole (DAPI; blue) in stenosed kidney from Sox6-WT mice. Similarly, the lower panel shows the expression of renin and Sox6 in stenosed kidney from Sox6-KO mice. Circle depicts glomeruli location,  $N=4$ . (B) Quantification of 300 glomeruli per sample with colocalization of renin and Sox6 expression along the afferent arteriole.  $N$  stenosed WT=5,  $N$  stenosed KO=5,  $N$  sham WT=4,  $N$  sham KO=5. Data are presented as the mean  $\pm$  SEM.  $P$  value calculated with two-way ANOVA followed by Tukey *post hoc* test. \*\*\*\* $P < 0.0001$ , comparing all samples to Sox6-WT mice that underwent RAS stenosis. (C) Specific KO of Sox6 in renin-expressing cells inhibits renin expression in connecting tubules (CNTs) and collecting ducts (CDs) in the stenosed kidney during RAS stenosis. Three days after surgery, kidneys were harvested, fixed, and immunohistochemistry was performed for renin and Aq2 colocalization in CNTs and CDs. Representative fluorescence microscopy images are shown. Upper panel shows the expression of renin (green), aquaporin 2 (Aq2, red), and DAPI (blue) in stenosed kidney from Sox6-WT mice. Similarly, lower panel shows the expression of renin (green) and Aq2 (red) in stenosed kidney from Sox6-KO mice.  $N$  stenosed WT=5,  $N$  stenosed KO=6,  $N$  sham WT=5,  $N$  sham KO=6. Arrowhead shows renin-expressing cells; \* shows the colocalization of renin and Sox6. Scale bar, 50  $\mu$ m. Original magnification,  $\times 60$ . AfAr, Afferent arteriole; G, glomeruli.



**Figure 5. | KO of Sox6 in Ren1d+ cells halts renin increases in mRNA expression and recruitment of juxtaglomerular cells along the afferent arteriole in the stenosed kidney.** Green staining represents renin mRNA expression; magenta represents expression of Sox6 mRNA; red, expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) mRNA; blue, nuclei. Representative microscopy images of stenosed kidneys from (A) Sox6-WT mice, and (B) Sox6-KO mice. Scale bar, 10  $\mu$ m. Original magnification,  $\times 90$ . (C) Quantification (300 glomeruli per kidney) of *in situ* hybridization analysis of renin mRNA expression along the afferent arteriole. *N* stenosed WT=6, *N* stenosed KO=5, *N* sham WT=6, *N* sham KO=5. Data are presented as the mean  $\pm$  SEM. *P* calculated with two-way ANOVA followed by Tukey *post hoc* test. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , comparing all samples with Sox6-WT mice that underwent RAStenosis. AfAr, afferent arteriole; G, glomerulus.

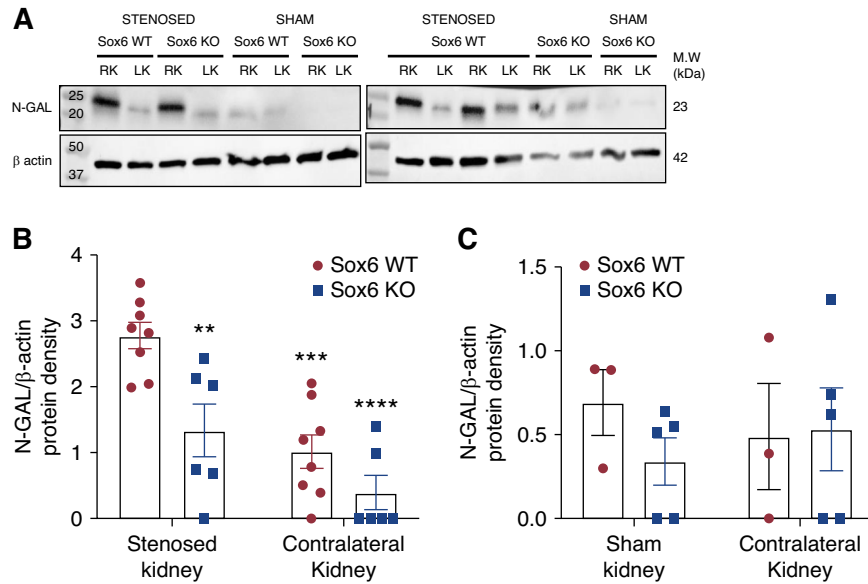
### Effect of Knocking Out Sox6 on Urine Creatinine Clearance during Renovascular Hypertension Induced by RAStenosis

Urine creatinine clearance determines kidney injury and function (37). Urine creatinine clearance after stenosis was significantly lower in Sox6-WT compared with Sox6-KO mice (Figure 7, A and B). We analyzed the data on the basis of sex and found no differences due to this biologic variable (Supplemental Figure 12). The levels of creatinine clearance were similar in Sox6-WT sham and Sox6-KO

sham mice, and were higher than the levels in the Sox6-WT stenosed mice (Figure 7, A and B). Furthermore, creatinine clearance in Sox6-KO stenosed mice was similar to that in the sham-operated mice (Figure 7, A and B).

### Discussion

In this study, we used the Sox6-KO mouse model to determine the function of Sox6 in renovascular hypertension



**Figure 6. | Specific KO of Sox6 in renin-expressing cells protects kidney against injury.** Neutrophil gelatinase-associated lipocalin (NGAL) was measured using Western blot in stenosed and sham mice. Three days after surgery, kidneys were harvested, and Western blot was performed. (A) Representative Western blots showing levels of NGAL expression in stenosed and sham animals.  $\beta$ -Actin was used as a loading control. (B) Densitometric analysis of NGAL protein bands from stenosed mice. (C) Densitometric analysis of NGAL protein bands from sham mice.  $N$  stenosed WT=8,  $N$  stenosed KO=6,  $N$  sham WT=3,  $N$  sham KO=5. Data are presented as the mean  $\pm$  SEM.  $P$  calculated with two-way ANOVA followed by Tukey *post hoc* test. \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001, comparing all samples with Sox6-WT mice that underwent RAS-tenosis. LK, left kidney; RK, right kidney.

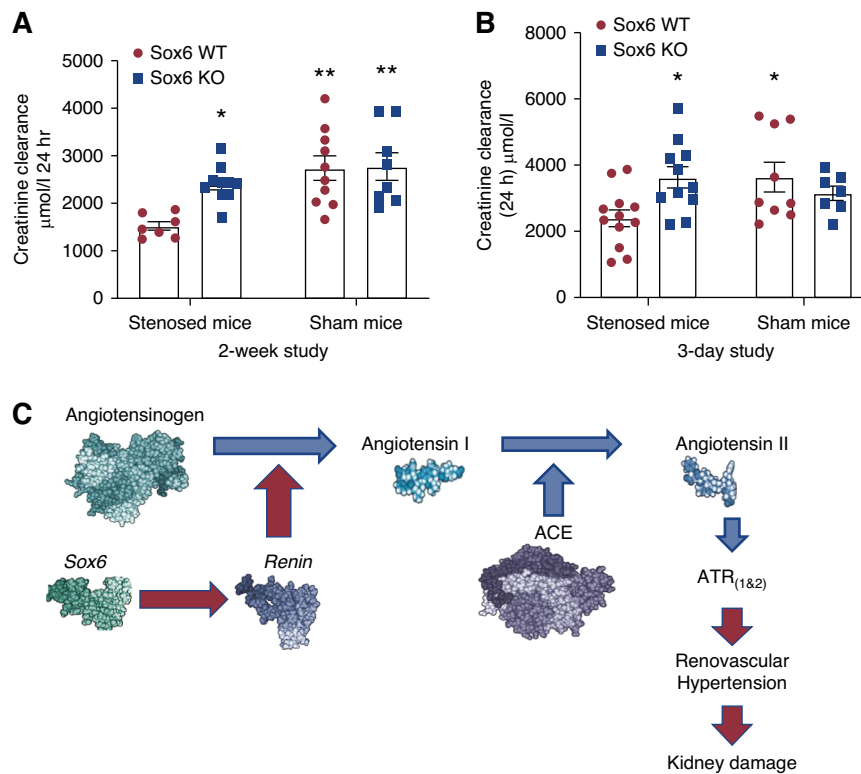
and kidney injury induced by RAS-tenosis. We used a modified 2K1C mouse model (7). A number of previous studies using various animal models have shown that stenosis in the renal artery is a strong stimulator of renin overexpression and release. In turn, renin promotes kidney injury (7,38). Renin, being the rate-limiting enzyme in RAAS, is considered the key driver of renovascular hypertension (12,39). Our data indicate that renal renin and prorenin overexpression and recruitment of JG cells along the afferent arteriole were inhibited in the stenosed kidney from Sox6-KO mice, and these mice were protected against renovascular hypertension and kidney injury.

RAS-tenosis causes an increase in expression of prorenin and renin in the kidney of WT mice, and knocking out Sox6 in Ren1d+ cells halted this increase, resulting in inhibition of hypertension and kidney injury. Sox6 KO in renin-expressing cells reduced both prorenin and renin expression, which inhibited the renin-mediated production of Ang II and ameliorated the renovascular hypertension and kidney injury induced during RAS-tenosis.

Renin is synthesized as preprorenin, which is transferred into the endoplasmic reticulum, where the signal peptide is cleaved off, and then prorenin is directed to the Golgi apparatus (40–43). Protogranules containing prorenin and proteases (prohormone convertases, cathepsin B), in an acidic environment (pH 4–6), are required to cleave the prosegment off from the trans-Golgi network. These protogranules will become renin granules for the regulated pathway (40,42,44). Renin is secreted by a regulated pathway in dense-core vesicles, whereas prorenin is secreted by both constitutive and regulated pathways (41). The sorting of renin to the regulated pathway is not very efficient in the JG cells, and only

25% of produced renin is directed to the dense-core secretory granules, whereas 75% is secreted as prorenin in clear vesicles *via* the constitutive pathway. According to some reports, some prorenin is glycosylated and directed to dense-core secretory granules in regulated pathways, meaning 25% of the produced renin contains some proportion of prorenin in the secretory granules (40–42,45). Here, we show that, during RAS-tenosis, prorenin and renin protein expression increases in the kidneys of WT mice. The increase in prorenin correlates with its continuous release into circulation *via* the constitutive pathway (40–42). In the kidney, the majority of preprorenin synthesized in JG cells represents prorenin before its secretion into circulation, whether it is secreted by constitutive the pathway (in clear vesicles) or the regulated pathway (in dense-core vesicles). We found a significant increase in both renal prorenin and renin in RAS-tenosis. Concordantly, there was an increase in systemic BP, reflecting the effects of renal renin increase into circulating RAAS. Changes in renal perfusion cause prorenin and renin to be continuously synthesized during RAS-tenosis (42). All of these factors add to the evidence that prorenin is the predominant protein form processed and secreted by JG cells during renal stenosis and other chronic stimulation resulting in renin release. Moreover, our results are in line with recently published reports regarding the intensity and position of the prorenin and renin bands, because these researchers found greater band density for prorenin than renin in mouse and rat kidney tissues in Western blots (33,46,47).

We recently reported that ablation of Sox6 in Ren1d+ cells inhibited the increase in renin expression and recruitment of JG cells during sodium restriction and dehydration (17). Previous studies reported that this process involves



**Figure 7. | Specific KO of Sox6 in renin-expressing cells preserves creatinine clearance.** Creatinine was measured using a colorimetric kit, following the manufacturer's instructions (A) Creatinine analysis from 2-week study. *N* stenosed WT=7, *N* stenosed KO=10, *N* sham WT=10, *N* sham KO=8. (B) Creatinine analysis from 3-day study. *N* stenosed WT=12, *N* stenosed KO=11, *N* sham WT=9, *N* sham KO=7. Data are presented as the mean±SEM. *P* calculated with two-way ANOVA followed by Tukey *post hoc* test. \**P*<0.05, \*\**P*<0.01, comparing all samples with Sox6-WT mice that underwent RAStenosis. (C). Schematic representation of the function of the transcription factor Sox6 in the regulation of renin expression, which has an effect on hypertension and kidney damage during renovascular hypertension induced by RAStenosis. ACE, angiotensin-converting enzyme; ATR<sub>(1&2)</sub>, angiotensin type 1 and 2 receptors.

the transdifferentiation of vascular smooth muscle cells (VSMCs) (26). Moreover, we reported that the adult kidney harbors mesenchymal stem cells that differentiate into JG cells and participate in JG cell recruitment (35). In this study, fluorescent ISH data show that the number of renin-positive cells and recruitment of JG cells increased in stenosed kidneys from Sox6-WT mice, and that this was significantly inhibited in stenosed kidneys from Sox6-KO mice. ISH data also show the colocalization of renin and Sox6 expression in stenosed WT kidney, which was absent in stenosed KO kidney. ISH data were corroborated by IHC results. We used specific probes for  $\alpha$ -SMA and determined that VSMCs transdifferentiate into renin-positive cells and participate in JG cell recruitment. The transdifferentiation process was absent in stenosed kidney from Sox6-KO mice. These data indicate that Sox6 modulates the recruitment of JG cells induced by RAStenosis.

It is well established that, during renovascular hypertension, the function of the stenosed kidney deteriorates and excretion of nitrogenous waste products decreases due to glomeruli injury (48). As mentioned above, ablation of Sox6 in Ren1d+ cells inhibits the increase in renin expression, the key driver of renovascular hypertension, which produced kidney injury in RAStenosis. Our data also show that creatinine clearance was significantly diminished in

stenosed WT mice, but was preserved in Sox6-KO mice, suggesting the preservation of GFR and kidney function in Sox6-KO mice. Renovascular hypertension is known to damage the kidney due to changes in kidney perfusion and rarefaction of tissue, and is, therefore, associated with some changes in the expression of kidney injury markers (49). Because NGAL is the most studied biomarker of AKI (50), we measured NGAL expression in kidney tissues and found that NGAL expression was increased in stenosed kidneys from Sox6-WT mice and was inhibited in stenosed kidneys from Sox6-KO mice. These data suggest that knocking out Sox6 from renin-expressing cells is protective against kidney injury (NGAL expression) and preserves kidney function (urine creatinine clearance) in Sox6-KO mice during renovascular hypertension induced by RAStenosis.

The data presented in this report support a novel function for the transcription factor Sox6 in the regulation of renin and prorenin expression, BP, and kidney injury during renovascular hypertension induced by RAStenosis (Figure 7C). Moreover, analysis of data on the basis of sex indicates that the differences in the parameters presented here are due to Sox6's actions in the Ren1d+ cells and not due to sex. Future studies are needed to define the function of Sox6 regulation of RAStenosis-induced oxidative stress in the kidney. Defining the novel molecular pathway

controlled by Sox6 may lead to a new therapeutic target for the treatment of renovascular or resistant hypertension, kidney injury, and associated cardiovascular diseases.

#### Disclosures

All authors have nothing to disclose.

#### Funding

This research was supported by National Heart, Lung, and Blood Institute <https://doi.org/10.13039/100000050> Research Scientist Development grant 1K01HL135461 (to J.A. Gomez).

#### Acknowledgments

The authors would like to thank Dr. R. Ariel Gomez for kindly providing the Ren1dCre mice, and Dr. Monique Lefebvre from the Cleveland Clinic for generously providing the Sox6<sup>f/f</sup> transgenic mice.

#### Author Contributions

P. Barturen-Larrea, J.A. Gomez, L. Saavedra-Sánchez, and M. Saleem were responsible for data collection and investigation; P. Barturen-Larrea, L. Saavedra-Sánchez, and M. Saleem wrote the original draft; J.A. Gomez conceptualized the study, reviewed and edited the manuscript, provided supervision, and was responsible for funding acquisition, project administration, and resources; and J.A. Gomez and M. Saleem were responsible for formal analysis and methodology.

#### Supplemental Material

This article contains the following supplemental material online at <http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0002792020/-/DCSupplemental>.

Supplemental Figure 1. Competition assay with renin peptide, and recombinant prorenin and renin experiments using Western blot show the antibody specificity and positions of distinct bands of both proteins respectively.

Supplemental Figure 2. There are no significant differences in systolic BP between males and females within each group in stenosed mice.

Supplemental Figure 3. There are no significant differences in renin and prorenin expressions between males and females within each group in stenosed mice.

Supplemental Figure 4. There are no significant differences in renin and prorenin expressions between males and females within each group in stenosed mice in acute settings.

Supplemental Figure 5. Knock out of Sox6 in renin expressing cells does not affect renin expression in the contralateral kidneys during renal artery stenosis.

Supplemental Figure 6. Knock out of Sox6 in renin expressing cells inhibits renin expression.

Supplemental Figure 7. There are no significant differences in co-localization of renin and Sox6 expression between males and females within each group in stenosed mice.

Supplemental Figure 8. Knock out of Sox6 in renin expressing cells does not affect mRNA levels of renin expression in the contralateral kidneys during renal artery stenosis.

Supplemental Figure 9. Knock out of Sox6 in renin expressing cells inhibits renin mRNA expression.

Supplemental Figure 10. There are no significant differences in JG cell recruitment between males and females within each group in stenosed mice.

Supplemental Figure 11. There are no significant differences in JG cell recruitment between males and females within each group in stenosed mice.

Supplemental Figure 12. There are no significant differences in creatinine clearance between males and females within each group in stenosed mice.

#### References

- Olin JW, Melia M, Young JR, Graor RA, Risius B: Prevalence of atherosclerotic renal artery stenosis in patients with atherosclerosis elsewhere. *Am J Med* 88: 46N–51N, 1990
- Muntner P, Carey RM, Gidding S, Jones DW, Taler SJ, Wright Jr. JT, Whelton PK: Potential US population impact of the 2017 ACC/AHA high blood pressure guideline. *Circulation* 137: 109–118, 2018
- Caps MT, Perissinotto C, Zierler RE, Polissar NL, Bergelin RO, Tullis MJ, Cantwell-Gab K, Davidson RC, Strandness Jr. DE: Prospective study of atherosclerotic disease progression in the renal artery. *Circulation* 98: 2866–2872, 1998
- Crowley JJ, Santos RM, Peter RH, Puma JA, Schwab SJ, Phillips HR, Stack RS, Conlon PJ: Progression of renal artery stenosis in patients undergoing cardiac catheterization. *Am Heart J* 136: 913–918, 1998
- Goldblatt H, Lynch J, Hanzal RF, Summerville WW: Studies on experimental hypertension: I. The production of persistent elevation of systolic blood pressure by means of renal ischemia. *J Exp Med* 59: 347–379, 1934
- Katz YJ, Goldblatt H: Studies on experimental hypertension: XXI. The purification of renin. *J Exp Med* 78: 67–74, 1943
- Gollan F, Richardson E, Goldblatt H: Hypertension in the systemic blood of animals with experimental renal hypertension. *J Exp Med* 88: 389–400, 1948
- Tigerstedt R, Bergman PQ. Niere und Kreislauf1. *Scand Arch Physiol* 8: 223–271, 1898
- Black HR, Glickman MG, Schiff Jr. M, Pingoud EG: Renovascular hypertension: Pathophysiology, diagnosis, and treatment. *Yale J Biol Med* 51: 635–654, 1978PubMed
- Chou YH, Huang TM, Chu TS: Novel insights into acute kidney injury-chronic kidney disease continuum and the role of renin-angiotensin system. *J Formos Med Assoc* 116: 652–659, 2017
- Webster AC, Nagler EV, Morton RL, Masson P: Chronic kidney disease. *Lancet* 389: 1238–1252, 2017
- Covic A, Gusbeth-Tatomir P: The role of the renin-angiotensin-aldosterone system in renal artery stenosis, renovascular hypertension, and ischemic nephropathy: Diagnostic implications. *Prog Cardiovasc Dis* 52: 204–208, 2009
- Mui KW, Sleswijk M, van den Hout H, van Baal J, Navis G, Woittiez AJ: Incidental renal artery stenosis is an independent predictor of mortality in patients with peripheral vascular disease. *J Am Soc Nephrol* 17: 2069–2074, 2006
- Cooper CJ, Murphy TP, Cutlip DE, Jamerson K, Henrich W, Reid DM, Cohen DJ, Matsumoto AH, Steffes M, Jaff MR, Prince MR, Lewis EF, Tuttle KR, Shapiro JL, Rundback JH, Massaro JM, D'Agostino Sr. RB, Dworkin LD; CORAL Investigators: Stenting and medical therapy for atherosclerotic renal-artery stenosis. *N Engl J Med* 370: 13–22, 2014
- Mistry S, Ives N, Harding J, Fitzpatrick-Ellis K, Lipkin G, Kalra PA, Moss J, Wheatley K: Angioplasty and Stent for renal artery lesions (ASTRAL trial): Rationale, methods and results so far. *J Hum Hypertens* 21: 511–515, 2007
- Cooper CJ, Murphy TP, Matsumoto A, Steffes M, Cohen DJ, Jaff M, Kuntz R, Jamerson K, Reid D, Rosenfield K, Rundback J, D'Agostino R, Henrich W, Dworkin L: Stent revascularization for the prevention of cardiovascular and renal events among patients with renal artery stenosis and systolic hypertension: Rationale and design of the CORAL trial. *Am Heart J* 152: 59–66, 2006
- Saleem M, Hodgkinson CP, Xiao L, Gimenez-Bastida JA, Rasmussen ML, Foss J, Payne AJ, Mirotsoy M, Gama V, Dzau VJ, Gomez JA: Sox6 as a new modulator of renin expression in the kidney. *Am J Physiol Renal Physiol* 318: F285–F297, 2020PubMed

18. Saleem M, Barturen-Larrea P, Gomez JA: Emerging roles of Sox6 in the renal and cardiovascular system. *Physiol Rep* 8: e14604, 2020
19. Lefebvre V: The SoxD transcription factors—Sox5, Sox6, and Sox13—are key cell fate modulators. *Int J Biochem Cell Biol* 42: 429–432, 2010
20. Iguchi H, Ikeda Y, Okamura M, Tanaka T, Urashima Y, Ohguchi H, Takayasu S, Kojima N, Iwasaki S, Ohashi R, Jiang S, Hasegawa G, Ioka RX, Magoori K, Sumi K, Maejima T, Uchida A, Naito M, Osborne TF, Yanagisawa M, Yamamoto TT, Kodama T, Sakai J: SOX6 attenuates glucose-stimulated insulin secretion by repressing PDX1 transcriptional activity and is down-regulated in hyperinsulinemic obese mice. *J Biol Chem* 280: 37669–37680, 2005
21. Iguchi H, Urashima Y, Inagaki Y, Ikeda Y, Okamura M, Tanaka T, Uchida A, Yamamoto TT, Kodama T, Sakai J: SOX6 suppresses cyclin D1 promoter activity by interacting with beta-catenin and histone deacetylase 1, and its down-regulation induces pancreatic beta-cell proliferation. *J Biol Chem* 282: 19052–19061, 2007
22. Yamashita A, Ito M, Takamatsu N, Shiba T: Characterization of Solt, a novel SoxLZ/Sox6 binding protein expressed in adult mouse testis. *FEBS Lett* 481: 147–151, 2000
23. Martinez MF, Medrano S, Brown EA, Tufan T, Shang S, Bertoncello N, Guessoum O, Adli M, Belyea BC, Sequeira-Lopez MLS, Gomez RA: Super-enhancers maintain renin-expressing cell identity and memory to preserve multi-system homeostasis. *J Clin Invest* 128: 4787–4803, 2018
24. Ganesh SK, Tragante V, Guo W, Guo Y, Lanktree MB, Smith EN, Johnson T, Castillo BA, Barnard J, Baumert J, Chang YP, Elbers CC, Farrall M, Fischer ME, Franceschini N, Gaunt TR, Gho JM, Gieger C, Gong Y, Isaacs A, Kleber ME, Mateo Leach I, McDonough CW, Meijis MF, Mellander O, Molony CM, Nolte IM, Padmanabhan S, Price TS, Rajagopalan R, Shaffer J, Shah S, Shen H, Soranzo N, van der Most PJ, Van Iperen EP, Van Setten J, Vonk JM, Zhang L, Beitelshes AL, Berenson GS, Bhatt DL, Boer JM, Boerwinkle E, Burkley B, Burt A, Chakravarti A, Chen W, Cooper-Dehoff RM, Curtis SP, Dreisbach A, Duggan D, Ehret GB, Fabsitz RR, Fornage M, Fox E, Furlong CE, Gansevoort RT, Hofker MH, Hovingh GK, Kirkland SA, Kottke-Marchant K, Kutlar A, Lacroix AZ, Langae TY, Li YR, Lin H, Liu K, Maiwald S, Malik R, Murugesan G, Newton-Cheh C, O'Connell JR, Onland-Moret NC, Ouweland WH, Palmas W, Penninx BW, Pepine CJ, Pettinger M, Polak JF, Ramachandran VS, Ranchalis J, Redline S, Ridker PM, Rose LM, Schrnag H, Schork NJ, Shimbo D, Shuldiner AR, Srinivasan SR, Stolk RP, Taylor HA, Thorand B, Trip MD, van Duijn CM, Verschuren WM, Wijmenga C, Winkelmann BR, Wyatt S, Young JH, Boehm BO, Caulfield MJ, Chasman DI, Davidson KW, Doevendans PA, Fitzgerald GA, Gums JG, Hakonarson H, Hillege HL, Illig T, Jarvik GP, Johnson JA, Kastelein JJ, Koenig W, März W, Mitchell BD, Murray SS, Oldehinkel AJ, Rader DJ, Reilly MP, Reiner AP, Schadt EE, Silverstein RL, Snieder H, Stanton AV, Uitterlinden AG, van der Harst P, van der Schouw YT, Samani NJ, Johnson AD, Munroe PB, de Bakker PI, Zhu X, Levy D, Keating BJ, Asselbergs FW; CARDIOGRAM, METASTROKE; Lifelines Cohort Study: Loci influencing blood pressure identified using a cardiovascular gene-centric array [published correction appears in *Hum Mol Genet* 22: 3394–3395, 2013]. *Hum Mol Genet* 22: 1663–1678, 2013
25. Johnson T, Gaunt TR, Newhouse SJ, Padmanabhan S, Tomaszewski M, Kumari M, Morris RW, Tzoulaki I, O'Brien ET, Poulter NR, Sever P, Shields DC, Thom S, Wannamethee SG, Whincup PH, Brown MJ, Connell JM, Dobson RJ, Howard PJ, Mein CA, Onipinla A, Shaw-Hawkins S, Zhang Y, Davey Smith G, Day IN, Lawlor DA, Goodall AH, Fowkes FG, Abecasis GR, Elliott P, Gateva V, Braund PS, Burton PR, Nelson CP, Tobin MD, van der Harst P, Glorioso N, Neuvirth H, Salvi E, Staessen JA, Stucchi A, Devos N, Jeunemaitre X, Plouin PF, Tichet J, Juhanson P, Org E, Putku M, Söber S, Veldre G, Viigimaa M, Levinsson A, Rosengren A, Thelle DS, Hastie CE, Hedner T, Lee WK, Melander O, Wahlstrand B, Hardy R, Wong A, Cooper JA, Palmen J, Chen L, Stewart AF, Wells GA, Westra HJ, Wolfs MG, Clarke R, Franzosi MG, Goel A, Hamsten A, Lathrop M, Peden JF, Seedorf U, Watkins H, Ouweland WH, Sambrook J, Stephens J, Casas JP, Drenos F, Holmes MV, Kivimaki M, Shah S, Shah T, Talmud PJ, Whittaker J, Wallace C, Delles C, Laan M, Kuh D, Humphries SE, Nyberg F, Cusi D, Roberts R, Newton-Cheh C, Franke L, Stanton AV, Dominiczak AF, Farrall M, Hingorani AD, Samani NJ, Caulfield MJ, Munroe PB; Cardio-genetics Consortium; Global BPgen Consortium: Blood pressure loci identified with a gene-centric array. *Am J Hum Genet* 89: 688–700, 2011
26. Sequeira López ML, Pentz ES, Nomasa T, Smithies O, Gomez RA: Renin cells are precursors for multiple cell types that switch to the renin phenotype when homeostasis is threatened. *Dev Cell* 6: 719–728, 2004
27. Dumitriu B, Dy P, Smits P, Lefebvre V: Generation of mice harboring a Sox6 conditional null allele. *Genesis* 44: 219–224, 2006
28. Kurtz TW, Griffin KA, Bidani AK, Davisson RL, Hall JE; Subcommittee of Professional and Public Education of the American Heart Association: Recommendations for blood pressure measurement in humans and experimental animals. Part 2: Blood pressure measurement in experimental animals: A statement for professionals from the subcommittee of professional and public education of the American Heart Association council on high blood pressure research. *Hypertension* 45: 299–310, 2005
29. Lorenz JN, Lasko VM, Nieman ML, Damhoff T, Prasad V, Beierwaltes WH, Lingrel JB: Renovascular hypertension using a modified two-kidney, one-clip approach in mice is not dependent on the  $\alpha 1$  or  $\alpha 2$  Na-K-ATPase ouabain-binding site. *Am J Physiol Renal Physiol* 301: F615–F621, 2011
30. Saleem M, Barturen-Larrea P, Saavedra L, Gomez JA: A modified two kidney one clip mouse model of renin regulation in renal artery stenosis. *J Vis Exp* (164), 2020
31. Bowers LD, Wong ET: Kinetic serum creatinine assays. II. A critical evaluation and review. *Clin Chem* 26: 555–561, 1980
32. Cook JG; Association of clinical Biochemists' Scientific and Technica Committee: Factors influencing the assay of creatinine. *Ann Clin Biochem* 12: 219–232, 1975
33. Gonzalez AA, Liu L, Lara LS, Bourgeois CR, Ibaceta-Gonzalez C, Salinas-Parra N, Gogulamudi VR, Seth DM, Prieto MC: PKC- $\alpha$ -dependent augmentation of cAMP and CREB phosphorylation mediates the angiotensin II stimulation of renin in the collecting duct. *Am J Physiol Renal Physiol* 309: F880–F888, 2015
34. Salinas-Parra N, Reyes-Martínez C, Prieto MC, Gonzalez AA: Prostaglandin E<sub>2</sub> induces prorenin-dependent activation of (Pro)renin receptor and upregulation of cyclooxygenase-2 in collecting duct cells. *Am J Med Sci* 354: 310–318, 2017
35. Wang H, Gomez JA, Klein S, Zhang Z, Seidler B, Yang Y, Schmeckpeper J, Zhang L, Muramoto GG, Chute J, Pratt RE, Saur D, Mirosou M, Dzau VJ: Adult renal mesenchymal stem cell-like cells contribute to juxtaglomerular cell recruitment. *J Am Soc Nephrol* 24: 1263–1273, 2013
36. Kwon TH, Frøkiær J, Nielsen S: Regulation of aquaporin-2 in the kidney: A molecular mechanism of body-water homeostasis. *Kidney Res Clin Pract* 32: 96–102, 2013
37. Ma L, Liu Y, Landry NK, El-Achkar TM, Lieske JC, Wu XR: Point mutation in D8C domain of Tamm-Horsfall protein/uromodulin in transgenic mice causes progressive renal damage and hyperuricemia. *PLoS One* 12: e0186769, 2017
38. Johnson JA, Ichikawa S, Kurz KD, Fowler Jr. WL, Payne CG: Pressor responses to vasopressin in rabbits with 3-day renal artery stenosis. *Am J Physiol* 240: H862–H867, 1981
39. Durante A, Peretto G, Laricchia A, Ancona F, Spartera M, Mangieri A, Cianflone D: Role of the renin-angiotensin-aldosterone system in the pathogenesis of atherosclerosis. *Curr Pharm Des* 18: 981–1004, 2012
40. Galen FX, Devaux C, Houot AM, Menard J, Corvol P, Corvol MT, Gubler MC, Mounier F, Camilleri JP: Renin biosynthesis by human tumoral juxtaglomerular cells. Evidence for a renin precursor. *J Clin Invest* 73: 1144–1155, 1984
41. Pratt RE, Carleton JE, Richie JP, Heusser C, Dzau VJ: Human renin biosynthesis and secretion in normal and ischemic kidneys. *Proc Natl Acad Sci U S A* 84: 7837–7840, 1987

42. Schweda F, Friis U, Wagner C, Skott O, Kurtz A: Renin release. *Physiology (Bethesda)* 22: 310–319, 2007PubMed
43. Taugner R, Metz R: Development and fate of the secretory granules of juxtaglomerular epithelioid cells. *Cell Tissue Res* 246: 595–606, 1986
44. Acker GM, Galen FX, Devaux C, Foote S, Papernik E, Pesty A, Menard J, Corvol P: Human chorionic cells in primary culture: A model for renin biosynthesis. *J Clin Endocrinol Metab* 55: 902–909, 1982
45. Yokosawa H, Holladay LA, Inagami T, Haas E, Murakami K: Human renal renin. Complete purification and characterization. *J Biol Chem* 255: 3498–3502, 1980
46. Gonzalez AA, Cifuentes-Araneda F, Ibaceta-Gonzalez C, Gonzalez-Vergara A, Zamora L, Henriquez R, Rosales CB, Navar LG, Prieto MC: Vasopressin/V2 receptor stimulates renin synthesis in the collecting duct. *Am J Physiol Renal Physiol* 310: F284–F293, 2016
47. Gonzalez AA, Liu L, Lara LS, Seth DM, Navar LG, Prieto MC: Angiotensin II stimulates renin in inner medullary collecting duct cells via protein kinase C and independent of epithelial sodium channel and mineralocorticoid receptor activity. *Hypertension* 57: 594–599, 2011
48. Liu X, Mao Y, He X, Wang M, Gan L: Ultrastructural pathological features of unilateral renal artery stenosis in the rats. *Int J Clin Exp Pathol* 8: 4807–4814, 2015
49. Chade AR, Williams ML, Engel J, Guise E, Harvey TW: A translational model of chronic kidney disease in swine. *Am J Physiol Renal Physiol* 315: F364–F373, 2018
50. Singer E, Markó L, Paragas N, Barasch J, Dragun D, Müller DN, Budde K, Schmidt-Ott KM: Neutrophil gelatinase-associated lipocalin: Pathophysiology and clinical applications. *Acta Physiol (Oxf)* 207: 663–672, 2013

**Received:** May 6, 2020 **Accepted:** March 19, 2021