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 RAS, 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 RAS.

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, Ren1dcre/+ Sox6fl/fl (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 RAS and renovascular hypertension. BP was measured using the tail-cuff method. Renin, prorenin, Sox6, and NGAL expression 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 RAS compared with Sox6 WT (Ren1dcre/+ Sox6wt/wt). 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 RAS. These results point to a novel transcriptional regulatory network controlled by Sox6.

Introduction
Renal artery stenosis (RAS) 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). RAS 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 RAS (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
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 during embryonic development and adulthood (22). To define this novel function of Sox6, we used Ren1dcre/cre, Sox6fl/fl and Ren1dcre/cre; Sox6wt/wt 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 RAStenosis. 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.

**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-design 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 RAStenosis 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

**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).
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 × g for 10 minutes at room temperature (RT). Plasma was aliquoted in Eppendorf tubes, flash frozen in liquid nitrogen, and then stored at −80°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 10³. Prepared standards and diluted urine samples (15 μl each) were added to designated wells and the reaction was initiated by adding 150 μl 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

$$A_{final} - A_{initial} = \frac{C_{sample} \times V_{sample}}{V_{standard}}$$

where $A_{final}$ and $A_{initial}$ are the final and initial absorbance, $C_{sample}$ is the creatinine concentration of the sample, $V_{sample}$ is the volume of the sample, and $V_{standard}$ is the volume of the standard.
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-Techar (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 × 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% β-mercaptoethanol (catalog number 1610710; Bio-Rad). Samples containing 30 µg 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 β-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 β-actin (catalog number A1978; Sigma-Aldrich). Secondary antibody (Promega) dilutions were used at 1:500. The renin band position and specificity were 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 RAStenosis, 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 µm. 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 fluorescein isothiocyanate-conjugated secondary antibodies for 1 hour at RT. Anti-renin (10 µg/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 α-smooth muscle actin (α-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 µm. 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-Ren1-01, reference number 558571; Mm-Sox6-C2, reference number 472061-C3; Mm-α-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 α-SMA mRNA
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 α-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 ≤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 Ren1d1 cells was used, the Ren1dcre/Sox6fl/fl (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 1. Results in stenosed mice at a glance

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

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

Table 2. Results in sham mice at a glance

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

WT, wild type; KO, knockout; IHC, immunohistochemistry; ISH, in situ hybridization; JG, juxtaglomerular; NGAL, neutrophil gelatinase-associated lipocalin.
Specific KO of Sox6 in Ren1d+ cells inhibits renin expression in the stenosed kidney during RASTenosis. Two weeks after RASTenosis surgery, kidneys were harvested and Western blot was performed. (A) Representative Western blots images showing levels of prorenin/renin expression in stenosed mice. β-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±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 RASTenosis. LK, left kidney; RK, right kidney.
renin expression in the kidney during RASTenosis, 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-KO compared with Sox6-WT (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 renin and prorenin 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 RASTenosis

To determine the initial effects of Sox6 on renin gene expression during RASTenosis (Figure 3A), renin and prorenin expression levels were measured in kidney cortices after 3 days of RASTenosis, 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 renin and prorenin 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 RASTenosis

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 RASTenosis

To determine the expression of renin, Sox6, and α-SMA mRNA, fluorescent ISH was performed in kidneys after 3 days of RASTenosis. 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 RASTenosis

Next, we evaluated the effects of knocking out Sox6 in renin-expressing cells in RASTenosis-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. β-Actin was used as a loading control. Den- sitometric 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±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 RASenosis. 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±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 RASenosis. (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 RASenosis. 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 μm. Original magnification, ×60. AfAr, Afferent arteriole; G, glomeruli.
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.
and kidney injury induced by RASstenosis. 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.

RASstenosis 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 RASstenosis.

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). Protoplasts 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 protoplasts 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 RASstenosis, 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 RASstenosis. 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 RASstenosis (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...
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 α-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 expression, which has an effect on hypertension and kidney damage during renovascular hypertension induced by RAStenosis. ACE, angiotensin-converting enzyme; ATR(1&2), angiotensin type 1 and 2 receptors.
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.

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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.

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


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