Renin–Angiotensin System

Chronic Stimulation of Renin Cells Leads to Vascular Pathology

Masafumi Oka, Silvia Medrano, Maria Luisa S. Sequeira-López, R. Ariel Gómez

See Editorial Commentary, pp 35–37

Abstract—Experimental or spontaneous genomic mutations of the renin–angiotensin system or its pharmacological inhibition in early life leads to renal abnormalities, including poorly developed renal medulla, papillary atrophy, hydronephrosis, inability to concentrate the urine, polyuria, polydipsia, renal failure, and anemia. At the core of such complex phenotype is the presence of unique vascular abnormalities: the renal arterioles do not branch or elongate properly and they have disorganized, concentric hypertrophy. This lesion has been puzzling because it is often found in hypertensive individuals whereas mutant or pharmacologically inhibited animals are hypotensive. Remarkably, when renin cells are ablated with diphtheria toxin, the vascular hypertrophy does not occur, suggesting that renin cells per se may contribute to the vascular disease. To test this hypothesis, on a Ren1c−/− background, we generated mutant mice with reporter expression (Ren1c−/−;Ren1c-Cre;R26R.mTmG and Ren1c−/−;Ren1c-Cre;R26R.LacZ) to trace the fate of reninnull cells. To assess whether reninnull cells maintain their renin promoter active, we used Ren1c−/−;Ren1c-YFP mice that transcribe YFP (yellow fluorescent protein) directed by the renin promoter. We also followed the expression of Akr1b7 and miR-330-5p, markers of cells programmed for the renin phenotype. Contrary to what we expected, reninnull cells did not die or disappear. Instead, they survived, increased in number along the renal arterial tree, and maintained an active molecular memory of the myoepithelial renin phenotype. Furthermore, null cells of the renin lineage occupied the walls of the arteries and arterioles in a chaotic, directionless pattern directly contributing to the concentric arterial hypertrophy. (Hypertension. 2017;70:119-128. DOI: 10.1161/HYPERTENSIONAHA.117.09283.) • Online Data Supplement

Key Words: kidney ■ mice ■ mutation ■ phenotype ■ renin-angiotensin system

Mice homozygous for the Ren1c gene disruption (Ren1c−/−) display several morphological and physiological abnormalities, including poorly developed renal medulla, papillary atrophy, hydronephrosis, inability to concentrate the urine, polyuria, polydipsia, renal failure, and anemia.1 Underlying such complex phenotype is the presence of unique renal vascular abnormalities: the renal arterioles do not branch or elongate properly, and the affected vessels show concentric arteriolar hypertrophy.1 Similar vascular abnormalities are found in mice with genomic deletions of any of the genes of the renin–angiotensin system (RAS), including the angiotensinogen,2–5 angiotensin I–converting enzyme,6–8 angiotensin II type 1A and 1B receptor,9 and aldosterone synthase10 genes. Analogous abnormalities were previously found in animals treated early in life with RAS inhibitors.11–13 Equally important, humans with mutations of the RAS genes also display similar morphological abnormalities, including the hallmark of concentric arteriolar hypertrophy.14 This vascular lesion, although central to the phenotype, has been difficult to understand from a physiopathological point of view because it has usually been considered a hallmark of hypertensive vascular disease. However, in all the examples mentioned above, the subjects are hypotensive. An opportunity for understanding arose from experiments in which the renin cells were ablated in mice using diphtheria toxin genetically engineered to be expressed under the control of the renin gene (Ren1d-DTA mice).14 Because the arterial thickening disappeared with ablation of renin cells, we hypothesized that renin cell precursors contributed to the pathology. Normally, renin cells are identified by the detection of either renin or its mRNA. However, Ren1c−/− mice cannot make either of them. Because we could not mark/identify the reninnull cells, we were uncertain whether reninnull cells remained or disappeared from the kidneys of renin knockout mice. However, if the cells were present in the kidney even though they could not make renin, it may indicate that the molecular program for the renin phenotype may be still active although hidden to us because of the lack of tools to track the cells. We therefore genetically engineered several mouse lines to trace the fate of reninnull cells and their precursors and test the activity of the renin promoter in vivo using fluorescent reporter constructs. Using those tools and specific renin-independent antibodies and in situ hybridization, we tested whether reninnull cells (1) persist and maintain the program of the renin phenotype and (2) contribute directly to the vascular disease.

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Materials and Methods

Animals

Ren1c−/−;Ren1c−/−;Ren1c−/− mice were generated by crossing Ren1c−/− mice, generated by gene targeting, with mice harboring a Ren1c−/− transgene.15,16 The Ren1c−/− transgene contains all the regulatory regions necessary for tissue- and cell-specific expression of renin and YFP (yellow fluorescent protein) during normal development and in response to stimuli that regulate renin expression.16–18 Mice were significantly smaller (20.6±2.1 g) than control mice (22.1±2.7 g). The growth effects of the renin deletion were more pronounced in the kidneys than on total somatic growth as evidenced by a much lower kidney weight to body weight ratio in Ren1c−/− mice versus Ren1c−/− mice (Table). Interestingly, Ren1c−/− mice had splenomegaly as indicated by the assessment of spleen weight corrected for total somatic weight (Table). Blood chemistries revealed that Ren1c−/− mice had elevated creatinine (Ren1c−/− 0.52±0.04 vs Ren1c−/− 0.42±0.04 mg/dL; P<0.0001), blood urea nitrogen (Ren1c−/− 90.9±16.5 mg/dL vs Ren1c−/− 22.5±3.4 mg/dL; P<0.0001), normocytic anemia (hemoglobin, Ren1c−/− 10.0±1.1 g/dL vs Ren1c−/− 13.6±0.6 g/dL; P<0.0001), and mean corpuscular volume (Ren1c−/− 55.8±1.6 fL vs Ren1c−/− 56.6±1.5 fL; P=0.054). Thus, at 2 months of age, Ren1c−/− mice already displayed clear signs of chronic renal failure and anemia.

Distribution and Activity of Renin Null Cells in the Kidneys of Ren1c−/− Mice

We first performed renin immunostaining in kidney sections from control and Ren1 knockout mice. Whereas in control Ren1−/− mice, renin was detected in the juxtaglomerular regions of the renal cortex, in Ren1−/− mice, as expected, no renin was observed (Figure 1A and 1B). To determine whether reninnull cells persisted in Ren1−/− mice and whether those cells were actively attempting to make renin, we studied Ren1−/−;Ren1−/− mice. In these mice, YFP permits the identification, localization, and counting of cells with an active renin promoter even when the cells are unable to make renin.15,16 Whereas in control mice, YFP + cells were limited to the juxtaglomerular area, in Ren1−/−;Ren1−/− mice, YFP + cells were observed throughout the kidney arterial tree, including afferent arterioles, interlobular arteries, arcuate arteries, and corticomedullary arteries (Figure 1C and 1D). The intensity of YFP expression in afferent arterioles was stronger than that in larger arteries. To determine whether the expanded distribution of YFP expression in Ren1−/− was reflected in an increased number of YFP + cells, we counted YFP + cells in control and mutant mice. As shown in Figure 1E, the number of YFP + cells in the kidneys of Ren1−/− mice was increased compared to control mice (Table). Results are presented as means±SD. Statistical significance was determined using either Student t test or nonparametric tests as needed.

Results

Ren1c−/− Mice Have Growth Retardation, Renal Failure, and Anemia

In situ hybridization was performed in 4% paraformaldehyde-, paraffin-embedded kidney sections using a digoxigenin-labeled locked nucleic acid probe (Exiqon, Woburn, MA) specific for mouse mir-330-5p.

In Situ Hybridization

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X-Gal Staining

To reveal the distribution of cells from the renin and smooth muscle lineage, we performed X-gal staining in Ren1−/−;Ren1−/−;Ren1−/− mice, as previously described.15

Statistical Analysis

Results were presented as means±SD. Statistical significance was determined using either Student t test or nonparametric tests as needed.

Table. Ren1c−/− Mice Have Growth Retardation, Renal Failure, and Anemia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ren1c−/−</th>
<th>Ren1c−/−</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>22.1±2.7 (26)</td>
<td>20.6±2.1 (27)*</td>
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<tr>
<td>Total kidney to body weight, mg/g</td>
<td>13.1±1.8 (26)</td>
<td>9.5±1.4 (27)†</td>
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<td>Spleen to body weight, mg/g</td>
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<td>4.6±1.0 (27)†</td>
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<td>2.06±1.33 (21)</td>
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<td>Hemoglobin, g/dL</td>
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<td>10.0±1.1 (21)†</td>
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<tr>
<td>Platelet, k/µL</td>
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<td>561±120 (21)</td>
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<tr>
<td>Blood urea nitrogen, mg/dL</td>
<td>22.5±3.4 (26)</td>
<td>90.9±16.5 (26)†</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.42±0.04 (26)</td>
<td>0.52±0.04 (26)†</td>
</tr>
</tbody>
</table>

Values are means±SD; number of animals in parentheses.

*P<0.05 compared with Ren1c−/−.

†P<0.001 compared with Ren1c−/−.
of YFP+ cells was significantly higher in Ren1$^{-/-}$;Ren1$^{-/-}$-YFP (10810±7855) than in Ren1$^{+/+}$;Ren1$^{-/-}$-YFP (2902±1476; P<0.0001) mice. Thus, renin$^{null}$ cells persisted, increased in numbers, and their distribution along the renal arterial tree was expanded beyond the juxtaglomerular region.

**Histopathological Changes in the Kidneys of Ren1$^{-/-}$ Mice**

To define the extent of histopathological differences between Ren1$^{+/+}$ and Ren1$^{-/-}$ mice, we performed immunostaining for αSMA, MHC (myosin heavy chain 11), and PECAM-1. Gross anatomic examination of the kidneys in Ren1$^{-/-}$ mice revealed a granular surface that was correlated histologically with marked interstitial fibrosis and tubular dilatation and atrophy (Figure 2B). The areas of fibrosis were positive for αSMA (Figure 2B). In addition to interstitial pathology, the kidneys from Ren1$^{-/-}$ mice displayed periglomerular fibrosis as detected by staining with the αSMA antibody (Figure 2B and 2D). Notably, the renal arteries and arterioles were thickened with cells that stained positive with the αSMA antibody (Figure 2D). The arteries and
arterioles of control Ren1c+/− mice did not show any abnormalities (Figure 2A and 2C). Similar staining patterns in the arterioles were observed with immunostaining for MHC (not shown), and PECAM-1 staining did not reveal any difference in the distribution and thickness of the endothelial cell layer between Ren1c+/− and Ren1c−/− mice (not shown).

**Ren1c−/− Mice Have Thicker Arterioles and Arteries Than Ren1c+/−**

As mentioned above, the kidney arterioles of Ren1c−/− mice displayed a unique type of concentric arteriolar hypertrophy. We quantified the arteriolar wall thickness of afferent arterioles in slides stained for αSMA (Figure 2A through 2E). Ren1−/− mice had thicker arteriolar walls than Ren1+/− mice (14.3±3.8 versus 8.26±2.5 μm; P<0.0001; Figure 2E). Given that the actual size and plane of cut of individual arterioles are somewhat variable, we also analyzed the data using relative frequency distribution histograms. Figure 2F shows that the distribution curve corresponding to the Ren1−/− animals is displaced to the right indicating that a higher proportion of vessels possesses thicker walls than in control mice.
of ≥20 μm: mostly arcuate and corticomedullary arteries. Ren1c−/− mice had thicker arteries than Ren1c+/− (Ren1c+/− 29.2±11.1 μm versus Ren1c−/− 42.1±11.1 μm; P<0.0001; Figure S1 in the online-only Data Supplement). Again, the frequency distribution histogram clearly showed that a higher proportion of arterial vessels in Ren1c−/− mice possessed thicker walls than in Ren1c+/− mice (Figure S1). Occasionally, the walls of larger arteries in Ren1c−/− were irregular, protruding toward the lumen of the vessel (Figure S1).

**Renin Null Cells Express Akr1b7 and miR-330-5p, Markers of Cells Programmed for the Renin Phenotype**

We have previously shown that Akr1b7 is a novel marker of cells programmed for the renin phenotype. In fact, expression of Akr1b7 is maintained even when the cells are unable to synthesize renin, suggesting that Akr1b7 expression is part of the genetic program of the renin phenotype (Figure 3). Akr1b7-positive cells coincided with cells expressing αSMA in the outer layers of the arteriole (Figure 3A and 3B, top). In some vessels, Akr1b7+ cells were also distributed deeper inside the arteriolar wall (Figure 3A and 3B, bottom). Few Akr1b7+ cells were observed in areas of periglomerular fibrosis. As expected in those areas, αSMA expression was prominent (Figure 3A and 3B, bottom). αSMA and Akr1b7+ positive cells were also present in portions of the intraglomerular and extraglomerular mesangial areas (not shown).

Next, we performed in situ hybridization staining for miR-330-5p, a renin cell marker expressed under conditions known to induce renin expression along the kidney vasculature. In contrast to Ren1c+/− controls (Figure 3C), miR-330-5p was highly expressed in the vessel walls and inside glomeruli in Ren1c−/− kidneys, consistent with its presence when the renin program is activated (Figure 3D).

**Distribution of Cells From the Renin Lineage in Ren1c−/− Mice**

We have reported that during normal development, renin precursors and their descendants are distributed along large
intrarenal arteries, afferent arterioles, and inside glomeruli. To define the contribution of cells from the renin lineage to the thickening of arterial walls, we performed fate tracing using Ren1c−/−;Ren1c-Cre;R26R.mTmG mice (Figure 4). In control Ren1c+/− mice, cells from the renin lineage were found in the afferent arterioles including its juxtaglomerular area (A, top). In Ren1c−/− mice, renin lineage cells were found surrounding arterioles and around glomeruli (arrows; B, top). In larger vessels, cells from the renin lineage were found throughout the arterial walls both in Ren1c+/− control and Ren1c−/− animals, but the intramural arrangement of the cells is markedly different (A, B, bottom). In control animals, cells from the renin lineage arranged themselves in an organized circular pattern (A, bottom), and cells from knockout animals were disorganized without a clear directional pattern (B, bottom, D). Labeled in green (GFP+) are cells from the renin lineage. Labeled in red (RFP+, red fluorescent protein) are nonrenin lineage cells. A similar disorganized pattern of renin null cells is also observed when examining kidneys using the Lac-Z reporter. Stained in blue are cells from the renin lineage. Scale bars, 75 µm (A, B, top); 100 µm (A, B, bottom; C); 50 µm (D).

Figure 4. Distribution of cells from the renin lineage in Ren1c−/− mice. In control Ren1c+/− kidneys, cells from the renin lineage (GFP+, green fluorescent protein) are found in the afferent arterioles including its juxtaglomerular area (A, top). In Ren1c−/− mice, renin lineage cells are found surrounding arterioles and around glomeruli (arrows; B, top). In larger vessels, cells from the renin lineage are found throughout the arterial walls both in Ren1c+/− control and Ren1c−/− animals, but the intramural arrangement of the cells is markedly different (A, B, bottom). In control animals, cells from the renin lineage arranged themselves in an organized circular pattern (A, bottom), and cells from knockout animals were disorganized without a clear directional pattern (B, bottom, D). Labeled in green (GFP+) are cells from the renin lineage. Labeled in red (RFP+, red fluorescent protein) are nonrenin lineage cells. C, A similar disorganized pattern of renin null cells is also observed when examining kidneys using the Lac-Z reporter. Stained in blue are cells from the renin lineage. Scale bars, 75 µm (A, B, top); 100 µm (A, B, bottom; C); 50 µm (D).

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To define whether cells originating from endothelial progenitors could physically become part of the thickened arterioles, we studied EC-SCL-CreERT2;R26R.mTmG mice. We have shown that in these mice, expression of Cre and therefore green fluorescent protein is confined to cells of the endothelial progeny, including precursors and their descendants. Results showed that the distribution of cells from the endothelial lineage was similar between Ren1c+/− and Ren1c−/− mice (not shown). Thus, endothelial cells did not contribute to the thickening of the vessels. Furthermore, no thickening of the endothelial layer per se was found in blood vessels from Ren1c−/− mice (not shown).

Discussion

The present series of studies show that in renin knockout mice (1) there is concentric hypertrophy of the kidney arterioles leading to advanced kidney disease; (2) the cells programmed for the renin phenotype survive and increase in numbers along the renal arterial tree; (3) those reninnull cells maintain the molecular program of the renin phenotype; and (4) reninnull cells integrate in a chaotic, disorderly manner inside the vessel wall, thus contributing to the vascular disease.
Pervasive Phenotype: Concentric Vascular Hypertrophy and Kidney Disease

Ren1c−/− mice showed a distinct phenotype characterized by diminished kidney size, tubular atrophy and dilatation, marked periglomerular and interstitial fibrosis, and severe vascular disease. These morphological findings were accompanied by advanced renal failure, anemia, and splenomegaly. We speculate that the normocytic anemia is because of chronic renal failure and inability to synthesize or respond to erythropoietin as it has been suggested in patients with end-stage renal disease. Given that the mouse spleen retains hematopoietic capabilities throughout life, we assume that the splenomegaly is the result of a failed attempt to compensate for the anemia. Those findings are in agreement with previous work from several laboratories.1,29

With regards to the arterial lesions, we have shown that deletion of renin solely in the vasculature fully reproduces the entire morphological, physiological, and pathological manifestations (including the renal failure, hydropnephrosis, and anemia) observed in renin knockout mice.28 Deletion of renin in the tubular compartment does not lead to any obvious abnormalities. Thus, vascular renin is necessary to maintain the structural and physiological integrity of the kidney.

The vascular disease observed in our Ren1c−/− mice was characterized by the presence of concentric and disorganized layers of renin lineage and smooth muscle cells that thickened the vessel walls and in some arterioles significantly narrowed their lumens. This lesion is not found solely in renin knockout mice. It is found in the kidneys of all the RAS gene knockout mice studied so far.30 The same vascular lesion is also encountered in the kidneys of patients harboring mutations of the RAS genes13 and in the kidneys of animals subjected to pharmacological inhibition of the RAS in early life12,32 or during the treatment of severe hypertension in adult rats.13 This concentric vascular hypertrophy has been difficult to explain from a pathophysiological point of view because it resembles a similar lesion found in subjects with severe and chronic hypertension.33 But animals and patients harboring mutations of the RAS genes or exposed to pharmacological inhibition of the RAS have low arterial pressure. It is well known that angiotensin is an angiogenic factor that could have contributed to the arteriolar thickening.34 However, angiotensins I and II are undetectable in the kidneys of all angiotensin harboring mutations of the RAS genes or expressed in mice with combined deletion (or inhibition) of angiotensin type 1A and type 1B receptors.9 In aggregate, those results indicate that in our study, the vascular lesions are unlikely to be because of angiotensin actions in the vasculature. Interestingly, when we ablated the renin cells by targeting diphertheria toxin into the renin gene,14 the animals did not develop the arteriolar hypertrophy, suggesting that renin cells, directly, physically, or otherwise, were responsible for the vascular disease. For this hypothesis to be plausible, as discussed below, it required at a minimum that renin cells persisted in the kidney vasculature of Ren1c−/− mice.

Cells Programmed for the Renin Phenotype Survive, Increase in Number Along the Renal Vasculature, and Maintain the Program for the Renin Phenotype

At first glance, it seemed conceivable that when cells are unable to perform their main function or to manufacture the protein that characterizes them, they would either undergo apoptosis or would not be replaced as the cells naturally aged and died.35–40 However, we did not find evidence of apoptosis. Instead and more importantly, using genetic fate tracing and immunostaining for Akr1b7 and in situ hybridization for miR-330-5p, we found that the reninnull cells did not disappear. Instead, the cells survived and persisted throughout the kidney vasculature. Expression of Akr1b7, a marker of cells programmed for the renin phenotype, is of particular significance and deserves some comment: aldo-keto-reductase has been proposed to detoxify endocrine cells from harmful aldehydes—generated during periods of high protein synthesis—to less toxic alcohols. The enzyme is highly enriched, almost as much as renin, in microarrays obtained from renin-synthesizing cells.15 Akr1b7 is expressed with renin throughout development of renin-expressing cells and when differentiated, formerly renin-expressing cells (renin cell descendants) along the kidney vasculature re-enact the renin phenotype in response to physiological threats to homeostasis. This pattern of coexpression persists in extreme pathological situations,18 and both enzymes, renin and Akr1b7, are regulated by cAMP in vitro and in vivo. In addition, we found that Ren1c−/− mice expressed miR-330-5p along the kidney arterial tree. We have previously shown that expression of miR-330-5p occurs in juxtaglomerular cells when animals are exposed to angiotensin I–converting enzyme inhibitors and sodium depletion,42 2 manipulations known to induce renin expression along the kidney vasculature. Those findings together with the results of the present experiments indicate that Akr1b7 and miR-330-5p (in addition to renin) are part of the conserved program of the renin cell phenotype.29,43 Furthermore, such genetic program is highly active in reninnull cells.

To provide further proof that the reninnull cells were attempting to express renin, we studied mice harboring a transgene composed of the regulatory regions of the renin gene driving expression of YFP. Results showed that YFP-expressing cells were numerous and observed throughout the kidney arterial tree, including large corticomedullary vessels. Thus, reninnull cells not only persisted but also their distribution and number along the renal arterial tree were expanded in a pattern that replicated the distribution of renin cells during fetal development and in response to physiological stress in adult animals.17,20,44–46 The underlying mechanisms responsible for the reninnull cells’ attempt to maintain the endocrine phenotype of the native renin cell are intriguing. Renin cells are sensors designed to receive and respond to signals that convey the composition and volume of the extracellular fluid and the level of blood pressure. Under normal circumstances, renin synthesis and release are stimulated by a decrease in perfusion pressure, a decrease in sodium chloride at the level of the macula densa, and when the β-adrenergic receptors are stimulated by sympathetic activation.47 In addition, renin synthesis and release are normally inhibited by angiotensin II.48
Although not formally studied, in renin knockout mice (which are hypotensive, dehydrated, and lacking angiotensin), several or all of the major mechanisms that control renin synthesis and release are potentially activated in them, in an attempt to restore homeostasis. How those signals are transmitted to the genome to activate the renin gene and to restore the full endocrine phenotype of the renin cell is not fully clear although several key signaling mechanisms including but not limited to cAMP pathway, Notch receptors, and connexins are known be involved (reviewed in 31).

Reninnull Cells Contribute to Vascular Pathology

In addition to encircling the arterioles, the intramural arrangement of the reninnull cells was markedly different in knockout versus control animals: whereas in control animals, cells from the renin lineage were arranged in an organized circular pattern, cells from knockout animals were disorganized without a clear directional pattern. A similar disorganized pattern of reninnull cells was observed whether the analysis was done using green fluorescent protein or Lac-Z reporting mice. The reasons for this particular intramural distribution are not clear and remain to be investigated. Nevertheless, the results of the present work suggest that renin cells per se contribute physically to the vascular hypertrophy. The results, however, do not exclude the distinct possibility that either the reninnull cells that encircle the vessels or those located intramurally may produce some factor(s) that stimulated the growth of adjacent smooth muscle cells. In fact, from previous microarray data, we know that cells that are so continuously stimulated produce at least 14 different types of growth factors with the capability to induce smooth muscle growth.15 Further work in this area will be necessary to identify the factor(s) involved.

A question remains about the possible mechanisms underlying such seemingly nonhomeostatic response from renin cells. During normal embryonic development, renin cells are distributed extensively along and throughout the renal arterial tree.31 Given that Ren1c−/− mice have a constant threat to homeostasis that becomes even more intense in extrauterine life (dehydration and hypotension), we hypothesize that the renin null cells are constantly stimulated to attempt renin synthesis. In the process, the cells are unable to mature and fully differentiate into smooth muscle cells, and as a consequence, the reninnull cells and their progenitors retain their fetal characteristics and a molecular program driven not only to produce renin but also vascular growth because it occurs in early life (Figure 5).31 Further work will be needed to define whether the repertoire of genes expressed by reninnull cells is similar to the pattern found in fetal-embryonic cells. More importantly, whether chronic pharmacological inhibition of the RAS in adult life leads to similar arterial and renal diseases needs to be determined. Given the widespread use of and efficacy of RAS inhibition in the treatment of

![Figure 5. Schematic of Reninnull cells contribution to vascular pathology. During normal development, renin precursors and their descendants are present along large intrarenal arteries, afferent arterioles, and inside glomeruli. In the normal adult mouse, renin cells become confined to the juxtaglomerular area. When the renin gene is knocked out, mice exhibit concentric hypertrophy of the kidney arterioles, leading to advanced kidney disease. The cells programmed for the renin phenotype survive and increase in numbers along the renal arterial tree. Those reninnull cells maintain the molecular program of the renin phenotype, that is expression of renin cell markers Akr1b7, miR-330-5p, and YFP, and integrate in a disorderly manner inside the vessel wall, thus contributing to the vascular disease. Thus, renin null cells not only persist but also their distribution and number along the renal arterial tree are expanded in a pattern that replicates the distribution of renin cells during fetal development and in response to physiological stress in adult animals. Akr1b7 indicates aldo-keto reductase 1b7; EC, endothelial cell; GFP, green fluorescent protein; JG cell, juxtaglomerular cell; RPC, renin progenitor cell; SMC, smooth muscle cell; WT, wild type; and YFP, yellow fluorescent protein.](http://hyper.ahajournals.org/content/suppl/2017/07/16/HYPERTENSION.117.134196.DC1.jpg)
hypertension and other diseases and the pervasive vascular pathology that occurs when the RAS is ablated, it would be important to determine whether prolonged, full-blown, and aggressive inhibition of the RAS in humans with hypertension have similar effects or not.

Perspectives

Our results demonstrate that when concentric vascular hypertrophy develops as a result of deletion of renin gene in the mouse, renin\textsuperscript{null} cells survive, increase in number along the renal arterial tree, and maintain an active molecular memory of the renin phenotype. Furthermore, null cells of the renin lineage also occupy the walls of the arteries and arterioles in a chaotic, directionless pattern directly contributing to the concentric arterial hypertrophy. These observations stress the importance of determining whether prolonged and aggressive pharmacological inhibitions of the RAS leads to similar arterial and renal diseases.

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Disclosures

None.

References

This study demonstrates that in renin knockout mice:
- Renin cells persist and maintain the program of the renin phenotype.
- Renin cells per se contribute to the vascular renal hypertrophy in renin knockout mice.

This study points at renin lineage cells as contributors to vascular renal hypertrophy. Understanding the underlying mechanisms is important because of the use of prolonged pharmacological inhibition of the renin–angiotensin system in humans with hypertension.
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CHRONIC STIMULATION OF RENIN CELLS LEADS TO VASCULAR PATHOLOGY

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Supplemental Materials and Methods

Animals
To identify renin-null cells in renin knock out mice, we generated Ren1c−/−;Ren1c-YFP mice. The mice were generated by initially crossing Ren1c−/− mice, generated by gene targeting 1 with mice harboring a Ren1c−YFP transgene 2, 3, followed by inter-crossing of heterozygous Ren1c+/−;Ren1c-YFP mice. The Ren1c−YFP transgene contains all the regulatory regions 4.9 Kb- upstream of the start site of the renin gene linked to YFP. This sequence is responsible for accurate tissue and cell specific expression of renin and YFP during normal development and in response to stimuli that regulate renin expression such as hypotension or lack of angiotensin 3, 4. In these mice when the cells attempt to transcribe renin (which is not possible because the renin coding regions are absent), they transcribe YFP, effectively labeling cells with an active renin promoter. We have shown that in Ren1c+/−;Ren1c-YFP mice, YFP labels renin cells in correspondence with the expression of renin and its mRNA throughout development and in response to physiological manipulations that alter the number of renin synthesizing cells 3, 5. To follow the fate of reninnull cells, we generated Ren1c−/−;Ren1c-Cre;R26R.LacZ mice and Ren1c+/−;Ren1c-Cre;R26R.LacZ mice using the following strategy. First, Ren1c+/− mice were crossed to Ren1c-Cre or R26R.LacZ mice respectively. The Ren1c-cre mice harbor a transgene containing the same 4.9 Kb of the renin promoter as in the Ren1c-YFP mice described above- governing the expression of cre recombinase. 2, 3 Then Ren1c+/−;Ren1c-Cre mice were crossed to Ren1c+/−;R26R.LacZ mice 6. Expression of LacZ was detected using the Xgal reaction whereby cells expressing β-galactosidase acquire a characteristic blue color due to the generation of the insoluble indigo compound generated during the enzymatic action of β-galactosidase on the X-gal substrate, 5'-Bromo-4'-Chloro-galactopyranoside 7, 8. Using this strategy, all renin cell precursors that have expressed renin and their descendants are labeled blue regardless of whether the cells have changed phenotype or whether renin expression has ceased 9. Similarly, we generated Ren1c+/−;Ren1c-Cre;R26R.mTmG mice. In these mice, before recombination occurs, all cells are RFP positive. Upon recombination, cells from the renin lineage become GFP positive 10.

All animals were handled in accordance with the National institutes of Health guidelines for the care and use of experimental animals, and the study was approved by the Institutional Animal Care and Use Committee of the University of Virginia.

Blood chemistry measurements
Animals were anesthetized and blood was collected via intracardiac puncture and placed either into tubes containing EDTA or heparinized plasma separator tubes (Microtainer), as previously described 11. A basic metabolic panel for blood urea nitrogen (BUN), creatinine, potassium, sodium, calcium, glucose, chloride, and CO2 was performed by the University of Virginia Hospital laboratory.

Morphometric measurements
In each animal, the total kidney- and total spleen-to-body weight ratios were derived by dividing the total weight of both kidneys or spleen in milligrams by the body weight in grams, respectively. Measurements of arteriolar wall thickness were done using a light microscope and the Leica MM.AF® version1.5 software. For measurements of arterial
wall thickness, the external and internal diameters of arteries were measured in alpha-smooth muscle actin (αSMA) stained sections.

**Quantification of YFP cell number in Ren1c-YFP mice**
To measure the number of YFP positive cells in Ren1c-YFP mice, we isolated YFP cells using fluorescent activated cell sorting (FACS). Kidneys were harvested and their medulla removed. Thereafter, the kidneys were minced with a razor blade, disassociated with enzymatic solution (Collagenase A, 1 mg/ml; Trypsin, 2.5 mg/ml and DNase, 0.21 mg/ml) for 15 minutes at 37 °C. The top layer of the supernatant containing the cells was collected. The disassociation and supernatant collection were cycled three times. After gently passing through a 100 µm and a 40 µm strainer, the cells were suspended in Dulbecco’s buffer (Dulbecco’s PBS, 9.59 mL; DNAse I, 2.1 mg; FBS, 100 µL and 0.5 M EDTA, 100 µL) and exposed to DAPI, 5 µg/ml. All living cells labeled with YFP not staining positive with DAPI were sorted and counted. FACS sorting was performed at the UVA Flow Cytometry core laboratory using a Becton Dickinson Influx cell sorter.

**Immunohistochemistry**
To study the distribution of renin cells, vascular smooth muscle cells and endothelial cells, kidneys were harvested from adult mice at two months of age. Then, Bouins-fixed and paraffin-embedded kidneys were processed for immunostaining, as previously described. Five µm sections were exposed to our anti-mouse renin polyclonal antibody made in rabbit (1:500 dilution), α- a mouse monoclonal anti-αSMA-specific antibody isotype Ig2a (Sigma, St. Louis, MO, 1:10,000 dilution), goat polyclonal anti AKR1B7 antibody (Santa Cruz Biotechnology; 1:200 dilution) and rabbit polyclonal anti PECAM-1 antibody (Santa Cruz Biotechnology, 1:1,000 dilution). Kidneys sections were examined under a microscope (Leica DFC 480) and imaged with a digital camera (Leica DFC310 FX).

**In Situ Hybridization**
Kidneys were removed from Ren1c/− and Ren1c+− mice, immediately fixed in 4% paraformaldehyde at 4°C for 24 hours, and embedded in paraffin. In situ hybridization was performed on 5-mm-thick sections. Sections were deparaffinized, fixed in 4% paraformaldehyde/phosphate-buffered saline, acetylated, and incubated in hybridization solution consisting of 50% formamide, 5x standard saline citrate, 50 mg/mL tRNA, 1% SDS, and 5 mg/mL heparin at room temperature for 1 hour. Hybridization was conducted at 37°C for 18 hours using 40 nmol/L digoxigenin-labeled locked nucleic acid probe (Exiqon, Woburn, MA) specific for mouse miR-330-5p in hybridization solution. Sections were washed sequentially once with 5x standard saline citrate at 37°C, three times with 0.2x standard saline citrate at 40°C, and once with 0.2x standard saline citrate at room temperature. Sites of hybridization were detected using alkaline phosphatase-conjugated digoxigenin antibody (Roche Diagnostics Corp., Indianapolis, IN) at a 1:4000 dilution, followed by BM Purple AP substrate color development (Roche). Negative controls were performed by omitting the probe in the hybridization step and by using a commercial nontargeting miRNA probe.

**X-gal staining**
To reveal the distribution of cells from the renin and smooth muscle lineage, we performed X-gal staining in Ren1c-Cre;R26R.LacZ and SM22α-Cre;R26R.LacZ mice, as previously described. In brief, kidneys were fixed for one hour in 4% paraformaldehyde. Then, the fixed kidneys were immersed in 30% sucrose in PBS overnight and kept at -80°C until used. Cryosections (7µm) were obtained using a Leica Cryocut 1800 cryostat and subjected to the X-gal reaction. The sections were placed in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Fisher Biotech) in dimethylformamide) overnight in the dark at 37°C. The sections were washed three times for 15 min each in PBS, post-fixed in 4% paraformaldehyde for one hour in the dark at 4°C, counterstained (Nuclear Fast Red, Sigma-Aldrich), dehydrated in graded alcohols to xylenes, and mounted with Permount.

**Statistical analysis**

Results are presented as means ± SD. Statistical significance was determined using either Student’s t-test or non-parametric tests as needed. A p value less than 0.05 was considered significant.

**References**


9. Sequeira Lopez 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*. 2004;6:719-728.


Figure S1. Wall thickness of larger arteries. Analysis of the wall thickness of larger arteries with an internal diameter of 20 µm or more: mostly arcuate and corticomedullary arteries performed in kidney sections immunostained for αSMA. Larger arteries in Ren1c−/− kidneys exhibit thicker walls (B) compared to Ren1c+/− kidneys (A). Occasionally, the walls of large arteries in Ren1c−/− were irregular, protruding towards the lumen of the vessel (B, arrows). Scale bars: 75 µm. C. Quantification of wall thickness shows that Ren1c−/− mice have thicker arteries than Ren1c+/− (Ren1c+/−; 29.2 ± 11.1 µm versus Ren1c−/−; 42.1 ± 11.1 µm, ***P<0.0001 . The frequency distribution histogram clearly shows that a higher proportion of arterial vessels in Ren1c−/− mice possess thicker walls than in Ren1c+/− mice (D).