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# CHRONIC STIMULATION OF RENIN CELLS LEADS TO VASCULAR PATHOLOGY

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## 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 $Ren1^{c-/-}$ background, we generated mutant mice with reporter expression ($Ren1^{c-/-}$;$Ren1^{c}$-*Cre;R26R.mTmG*; and $Ren1^{c-/-}$;$Ren1^{c}$-*Cre;R26R.LacZ* ) to trace the fate of $renin^{null}$ cells. To assess whether $renin^{null}$ cells maintain their renin promoter active, we used $Ren1^{c-/-}$;$Ren1^{c}$-*YFP* mice which transcribe YFP 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, $renin^{null}$ 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 myo-epitheliod renin phenotype. Further, 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.

### Keywords

Concentric arterial hypertrophy; molecular memory; cell fate; renin null cells; renin-angiotensin system

## Introduction

Mice homozygous for the $Ren1^{c}$ gene disruption ($Ren1^{c-/-}$) display a number of morphological and physiological abnormalities including poorly developed renal medulla,

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None

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 (AGT)[2,3–5] angiotensin I converting enzyme (ACE), [6–8] angiotensin II type 1A and 1B receptor (Agtr1a/1b) [9] and aldosterone synthase (AS) [10] genes. Analogous abnormalities were previously found in animals treated early in life with RAS inhibitors [11, 12]. Equally important, humans with mutations of the RAS genes also display similar morphological abnormalities including the hallmark of concentric arteriolar hypertrophy [13]. 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, $Ren1^{c-/-}$ mice cannot make either of them. Because we could not mark/identify the $renin^{null}$ cells, we were uncertain whether $renin^{null}$ cells remained or disappeared from the kidneys of *renin* knock out 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 due to the lack of tools to track the cells. We therefore genetically engineered several mouse lines to trace the fate of $renin^{null}$ 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 $renin^{null}$ cells: i) persist and maintain the program of the renin phenotype and ii) contribute directly to the vascular disease.

## Materials and Methods

### Animals

$Ren1^{c-/-}$;$Ren1^{c}$-*YFP* mice were generated by crossing $Ren1^{c-/-}$ mice, generated by gene targeting [1] with mice harboring a $Ren1^{c}$-*YFP* transgene [15, 16]. The $Ren1^{c}$-*YFP* transgene contains all the regulatory regions necessary for tissue and cell specific expression of *renin* and YFP during normal development and in response to stimuli that regulate *renin* expression [16, 17, 18]. $Ren1^{c-/-}$;$Ren1^{c}$-*Cre;R26R.LacZ* and $Ren1^{c+/-}$;$Ren1^{c}$-*Cre;R26R.LacZ* mice we generated by crossing $Ren1^{c-/-}$ mice to $Ren1^{c}$-*Cre* or *R26R.LacZ* mice respectively. The $Ren1^{c}$-*cre* mice harbor a transgene containing the same 4.9 Kb of the renin promoter-as in the $Ren1^{c}$-*YFP* mice described above-governing the expression of cre recombinase. [15, 16] Then $Ren1^{c+/-}$;$Ren1^{c}$-*Cre* mice were crossed to $Ren1^{c+/-}$;*R26R.LacZ* mice [19]. *Expression of LacZ* was detected using the Xgal reaction [20 21]. Using this strategy, all renin cell precursors are permanently labeled blue [22]. Similarly, we generated

*Ren1*$^{c-/-}$*;Ren1*$^{c}$*-Cre;R26R.mTmG* mice, in which upon recombination, cells from the renin lineage become GFP positive [23].

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 a blood metabolic panel was conducted as previously described [14].

### Morphometric measurements

To determine 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 *Ren1*$^{c}$*-YFP* mice, we isolated YFP cells from kidneys using fluorescent activated cell sorting (FACS). FACS sorting was performed at the UVA Flow Cytometry core laboratory using a Becton Dickinson Influx cell sorter.

### Immunohistochemistry

Immunostaining for renin αSMA, AKR1B7 and PECAM was performed in kidneys harvested from 2-month-old mice as previously described [24, 25, 26, 15, 18, 27].

### In Situ Hybridization

*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.

### X-gal staining

To reveal the distribution of cells from the renin and smooth muscle lineage, we performed X-gal staining in *Ren1*$^{c}$*-Cre;R26R.LacZ* and *SM22*α*-Cre;R26R.LacZ* mice, as previously described [28].

### Statistical analysis

Results are presented as means ± SD. Statistical significance was determined using either Student's *t*-test or non-parametric tests as needed.

## Results

### Ren1c−/− mice have growth retardation, renal failure and anemia

We compared the phenotypes of *Ren1*$^{c-/-}$ mice (*n=27*) and *Ren1c*$^{+/-}$ mice (*n=26*) at 2 months of age (Table 1). *Ren1*$^{c-/-}$ mice were significantly smaller (20.6 ± 2.1 g) than control *Ren1c*$^{+/-}$ (22.1 ± 2.7 g) mice. 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 than in *Ren1c*$^{+/-}$ mice (Table 1). Interestingly, *Ren1c*$^{-/-}$ had splenomegaly as indicated by the assessment of spleen weight corrected for total somatic weight (Table 1). Blood chemistries revealed that *Ren1c*$^{-/-}$ mice had elevated creatinine (*Ren1c*$^{-/-}$ 0.52 ± 0.04 mg/dL versus *Ren1c*$^{+/-}$ 0.42 ± 0.04 mg/dL, $p<0.0001$), and blood urea nitrogen (*Ren1c*$^{-/-}$; 90.9 ± 16.5 mg/dL versus *Ren1c*$^{+/-}$; 22.5 ± 3.4 mg/dL, $p<0.0001$) and normocytic anemia (Hemoglobin, *Ren1c*$^{-/-}$ 10.0 ± 1.1 g/dL versus *Ren1c*$^{+/-}$ 13.6 ± 0.6 g/dL, $p<0.0001$. MCV, *Ren1c*$^{-/-}$; 55.8 ± 1.6 fL versus *Ren1*$^{c+/-}$; 56.6 ± 1.5 fL, $P=0.054$). Thus, at two 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 knock out mice. Whereas in control *Ren1*$^{c+/-}$ mice, renin was detected in the juxtaglomerular regions of the renal cortex, in *Ren1c*$^{-/-}$ mice, as expected, no renin was observed (Figure 1A and B). To determine whether *renin*$^{null}$ cells persisted in *Ren1c*$^{-/-}$ mice and whether those cells were actively attempting to make renin, we studied *Ren1c*$^{-/-}$*;Ren1*$^{c}$*-YFP* mice. In these mice, YFP permits the identification, distribution, and counting of cells with an active renin promoter even when the cells are unable to make renin [1516]. Whereas in control mice YFP+ cells were limited to the juxtaglomerular (JG) area, in *Ren1c*$^{-/-}$*;Ren1*$^{c}$*-YFP* mice, YFP+ cells were observed throughout the kidney arterial tree including afferent arterioles, interlobular arteries, arcuate arteries, and cortico-medullary arteries (Figure 1C and D). 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 *Ren1c*$^{-/-}$ mice 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 was significantly higher in *Ren1*$^{c-/-}$*;Ren1*$^{c}$*-YFP* (10,810 ± 7,855) than in *Ren1*$^{c+/-}$*;Ren1*$^{c}$*-YFP* (2,902 ± 1,476, $p<0.0001$) 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 Ren1c$^{-/-}$ mice

To define the extent of histo-pathological differences between *Ren1*$^{c+/-}$ and *Ren1*$^{c-/-}$ mice, we performed immunostaining for αSMA, MHC and PECAM-1. Gross anatomical examination of the kidneys in *Ren1c*$^{-/-}$ 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*$^{c-/-}$ mice displayed peri-glomerular fibrosis as detected by staining with the smooth muscle antibody (Figure 2B and D). Notably, the renal arteries and arterioles were thickened with cells that stained positive with the smooth muscle antibody (Figure 2D). The arteries and arterioles of control *Ren1*$^{c+/-}$ mice did not show any abnormalities (Figure 2A and C). Similar staining patterns in the arterioles were observed with immunostaining for MHC (not shown), PECAM-1 staining did not reveal any difference in the distribution and thickness of the endothelial cell layer between *Ren1*$^{c+/-}$ and *Ren1*$^{c-/-}$ mice (not shown).

### Ren1$^{c-/-}$ mice have thicker arterioles and arteries than Ren1$^{c+/-}$

As mentioned above, the kidney arterioles of *Ren1*$^{c-/-}$ mice displayed a unique type of concentric arteriolar hypertrophy. We quantified the arteriolar wall thickness of afferent arterioles in slides stained for αSMA (Figures 2A–D; E). *Ren1*$^{c-/-}$ mice had thicker arteriolar walls than *Ren1*$^{c+/-}$ (14.3 ± 3.8 μm versus 8.26 ± 2.5 μm, *P<0.0001;* Figure 2E). Given that the actual size and plane of cut of individual arterioles is somewhat variable, we also analyzed the data using relative frequency distribution histograms. Figure 2F shows that the distribution curve corresponding to the *Ren1*$^{c-/-}$ animals was displaced to the right indicating that a higher proportion of vessels possessed thicker walls than in control mice. We also evaluated the wall thickness of larger arteries with an internal diameter of ≥20 μm: mostly arcuate and cortico-medullary arteries. *Ren1*$^{c-/-}$ mice had thicker arteries than *Ren1*$^{c+/-}$ (*Ren1*$^{c+/-}$; 29.2 ± 11.1 μm versus *Ren1*$^{c-/-}$; 42.1 ± 11.1 μm, *P<0.0001*; Figure S1). Again, the frequency distribution histogram clearly showed that a higher proportion of arterial vessels in *Ren1*$^{c-/-}$ mice possessed thicker walls than *in Ren1*$^{c+/-}$ *mice* (Figure S1). Occasionally, the walls of larger arteries in *Ren1*$^{c-/-}$ were irregular, protruding towards 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 [18]. 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 B, top). In some vessels though, AKR1B7+ cells were also distributed deeper inside the arteriolar wall (Figure 3 A and B bottom). Some AKR1B7+ cells were also observed in areas of peri glomerular fibrosis. As expected in those areas of peri glomerular fibrosis, αSMA expression was prominent. Occasionally, αSMA and AKR1B7 positive cells were also present in portions of the intra glomerular and extra glomerular mesangial areas (not shown).

Next, we performed *in situ* hybridization 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, miR-330-5p is highly expressed in the vessel walls and inside glomeruli in *Ren1c−/−* kidneys, consistent with its presence when the renin program is activated (Figure 3 C and D).

### Distribution of cells from the renin lineage in Ren1$^{c-/-}$ mice

We have reported that during normal development, renin precursors and/or their descendants are distributed along large intra-renal arteries, afferent arterioles and inside glomeruli [22]. To define the contribution of cells from the renin lineage to the thickening of arterial walls, we performed fate tracing using *Ren1*$^{c-/-}$*;Ren1*$^{c}$*-Cre;R26R.mTmG* mice (Figure 4). In control *Ren1*$^{c+/-}$ mice, cells from the renin lineage were found as expected in the afferent arterioles including its juxtaglomerular segment (Figure 4A, top). In *Ren1*$^{c-/-}$ mice, renin lineage cells were found surrounding arterioles and were also detected around glomeruli (Figure 4B, top). In larger vessels, cells from the renin lineage were found throughout the arterial walls both

in control (*Ren1*$^{c+/-}$) and renin knock out (*Ren1*$^{c-/-}$) animals (Figure 4A and B, bottom). However, the intramural arrangement of the cells was markedly different in knock out versus control animals: whereas in control animals, cells from the renin lineage arranged themselves in an organized circular pattern, cells from knock out animals were disorganized without a clear directional pattern (Figure 4B and D). A similar disorganized pattern of renin null cells was also observed when examining them using the *Lac-Z* reporter (Figure 4C).

To define whether cells originating form endothelial progenitors could physically become part of the thickened arterioles, we studied *EC-SCL-Cre*$^{ERT2}$*;R26R.mTmG* mice. We have shown that in these mice, expression of Cre and therefore GFP is confined to cells of the endothelial progeny, including precursors and their descendants[27]. Results showed that the distribution of cells from the endothelial lineage was similar between *Ren1c*$^{+/-}$ and *Ren1*$^{c-/-}$ mice (not shown). Thus, endothelial cells did not contribute to the thickening of the vessels. Further, no thickening of the endothelial layer *per se* was found in blood vessels from *Ren1*$^{c-/-}$ mice (not shown).

## Discussion

The present series of studies show that in *renin* knock out mice: i) there is concentric hypertrophy of the kidney arterioles leading to advanced kidney disease; ii) the cells programmed for the renin phenotype survive and increase in numbers along the renal arterial tree; iii) those *renin*$^{null}$ cells maintain the molecular program of the renin phenotype, and iv) *renin*$^{null}$ cells integrate in a chaotic, disorderly manner inside the vessel wall, thus contributing to the vascular disease.

### A pervasive phenotype: concentric vascular hypertrophy and kidney disease

*Ren1*$^{c-/-}$ 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 due to 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, hydronephrosis and anemia) observed in *renin* knock out mice.[26]. 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 *Ren1*$^{c-/-}$ 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* knock out mice. Indeed, it is found in the kidneys of all the RAS gene knock out mice studied so far [5, 30, 31] The same vascular lesion is also encountered in the kidneys of patients harboring mutations of the RAS genes [13] and in the kidneys of animals subjected to pharmacological inhibition of the RAS in early life [12, 32] or during the treatment of severe hypertension in adult rats [33]. 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.[34] 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 which could have contributed to the arteriolar thickening [35]. However, angiotensins I and II are undetectable in the circulation of Ren1c−/− mice [1]. Further, the same vascular lesions are found in animals with deletion of the source of all angiotensins, angiotensinogen −/− mice[2, 3, 5, 36]. The same lesions are also found in mice with combined deletion (or inhibition) of AT1A and AT1B angiotensin receptors[9]. In aggregate, those results indicate that in our study, the vascular lesions, are unlikely to be due to angiotensin actions in the vasculature. Interestingly, when we ablated the renin cells by targeting diphtheria 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 *Ren1*$^{c-/-}$ 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, 37–41] 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 *renin* $^{null}$ 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 reenact the renin phenotype in response to physiologic threats to homeostasis. This pattern of co-expression 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 Ren1$^{c-/-}$ mice expressed miR-330-5p along the kidney arterial tree. We have previously shown that expression of miR-330-5p occurs in JG cells when animals are exposed to ACE inhibitors and sodium depletion [42], two 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 [4329] Further, such genetic program is highly active in *renin*$^{null}$ cells.

To provide further proof that the *renin*$^{null}$ 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 cortico-medullary vessels. Thus, *renin*$^{null}$ cells not only persisted, their distribution and number along the renal arterial tree was expanded in a pattern that replicated the distribution of renin cells during fetal development and in response to physiological stress in adult animals [17, 29, 44–48]. The underlying mechanisms responsible for the *renin*$^{null}$ 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 is stimulated by a decrease in perfusion pressure, a decrease in sodium chloride at the level of the macula densa and when the beta-adrenergic receptors are stimulated by sympathetic activation[49]. In addition, renin synthesis and release is normally inhibited by angiotensin II [50]. Although not formally studied, in *renin* knock out 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]].

## Renin$^{null}$ cells contribute to vascular pathology

In addition to encircling the arterioles, the intramural arrangement of the *renin*$^{null}$ cells was markedly different in knock out versus control animals: whereas in control animals, cells from the renin lineage were arranged in an organized circular pattern, cells from knock out animals were disorganized without a clear directional pattern. A similar disorganized pattern of *renin*$^{null}$ cells was observed whether the analysis was done using GFP or LacZ 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 *renin*$^{null}$ cells that encircle the vessels and/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 fourteen 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 regarding the possible mechanisms underlying such seemingly non-homeostatic 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 which 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 *renin*$^{null}$ cells and/or their progenitors retain their fetal characteristics and a molecular program driven not only to produce renin but also vascular growth as it occurs in early life [51] (Figure 5). Further work will be needed to define whether the repertoire of genes expressed by *renin*$^{null}$ 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 disease needs to be determined. Given the widespread use of and efficacy of RAS inhibition in the treatment of 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 hyperthrophy develops as a result of deletion of renin gene in the mouse, *renin*$^{null}$ cells survive, increase in number along the renal arterial tree, and maintain an active molecular memory of the renin phenotype. Further, 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 inhibition of the RAS leads to similar arterial and renal disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Novelty and Significance

### What Is New?

This study demonstrates that in *renin* knock out mice:

- Renin cells persist and maintain the program of the renin phenotype.
- Renin cells *per se* contribute to the vascular renal hyperthrophy in renin knock out mice.

### What Is Relevant?

This study points at renin lineage cells as contributors to vascular renal hyperthrophy. Understanding the underlying mechanisms is important due to the use of prolonged pharmacological inhibition of the RAS in humans with hypertension.

### Summary

The study shows that in *renin* knock out mice: i) there is concentric hypertrophy of the kidney arterioles leading to advanced kidney disease; ii) renin lineage cells survive and increase in numbers along the renal arterial tree; iii) those $renin^{nul}$ cells maintain the molecular program of the renin phenotype, and iv) integrate in the vessel wall, thus contributing to the vascular disease.

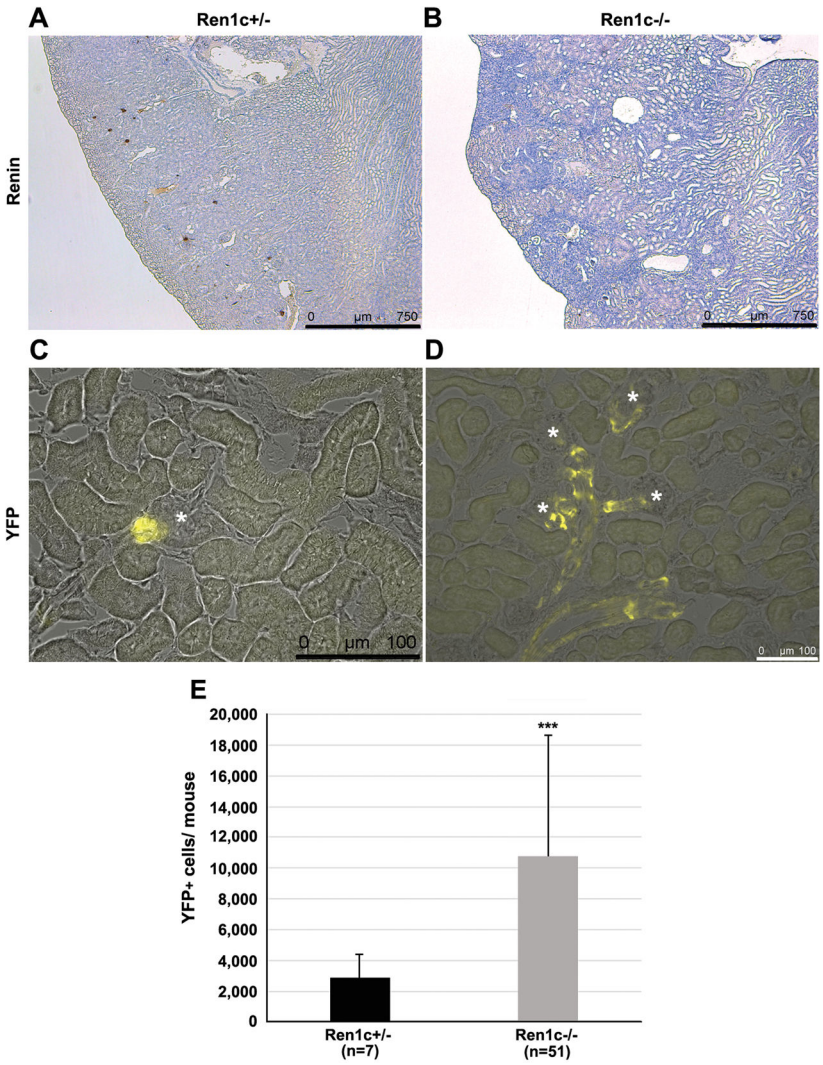


**Figure 1.**

Distribution of YFP + renin null cells in the kidneys of *Ren1*$^{c-/-}$ mice.

**A, B.** Low magnification view of kidneys from *Ren1*$^{c+/-}$ (**A**) and *Ren1*$^{c-/-}$ (**B**) mice immunostained for renin. Whereas renin expression is detected in the cortex of the *Ren1*$^{c+/-}$ kidney (**A**), as expected, no renin is observed in the *Ren1*$^{c-/-}$ kidney (**B**). Scale bars: 750 µm. **C-E**. YFP expression in kidneys from *Ren1*$^{c+/-}$ and *Ren1*$^{c-/-}$ mice. In control animals, YFP+ cells are restricted to the juxtaglomerular areas (**C**). In *Ren1*$^{c-/-}$ mice, YFP+ cells are observed throughout afferent arterioles, interlobular and arcuate arteries and glomeruli (**D**). Scale bar: 100 µm (*), glomeruli. **E.** Quantification of YFP+ cells in kidneys. The number of YFP+ cells is increased threefold in *Ren1*$^{c-/-}$ mice when compared to heterozygous controls (***$P$<0.0001).

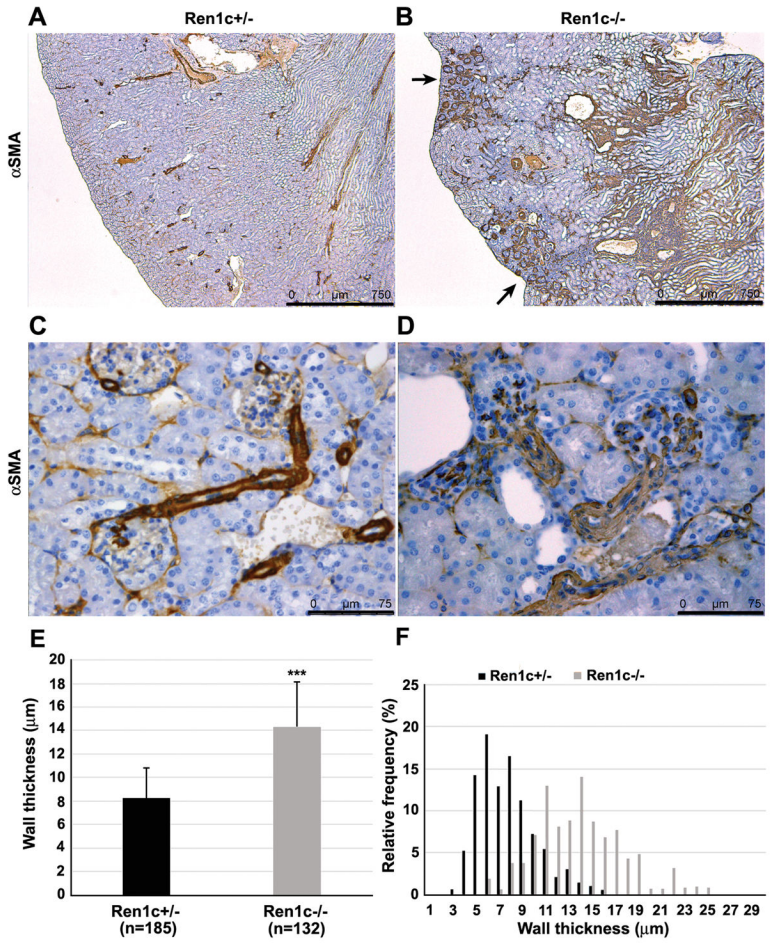


**Figure 2.**

Histology of kidneys from *Ren1*$^{c+/-}$ and *Ren1*$^{c-/-}$ mouse.

**A, B.** αSMA immunostaining shows that *Ren1*$^{c-/-}$ kidneys exhibit granular surface (arrows) correlated with marked interstitial and peri-glomerular fibrosis, tubular dilatation and atrophy. Scale bars: 750 μm. **C, D.** Higher magnificacion of kidney sections from control and *Ren1*$^{c-/-}$ mice immunostained for αSMA. *Ren1*$^{c-/-}$ kidneys show thicker intrarenal arterial walls than *Ren1*$^{c+/-}$ kidneys and and display interstitial fibrosis. Moreover, *Ren1*$^{c-/-}$ kidneys show αSMA expression in glomeruli and tubular expansions. Scale bars: 75 μm. **E, F.** Quantification of the wall thickness shows that *Ren1*$^{c-/-}$ mice have thicker arterioles than *Ren1*$^{c+/-}$ controls (**E,** ***$P$<0.0001). **F.** Relative frequency distribution histograms show that the distribution curve corresponding to the *Ren1*$^{c-/-}$ animals is displaced to the right indicating that a higher proportion of vessels possess thicker walls than in control mice.

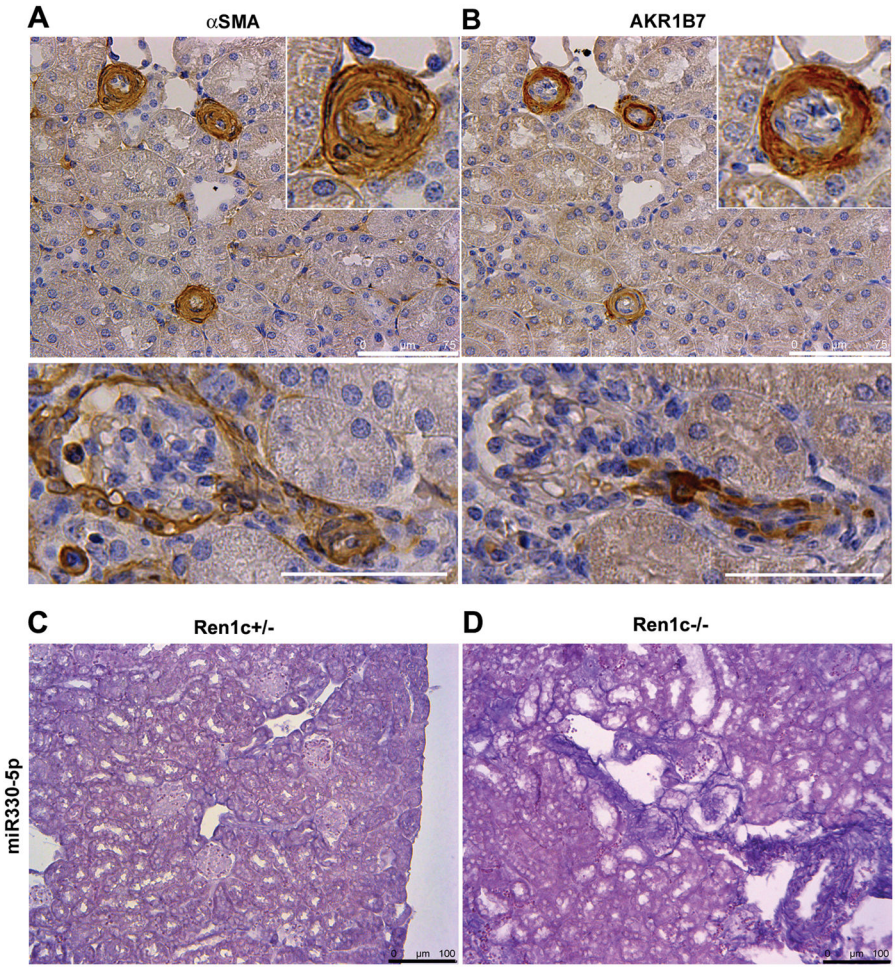


**Figure 3.**

Expression of renin cell markers in *Ren1*$^{c-/-}$ mice.

**A, B.** αSMA and AKR1B7 immunostaining of consecutive sections of *Ren1*$^{c-/-}$ kidneys. AKR1B7 is expressed even when the cells are unable to synthesize renin (**B**). AKR1B7 positive cells coincide with cells expressing αSMA in the outer layers of the arteriole (**A, B,** top). In some vessels, AKR1B7+ cells are also distributed deeper inside the arteriolar wall (**A, B,** bottom). Few AKR1B7+ cells are observed in areas of peri glomerular fibrosis. As expected in those areas, αSMA expression is prominent (**A, B**, bottom). Scale bars: 75 μm (top panels); 50 μm (bottom panels). **C, D.** *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 *Ren1*$^{c+/-}$ controls (**C**), *miR-330-5p* is highly expressed in the vessel walls and inside glomeruli in *Ren1*$^{c-/-}$ kidneys, consistent with its presence when the renin program is activated (**D**). Scale bars: 100 μm.

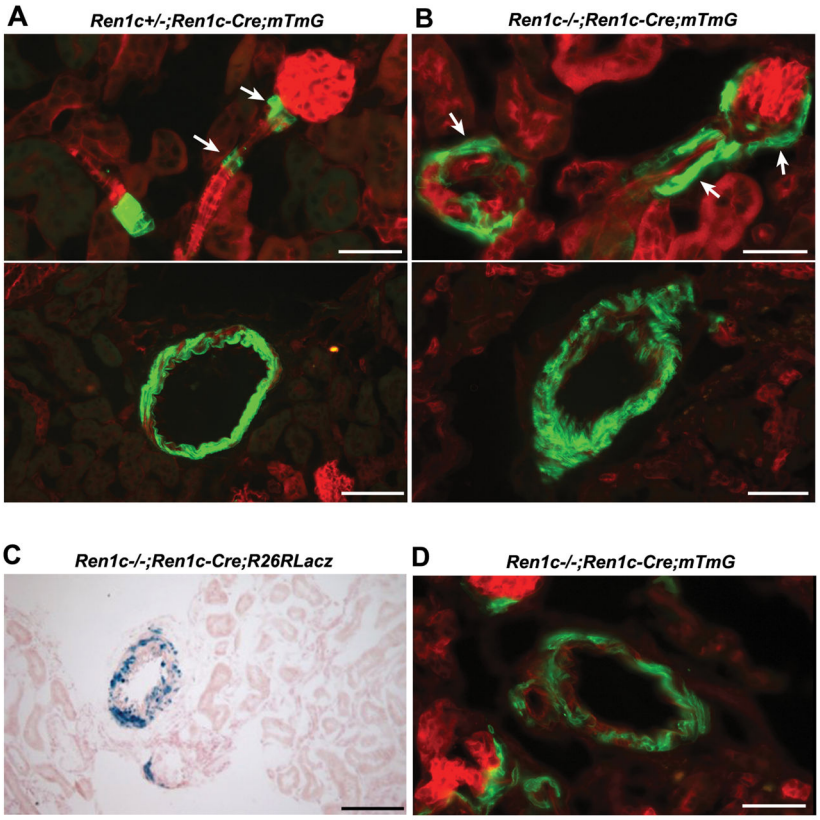


**Figure 4.**

Distribution of cells from the renin lineage in *Ren1*$^{c-/-}$ mice.

In control *Ren1*$^{c+/-}$ kidneys, cells from the renin lineage (GFP+) are found in the afferent arterioles including its juxtaglomerular area (**A,** top). In *Ren1*$^{c-/-}$ 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 *Ren1*$^{c+/-}$control and *Ren1*$^{c-/-}$ 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 knock out 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+) are non-renin 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**).

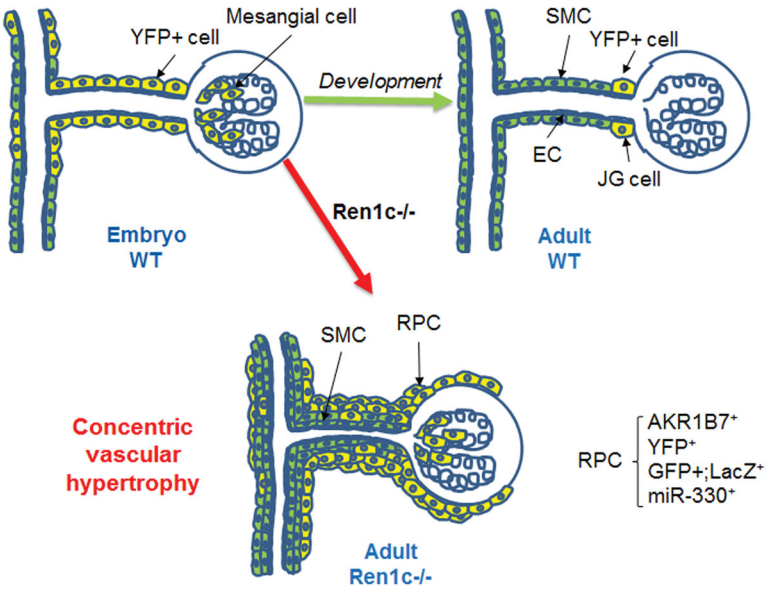


**Figure 5.**

Schematic of *Renin*$^{null}$ cells contribution to vascular pathology. During normal development, renin precursors and/or their descendants are present along large intra-renal 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. Those *renin*$^{null}$ cells maintain the molecular program of the renin phenotype, *i.e.* 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, their distribution and number along the renal arterial tree is expanded in a pattern that replicates the distribution of renin cells during fetal development and in response to physiological stress in adult animals. RPC: renin progenitor cell; SMC: smooth muscle cell; YFP: yellow fluorescent protein, GFP: green fluorescent protein; EC: endothelial cell; JG cell: juxtaglomerular cell.

**Table 1**

**Ren1c+/−**

mice have growth retardation, renal failure and anemia

| Parameter | *Ren1c+/−* | *Ren1c−/−* |
|---|---|---|
| **Body Weight (g)** | 22.1±2.7 (26) | 20.6±2.1 (27) * |
| **Total Kidney to Body weight (mg/g)** | 13.1±1.8 (26) | 9.5±1.4 (27) † |
| **Spleen to Body Weight (mg/g)** | 3.5±0.8 (26) | 4.6±1.0 (27) † |
| **White Blood Cell (k/uL)** | 1.78±1.25 (21) | 2.06±1.33 (21) |
| **Hemoglobin (g/dL)** | 13.6±0.6 (21) | 10.0±1.1 (21) † |
| **Platelet (k/uL)** | 616±138 (21) | 561±120 (21) |
| **Blood Urea Nitrogen (mg/dL)** | 22.5±3.4 (26) | 90.9±16.5 (26) † |
| **Creatinine (mg/dL)** | 0.42±0.04 (26) | 0.52±0.04 (26) † |

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

* $P<0.05$ compared to *Ren1c+/−*;

† $P<0.001$ compared to *Ren1c+/−*.