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Renin Cells Are Precursors for Multiple Cell Types that Switch to the Renin Phenotype When Homeostasis Is Threatened

Short Article

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Summary

Renin-synthesizing cells are crucial in the regulation of blood pressure and fluid-electrolyte homeostasis. Adult mammals subjected to manipulations that threaten homeostasis increase circulating renin by increasing the number of renin-expressing/-releasing cells. We hypothesize that the ability of adult cells to synthesize renin does not occur randomly in any cell type, depending instead on the cell's lineage. To determine the fate of renin-expressing cells, we generated knockin mice expressing cre recombinase in renin-expressing cells and crossed them with reporter mice. Results show that renin-expressing cells are precursors for a variety of cells that differentiate into non-renin-expressing cells such as smooth-muscle, epithelial, mesangial, and extrarenal cells. In the kidney, these cells retain the capability to synthesize renin when additional hormone is required to reestablish homeostasis: specific subpopulations of apparently differentiated cells are "held in reserve" to respond (repeatedly) by de-differentiating and expressing renin in response to stress, and re-differentiating when the crisis passes.

Introduction

The renin-expressing cell is a key regulatory element in the renin-angiotensin-aldosterone system. Renin-expressing cells are crucial in the regulation of blood pressure, renal hemodynamics, and fluid-electrolyte homeostasis. In adult mammals, these cells are located in the wall of the afferent arteriole (Figure 1A) at the entrance to the glomerulus, thus their name juxtaglomerular (JG) cells. JG cells are strategically located in the nephron, making contact with other structures of the juxtaglomerular apparatus (JGA) composed of the afferent and efferent arterioles, the macula densa cells of the distal tubule, and the extraglomerular mesangium that in turn is connected with glomerular mesangial cells (Figure 1A).

JG cells receive a variety of signals that very precisely regulate renin release. The basic mechanisms regulating renin release include: (1) the renal baroreceptor, whereby a decrease in perfusion pressure (hypotension or severe dehydration) is sensed by the JG cells, resulting in their release of renin; (2) the macula densa mechanism: a decrease in the concentration of sodium chloride (as in sodium depletion) in the distal tubule (Figure 1A) signals JG cells to release renin; (3) the β-adrenergic receptor mechanism: JG cells are densely innervated and stimulation of their β2 receptors (sympathetic discharge during hemorrhage or hypoxia) increases renin release. In most situations, several of these mechanisms act in concert, providing a potent stimulus for renin release.

Upon reaching the circulation, renin hydrolyzes its only known substrate angiotensinogen (Atg, produced in the liver) to generate angiotensin I (Ang I) (Figure 1B). In turn, Ang I is cleaved to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). Ang II is a powerful, direct vasoconstrictor, which also increases cardiac output by stimulating cardiac contractility and heart rate. The combined effects of vasoconstriction and increased cardiac output result in the reestablishment of blood pressure to normal (Figure 1B). In addition to enhancing sodium reabsorption via renal hemodynamic effects due to vasoconstriction, Ang II also enhances proximal and (via aldosterone release) distal tubular sodium reabsorption (Figure 1B). Ang II effects on the central nervous system and gut also result in water/NaCl reabsorption and expansion of the extracellular fluid volume (Figure 1B). Overall, the combined actions of Ang II result in normalization of blood pressure and the volume and composition of the extracellular fluid.

Ang II generated by the renin-angiotensin cascade in turn exerts a negative feedback effect on renin release, controlling its own production. Therefore, stimuli that prevent angiotensin generation (ACE inhibitors, Atg deletion) or action (Ang II receptors blockade or deletion) increase renin synthesis and release.

Renin-expressing cells have been considered as terminally differentiated because they synthesize a hormone (renin), are few in number, and are restricted to a JG localization. However, during embryonic development these cells are present in the undifferentiated metanephric mesenchyme before vascularization of the kidney has occurred and before the hemodynamic functions of renin are needed (Sequeira Lopez et al., 2001). Later in fetal life, renin-expressing cells are also found in large intrarenal arteries, inside the glomeruli and in the interstitium (Gomez et al., 1986; Sequeira Lopez et al., 2001). As maturation continues, the number of renin-expressing cells is reduced as they become progressively restricted to the classical JG localization found in the adult (Taugner and Hackenthal, 1989; Keeton and Campbell, 1980). This maturational pattern and the fact that renin expression precedes the appearance of markers characteristic of differentiated cells suggest that renin-expressing cells are not terminally differentiated and may act as precursors for other cell types including but not limited to JG cells (Sequeira Lopez et al., 2001).

The aforementioned developmental changes are relevant to the understanding of the phenomenon of recruitment (Gomez et al., 1990, 1988; Gomez and Norwood, 1995), a process whereby a threat to homeostasis (such as dehydration or hypotension) results in an increase in

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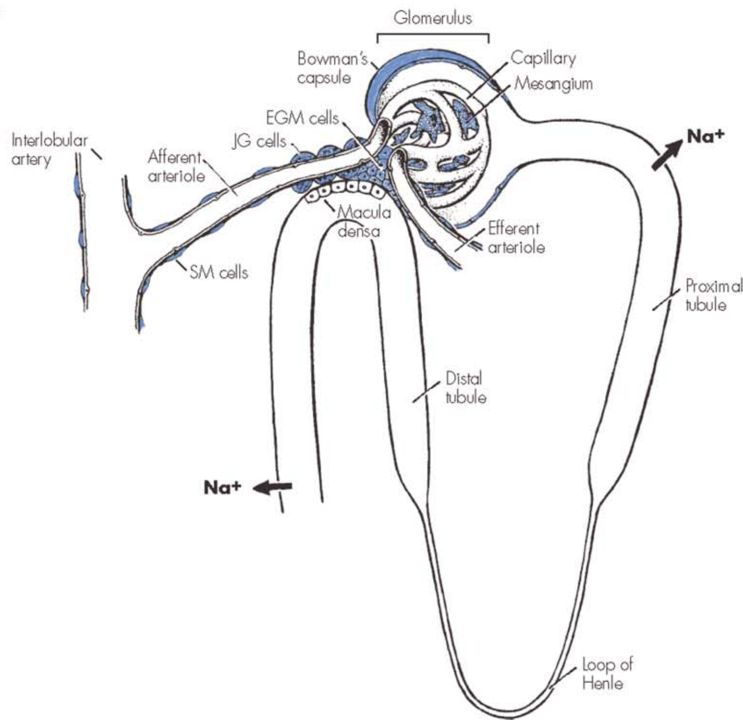

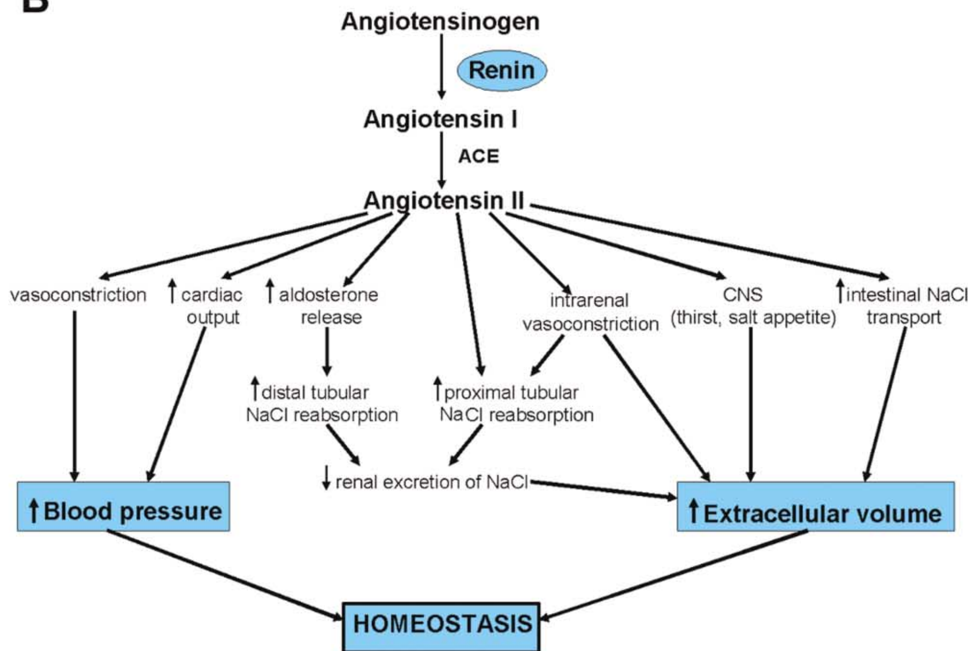

Figure 1. The Nephron and the Renin-Angiotensin System Cascade

(A) Schematic depiction of the adult nephron, the anatomical unit of the kidney, showing its vascular component, the glomerulus (where blood is filtered) and the proximal and distal tubular sites (where sodium reabsorption occurs). Blood enters the glomerulus through the afferent arteriole and exits it through the efferent arteriole. The balance between the afferent and efferent arteriolar resistance determines the amount of glomerular filtrate produced and also the proximal tubular sodium reabsorption. Distal tubular sodium reabsorption is regulated by aldosterone. Juxtaglomerular (JG) cells release renin in response to stimuli from neighboring structures and systemic influences. The blue color indicates cells that expressed renin in early life. EGM, extraglomerular mesangium; SM, smooth-muscle.

(B) Schematic of the renin-angiotensin system cascade and physiological functions of angiotensin II to maintain homeostasis. See text for details. ACE, angiotensin-converting enzyme; CNS, central nervous system.

the number of renin-expressing cells along the preglomerular arteries, inside the glomerulus, and in the kidney interstitium, resembling the embryonic and fetal pattern. These data suggest that adult renal vascular smooth-muscle cells and renal glomerular and interstitial cells have the plasticity to develop phenotypic characteristics of renin-expressing cells. We hypothesize that the ability of adult cells to synthesize renin does not occur randomly in any cell type but depends instead on the cell's lineage.

The fate of renin precursor cells in mammals has been almost impossible to study due to the lack of reagents and embryological techniques that allow these cells to be tracked along their differentiation pathways (Sequeira Lopez and Gomez, 2003). To define whether renin-expressing cells give rise to other cell types, we generated a mouse having cre recombinase under control of the renin locus and crossed it with reporter mice that after cre-mediated recombination express either β-gal (Soriano, 1999) or GFP (Novak et al., 2000) in the renin-expressing cell and its descendants, even if renin expression subsequently ceases, thus marking the renin cell lineage. We show that renin-expressing cells, in addition to JG cells, also differentiate into non-renin-expressing cells. Furthermore, using manipulations known to recruit renin-expressing cells we demonstrate that those adult cells that differentiated from cells that previously expressed renin retain the capability to synthesize renin when more hormone is required to reestablish homeostasis.

Results

Using homologous recombination, we inserted cre recombinase into the *Ren1^d* locus of strain 129 ES cells (Figures 2A–2E). The resulting mouse (Ren1^d-cre) is normal and expresses cre recombinase in all renin-expressing cells. To mark cells that express (or had previously expressed) renin, we crossed these mice (Ren1^d-cre) to the R26R and Z/EG reporter mice which express either β-galactosidase (β-gal) or GFP, respectively, after cre-mediated recombination (Figure 2F). Histological sections of kidneys from the offspring of Ren1^d-cre mice crossed to R26R reporter mice (Ren1^d-cre;R26R) were subjected to the X-gal reaction. As shown in Figures 2G–2K, β-gal (blue) is expressed not only in JG cells but also along the rest of the afferent arterioles (Figure 2H). β-gal is also broadly expressed in larger kidney vessels such as interlobular arteries (Figures 2G and 2H), arcuate arteries, the renal artery (Figure 2I), and its branches. This pattern of β-gal distribution is in agreement with the transient expression of renin in those sites during embryonic and fetal life (el Dahr et al., 1990; Taugner and Hackenthal, 1989; Gomez et al., 1986, 1989). Within the vessels, β-gal is observed in smooth-muscle cells in a striped pattern (Figures 2G–2I). β-gal is also expressed in the intraglomerular and extraglomerular mesangium (Figures 2J and 2K), in the Bowman's capsule (Figure 2K), and in a subset of proximal tubular cells (Figures 2J and 2K). The offspring of Ren1^d-cre mice crossed to the Z/EG reporter (Ren1^d-cre;Z/EG) had GFP expression in the same pattern as seen for β-gal in the Ren1^d-cre;R26R mice.

To address whether the increase in the number of renin-expressing cells that occurs when homeostasis is threatened is due to retransformation of cells that had previously expressed renin, we studied mice deficient in the angiotensinogen (Atg$^{-/-}$) gene and compared them to wild-type (Atg$^{+/+}$) mice. Figure 3 (A–F) shows the comparison of renin and β-gal distribution in the kidneys of Atg$^{+/+}$ and Atg$^{-/-}$ newborn mice. In Atg$^{+/+}$ mice, renin expression is limited to the JG cells and distal segments of the afferent arteriole (Figures 3A and 3D). However, in the Atg$^{-/-}$ mice renin extends beyond the JG area along the intrarrenal arterial tree (Figures 3B and 3E) in the same pattern as the one observed for β-gal in the Ren1^d-cre;R26R mice (Figures 3C and 3F). By 21 days of postnatal life, renin is further restricted to a few cells in the JG areas in Atg$^{+/+}$ mice (Figure 3G), whereas the Atg$^{-/-}$ mice express renin extensively along intrarenal arteries (Figure 3H). To quantify this further, we counted all cells that immunostained for renin along the kidney vasculature in kidney tissue sections of Atg$^{-/-}$ and Atg$^{+/+}$ mice at 21 days of postnatal life. The number of renin-positive cells in Atg$^{-/-}$ mice (1884 ± 120) was higher than in Atg$^{+/+}$ mice (214 ± 41) (Figure 3I, $p < 0.03$). Because renin-positive cells occupied practically all of the length of the arterioles, it is reasonable to assume that those cells that have reacquired the renin phenotype are cells that previously expressed renin such as smooth-muscle cells. This wide distribution of renin in the Atg$^{-/-}$ mice also corresponds to the expression of β-gal in the Ren1^d-cre;R26R mice (Figures 3C and 3F), indicating that recruitment of renin-expressing cells is accomplished by the cells that expressed renin during embryonic life.

To test this hypothesis, we subjected our Ren1^d-cre;R26R mice to manipulations that stimulate recruitment: a diet low in sodium chloride combined with captopril treatment. Whereas renin was restricted to the JG area in untreated animals (Figure 4A), in the treated ones renin extended throughout the length of the afferent arteriole (Figure 4B).

To verify that recruitment of renin-expressing cells occurred in cells that previously expressed renin, we performed double labeling studies (renin immunostaining and the X-gal reaction) in kidney sections of treated and untreated Ren1^d-cre;R26R mice (Figures 4C–4I). Figure 4C shows that untreated mice on a normal diet have immunostainable renin (brown) only in the JG cells whereas β-gal (blue) is observed throughout the length of the afferent arteriole. However, treated animals showed recruitment of renin along the preglomerular arteries (Figures 4D–4F), in the glomerular and extraglomerular mesangium (Figures 4F–4H), and in the glomerular capsule (Figure 4I), all areas that are positive for β-gal indicating that transformation of cells to the renin phenotype occurs in cells that previously expressed renin.

Additional double staining studies of Ren1^d-cre;R26R adult mouse kidneys with anti α-smooth-muscle actin antibody marked the vasculature and glomerular mesangium which coincided with β-gal expression (Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/6/5/719/DC1]), confirming that cells that expressed renin earlier in life became smooth-muscle cells of the renal arterial tree and mesangium.

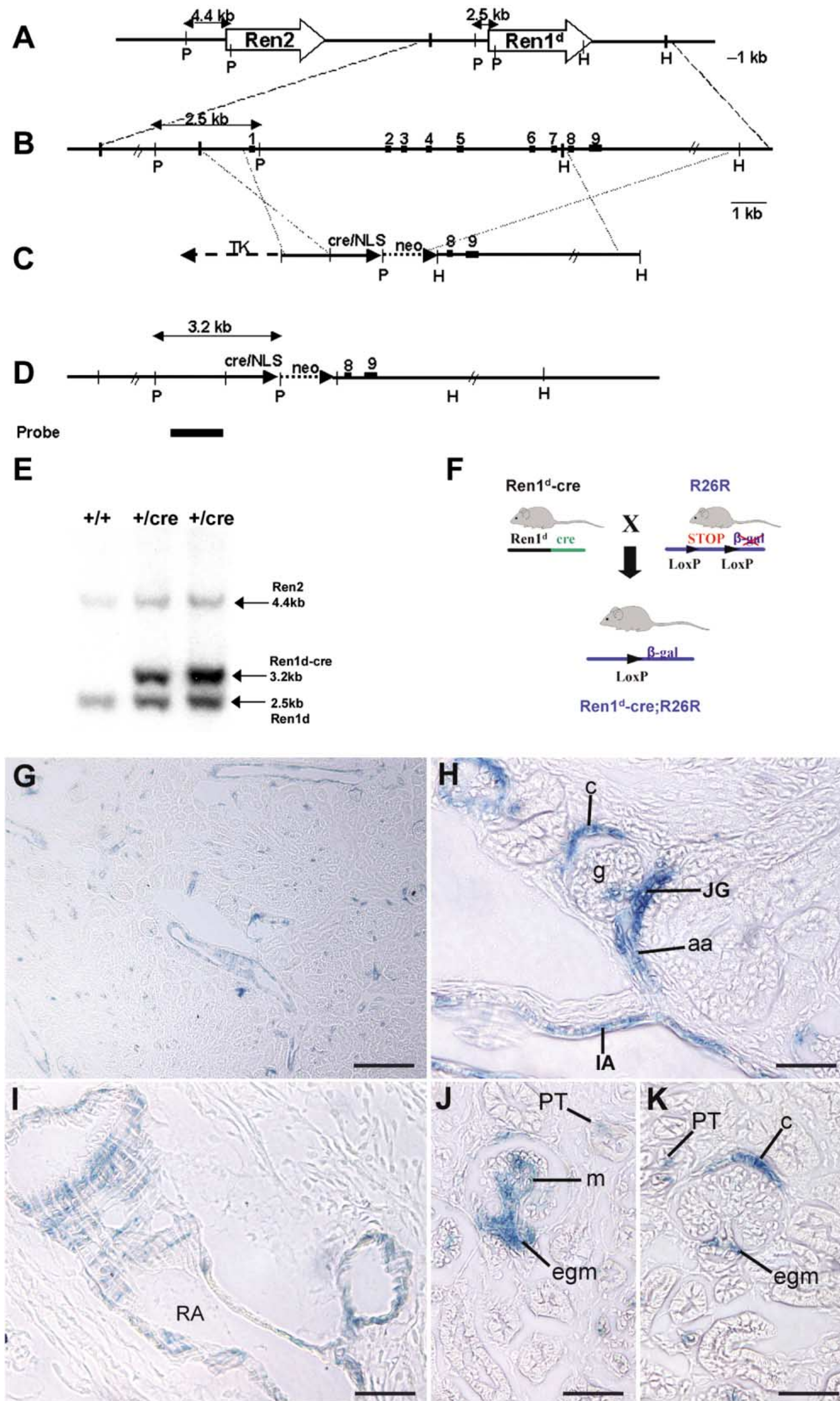

Figure 2. Marking the Lineage of Renin-Expressing Cells Reveals that They Are Progenitors for Smooth-Muscle, Mesangial, and Epithelial Cells in the Kidney

(A–E) Targeting cre to the renin gene. (A) The strain 129 mouse renin locus showing the two renin genes. Relevant restriction sites are shown: PstI (P) and HindIII (H). Genomic DNA fragments detected by the renin 5′ probe in *Ren1^d* and *Ren2* are shown above the map. Numbers indicate length in kb. (B) Expanded map of the *Ren1^d* gene showing intron/exon structure. Exons are the numbered black boxes. (C) Targeting construct showing 5′ and 3′ homology regions flanking cre and the locations of the neo resistance cassette and TK gene. Dashed lines show

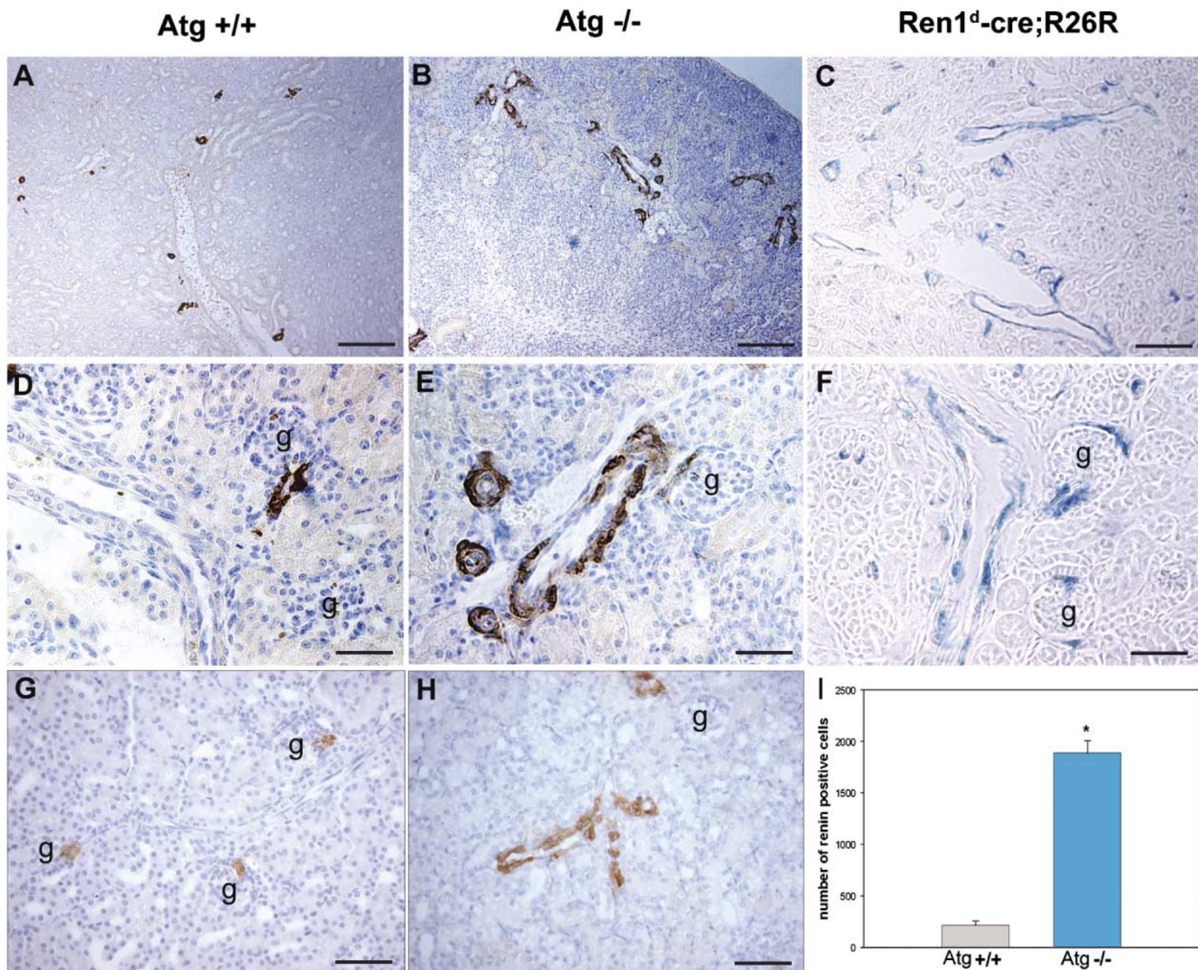

Figure 3. Angiotensinogen-Deficient Mice Recruit Renin-Expressing Cells

Renin distribution in $Atg^{+/+}$ (A, D, and G) and $Atg^{-/-}$ (B, E, and H) mouse kidneys assessed by immunostaining for renin (brown) compared to β-gal expression (blue) in $Ren1^d$-cre;R26R mice (C and F). Blue staining in (A), (B), (D), (E), (G), and (H) corresponds to counterstaining with hematoxylin. (A–F) 5-day-old mice, (G–I) 21-day-old mice.

(A) Renin immunostaining of a $Atg^{+/+}$ mouse kidney shows renin expression restricted to the juxtaglomerular areas and afferent arterioles.

(B) Kidneys from $Atg^{-/-}$ mice have extensive expression of renin along larger kidney arteries.

(C) β-gal in $Ren1^d$-cre;R26R mouse kidneys is present along the renal arterial tree marking the cells that previously expressed renin.

(D–F) Higher magnification of an afferent arteriole connecting to an interlobular artery. In the $Atg^{+/+}$ (D), renin is restricted to the JG area, whereas in $Atg^{-/-}$ (E), there is recruitment of renin-expressing cells along the arterial tree that coincides with β-gal expression in $Ren1^d$-cre;R26R (F) which have an intact Atg gene. g, glomerulus.

(G and H) Renin immunostaining of kidneys from 21-day-old mice shows the classical JG localization of renin in the $Atg^{+/+}$ kidney (G) and extensive recruitment of renin-expressing cells in the $Atg^{-/-}$ kidney (H).

(I) Bar graph showing number of renin-positive cells in $Atg^{+/+}$ and $Atg^{-/-}$ mouse kidneys at 21 days of age. * $P < 0.03$.

Scale bar: (A–C) 150 μm, (D–F) 37.5 μm, (G and H) 50 μm.

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the regions in which recombination takes place. (D) Targeted gene. Cre replaces exons 1–7 and part of intron 7 of the *Ren1^d* gene. Probe: 1.2 kb Renin 5′DNA. (E) Southern blot of PstI-digested DNA from wild-type (+/+) and cre targeted (+/cre) ES cells. The expected 2.5 kb *Ren1^d* and 4.4 kb *Ren2* genomic bands are present in +/+ and +/cre. In +/cre, the additional band at 3.2 kb confirms targeting of cre to *Ren1^d*. (F) Schematic of the cre-mediated fate-mapping system. $Ren1^d$-cre mice were mated with the reporter lines R26R (β-gal) and Z/EG (GFP). After cre-mediated excision of the LoxP flanked transcriptional stop sequence, the marker is permanently expressed in the descendent cells as illustrated for β-gal.

(G–K) Renin-expressing cells are progenitors for smooth-muscle, mesangial, and epithelial cells within the kidney. Sections of newborn kidneys from $Ren1^d$-cre;R26R mice stained for β-gal. (G) Low magnification showing widespread distribution of β-gal (blue) staining along the renal arterial tree and glomeruli. (H) β-gal staining in the JG cells (JG) within the afferent arteriole (aa), in the interlobular artery (IA) connected to it, inside the glomerulus (g), and in Bowman's capsule (c). (I) Renal artery (RA) showing the characteristic striped pattern that is usually seen with renin immunostaining in embryonic life. (J and K) Glomerular expression of β-gal: intraglomerular mesangium (m) and extraglomerular mesangium (egm), epithelial cells of Bowman's capsule (c), and some proximal tubular cells (PT).

Scale bars: (G) 150 μm, (H–K) 37.5 μm.

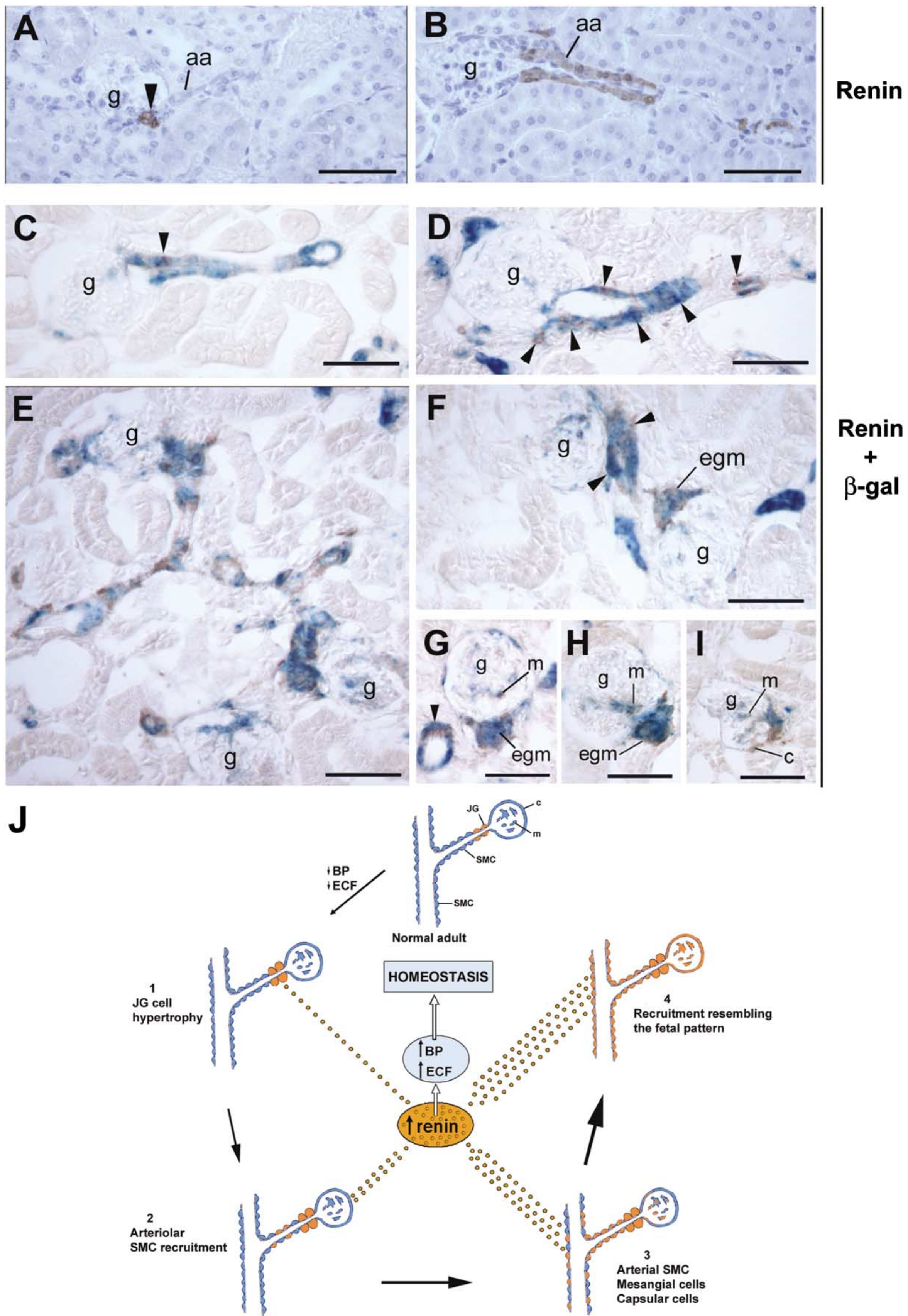

Figure 4. Recruitment of Renin-Expressing Cells in Ren1^{d}-cre;R26R Mice Subjected to Sodium Depletion and Captopril Treatment Corresponds to Cells that Previously Expressed Renin

Renin immunostaining (brown) in kidneys of untreated mice (A and C) and mice treated with sodium depletion plus captopril (B and D–I). (A and B) Paraffin sections counterstained with hematoxylin show that in an untreated animal (A) renin is restricted to a few JG cells (arrowhead)

The present studies allowed us to uncover a much wider distribution of cells that expressed renin earlier during development. In addition to the kidney, the adrenal glands also showed β-gal expression in a radial, variegated pattern (Supplemental Figure S2). Blue cells within the adrenal gland were observed in columns encompassing all zones of the cortex. In between cords of β-gal-positive cells there were patches of cells that did not express β-gal, suggesting that cells from the adrenal gland are composed of mixed and clonal types.

Leydig cells of the testis also showed β-gal expression (Supplemental Figure S2), indicating that they are capable of expressing renin. Sympathetic ganglia also showed high GFP expression (Supplemental Figure S2). Other extrarenal sites that showed cre-mediated β-gal or GFP expression include thymus, cartilage, and stomach (data not shown).

Discussion

To mark the renin cell lineage, we generated a mouse by homologous recombination that expresses cre recombinase under control of the *Ren1^d* locus in a two renin gene mouse (Figure 2A). A more detailed description of the renin genes and the reasons for choosing *Ren1^d* for targeting are provided in the Supplemental Data text, section one. Our results show that this model faithfully tracks renin cell fate both during development and in the retransformation to the renin phenotype that occurs during recruitment.

The Renin Cell Is a Precursor for Other Cell Types

This study shows that renin-expressing cells are not fully differentiated and that, in addition to JG cells, they are progenitors for other non-renin-expressing cells. Those non-renin-expressing cells differentiate into other cell types that retain the capability to synthesize renin when homeostasis is threatened.

We have previously shown that in the embryonic and fetal kidney renin-expressing cells are broadly distributed along the length of the intrarenal arterial tree and within the glomeruli (Gomez et al., 1986; Sequeira Lopez et al., 2001). As maturation ensues, renin expression is restricted in the adult animal to a few JG cells close to the glomerulus (Gomez and Norwood, 1995). It was unclear whether this decrease in the number of renin-expressing cells was due to differentiation into other cell types or other mechanisms. Because adult animals are capable of recruiting renin-expressing cells from outside the JG area when homeostasis is threatened, we hypothesized that in addition to JG cells, embryonic renin-expressing cells differentiate into non-renin-expressing cells such as arteriolar smooth-muscle and mesangial cells. Using a fate-mapping cre loxP system, we herein provide direct in vivo evidence that renin-expressing cells are precursors for other cell types in the kidney, including JG cells, smooth-muscle cells from the renal arterial tree, glomerular mesangium, and a subset of glomerular capsular and proximal tubular epithelial cells. During development, renin-expressing cells also act as precursors for cells in the adrenal glands, testis, sympathetic ganglia, cartilage, stomach, and thymus.

Because adult JG cells have myofilaments, it has been postulated that they derive from smooth-muscle cells (Taugner and Hackenthal, 1989). However, renin-expressing cells acquire the capacity to express smooth-muscle markers later in fetal life, at the time of arteriolar assembly (Sequeira Lopez et al., 2001). Those studies challenged the dogma that renin-expressing cells derived from smooth-muscle cells (Taugner and Hackenthal, 1989; Keeton and Campbell, 1980) and suggested instead that renin-expressing cells are capable of giving rise to vascular smooth-muscle and mesangial cells (Sequeira Lopez et al., 2001). This hypothesis is now confirmed by the results of the present study showing coexistence of smooth-muscle staining and β-gal staining along arteries and mesangium in the adult animal.

Renin Cell Descendants Switch to the Renin Phenotype When Homeostasis Is Threatened

The issue of renin cell fate is closely related to the phenomenon of recruitment, of central importance to the regulation of blood pressure and body fluid homeostasis. If an adult animal is subjected to manipulations that threaten homeostasis, such as hypotension, sodium depletion, and tissue hypoperfusion, circulating renin increases primarily due to an increase in the number of renin-expressing cells along the preglomerular arterioles (Gomez et al., 1988, 1990; Gomez and Norwood, 1995). The increased circulating renin eventually reestablishes blood pressure and sodium homeostasis. However, if the stimulus persists and the need for renin continues, additional smooth-muscle-like cells (glomerular mesangial cells) and epithelial cells of Bowman's capsule undergo metaplastic transformation and are thus "recruited" to synthesize renin in a pattern resembling that of the embryo (el Dahr et al., 1990; Taugner and Hackenthal, 1989; Gomez et al., 1986, 1989).

To define whether the increase in the number of renin-expressing cells that occurs when homeostasis is

in the afferent arteriole (aa) at the entrance to the glomerulus (g), whereas in a treated animal (B) there is recruitment of renin cells along the afferent arteriole.

(C–I) Frozen sections double labeled for β-gal (blue, X-gal reaction) and renin (brown, immunostaining) show that renin cells costain with β-gal. (C) Untreated mouse shows coincidence of renin and β-gal in a few JG cells (arrowhead) in the same location as in (A) with no renin along the rest of the blue arteriole. (D) Recruitment of renin showing coincidence of renin cells and β-gal along the afferent arteriole (arrowheads). (E) Extensive recruitment of renin within the kidney vasculature. Renin-positive cells correspond to cells with β-gal expression. (F–I) Recruitment of renin in arterioles ([F] and [G], arrowheads), in the extraglomerular mesangium ([F]–[H], egm), in the intraglomerular mesangium ([G] and [H], m) and in the glomerular capsule ([I], c). Scale bar: 50 μm.

(J) Conceptualization of the phenomenon of recruitment in the kidney. See Discussion for details. Recruitment of renin expression (orange) always occurs in cells that previously expressed renin (blue) during development. Blood pressure, BP; extracellular fluid, ECF; smooth-muscle cells, SMC.

threatened is due to recruitment/retransformation of cells that had previously expressed renin, we studied two different animal models. The first model consisted of mice with targeted deletion of the Atg gene (Kim et al., 1999). Homozygous Atg$^{-/-}$ mice cannot make Ang II (which normally exerts a negative feedback on renin expression and release) and are hypotensive, two well-known mechanisms to stimulate renin release and recruitment of renin-expressing cells. Kidneys from Atg$^{-/-}$ animals develop morphological abnormalities over time (described in the Supplemental Data text, section 2). This model is unique in that the stimulus for increased renin expression exists throughout the duration of the animal's life. The present study shows that these mice have a 10-fold increase in the number of renin-expressing cells along the kidney vasculature in an attempt to reestablish blood pressure. The location of renin-expressing cells in Atg$^{-/-}$ mice is similar to that in the fetal kidneys of wild-type mice, and also similar to the location of β-gal-positive cells in adult Ren1^d-cre;R26R mice. Because renin-expressing cells in adult Atg$^{-/-}$ mice occupy the same anatomical location that in the wild-type adult contains arteriolar smooth-muscle and glomerular mesangial cells, it is likely that recruitment of renin-expressing cells occurred by retransformation of cells that differentiated from embryonic renin-expressing cells. To test this hypothesis, we subjected our Ren1^d-cre;R26R mice to a low-sodium diet and treatment with an ACE inhibitor (captopril). Both manipulations are known to induce recruitment of renin-expressing cells along preglomerular arteries and occasionally in the glomerulus. We observed recruitment of renin-expressing cells along preglomerular arteries, in the glomerular capsule and in the extraglomerular and glomerular mesangium. Costaining for renin and β-gal showed a clear correspondence between recruited cells and those that also express β-gal. The coexpression of renin and β-gal in the same cells indicates that recruitment occurs by retransformation of cells that previously expressed renin. The experiments confirm our hypothesis that recruitment of renin-expressing cells is determined by the developmental history of the cells, which retain the memory to re-express the renin gene under physiological stress.

The presence of β-gal in the Bowman's capsule and proximal tubular cells deserves some comment. Recently, we detected renin-positive cells in the epithelial cells of the Bowman's capsule of adult mice treated with enalapril (our unpublished data), and others (Chen et al., 1994) have described the presence of renin mRNA, although at low levels (1/500th of the JG cells) in proximal tubular cells of the rat kidney. Furthermore, immunoreactive renin has been detected in proximal tubular cells in human embryos delivered by prostaglandin-induced abortion (Celio et al., 1985). These studies suggest a previously unsuspected lineage relationship between renin-expressing cells and a subset of epithelial cells both from the glomerular capsule and proximal tubules, indicating that those cells derive from renin-expressing cells.

The expression of β-gal and GFP in the adrenal cortex of the Ren1^d-cre;R26R and Ren1^d-cre;Z/EG mice reported in this study is an exciting finding because it reveals an extrarenal site with persistence of cells that expressed renin in fetal life. Renin is transiently expressed at high levels in the E15 adrenal gland and is markedly downregulated in late fetal life with undetectable levels at birth and thereafter during postnatal life (Kon et al., 1990; Pentz et al., 2001). The fate of renin-expressing cells in the fetal adrenal gland was unknown. The fact that we observe cells that expressed renin in radial columns extending from the outer cortex to the inner cortex supports the concept that cells in the same columns belong to the same lineage (Kataoka et al., 1996; Morley et al., 1996). The present study shows that cells that transiently expressed renin in the fetal adrenal gland differentiated and contributed to cells in all layers of the adrenal cortex.

The present study also showed evidence for the expression of renin in a very specific location within the testis, the interstitial cells of Leydig. Our findings are in agreement with another study showing the presence of renin in the rat testis (Deschepper et al., 1986). Two populations of Leydig cells have been described: the adult Leydig cells that seem to originate from peritubular mesenchymal cells (Siril Ariyaratne et al., 2000) and the fetal Leydig cells that are responsible for early masculinization of the embryo. The precise origin of fetal cells is unclear. It is believed that they originate from the mesonephros or the coelomic epithelium. It has also been suggested that Leydig cells and adrenal cortical cells may share a common origin with the mesonephros (Hatano et al., 1996), a tissue known to express renin in several animal species (Celio et al., 1985; Egerer et al., 1984). The presence of β-gal and GFP-positive cells in the adrenal, testis, and kidney supports the possibility that these organs share cells with a common lineage and/or origin.

An interesting question is whether recruitment can occur in extrarenal tissues such as the adrenal gland and testis. Although our own experiments in sodium-depleted and captopril-treated Ren1^d-cre;R26R mice did not show recruitment of renin-positive cells in the adrenal or testis, Naruse et al. showed that bilateral nephrectomy plus bilateral submandibular gland removal results in expression of adrenal renin in a pattern not dissimilar from that one found in our study (Naruse et al., 1984), suggesting that under certain conditions, recruitment of renin-expressing cells is not limited to the kidneys and can involve extrarenal tissues.

In summary, we used homologous recombination to develop a mouse model that expresses cre recombinase under the control of the *Ren1^d* locus. The model allowed us to establish the renin-expressing cell as a progenitor for other cell types and helped us formulate the basis for recruitment, the reacquisition of the renin phenotype when homeostasis is threatened. A conceptualization of the phenomenon of recruitment within the kidney is shown in Figure 4J. In response to a challenge to homeostasis that reduces blood pressure or changes the composition and/or volume of the extracellular fluid (as in dehydration, hypotension, and hemorrhage), JG cells first undergo hypertrophy (1) with a modest increase in renin release. If the stimulus continues and/or homeostasis is not reestablished, there is a progressive recruitment of renin-expressing cells from smooth-muscle cells (SMC) along the afferent arterioles (2) that eventually expands to arterial SMC, glomerular, and interstitial cells (3). As a result, circulating renin increases to

reestablish blood pressure and fluid and electrolyte homeostasis. If blood pressure and the composition of the extracellular fluid are not normalized, recruitment may become global, resembling the fetal pattern (4). Recruited cells are descendants of cells that expressed renin during development. Depending on the nature and intensity of the stimulus, recruitment of renin-expressing cells may occur in extrarenal sites such as the adrenal gland.

Overall, these studies suggest that the range of responses in the adult is determined and constrained by the developmental history of our cells.

The mice that we have generated should also be extremely valuable to delete genes specifically in the renin-expressing cell and therefore determine the precise cellular function of those genes independently of systemic influences.

Experimental Procedures

Generation of $Ren1^d$-cre Targeting Mouse

Targeting cre to the renin gene is depicted in Figure 2. The cre recombinase cDNA was obtained from pACNcre (gift of Kirk Thomas and Mario Capecchi) (Bunting et al., 1999). To facilitate cloning, ACNcre was amplified by PCR using a proofreading DNA polymerase (Vent DNA polymerase; New England Biolabs, Beverly, MA) with primers which added an NcoI site at the 5′ end. The *Ren1^d* targeting DNA fragments (1242 bp 5′ and 8.9 kb 3′) were those previously used in targeting GFP to the *Ren1^d* locus (Pentz et al., 2001). The *Ren1^d* 5′ fragment was cloned into the NcoI site of cre, and the resulting $Ren1^d$-cre 5′ targeting fragment was cloned 5′ to a Neo^R gene selectable marker in the targeting vector osdupdel. The 3′ targeting fragment was cloned 3′ to the Neo^R marker. The loxP flanked Neo^R gene in the original osdupdel vector was replaced with the SV40 enhancer/promoter-driven Neo^R gene from pIRES (BD Biosciences, La Jolla, CA).

Gene targeting was carried out using conventional procedures (Kim et al., 1995) in TC1 ES cells (a gift from Dr. Philip Leder). Targeted colonies were provisionally identified by PCR analysis (Kim and Smithies, 1988) using a 5′ primer (5′ GCCAGGGTCAGGT CACTTC) located upstream of any sequences in the targeting vector and 3′ primer (5′ GCAGGCAAATTTTGGTGTACGGTCA) located in cre. This PCR reaction generated a product (1.2 kb) only from correctly targeted DNA. PCR-positive colonies were expanded, genomic DNA was extracted, and Southern blots of PstI-digested DNA were hybridized with a 5′ genomic DNA probe (the *Ren1^d* 5′ targeting DNA fragment) to confirm the homologous recombination.

ES cells from one targeted line were injected into C57Bl/6J blastocysts to produce chimeras, which were crossed to C57Bl/6J and 129SvEv females (Taconic, Germantown, NY). Genotyping of the mice was by PCR of DNA from tail biopsies amplified with the same primers used to detect targeted clones.

Mice

The reporter lines used were R26R (Soriano, 1999) and the Z/EG double reporter (Novak et al., 2000) which ubiquitously express β-gal or GFP, respectively, in a cre-inducible manner. Angiotensinogen −/− (Kim et al., 1995) and wild-type mice were used to compare renin protein expression within the kidney.

Recruitment

To elicit recruitment of renin-expressing cells, 5-week-old $Ren1^d$-cre;R26R mice were administered a low-sodium diet (0.05%, Harlan, Madison, WI) plus captopril (Sigma, St. Louis, MO) in the drinking water (0.5 g/l) for 12 days ($n = 8$ treated animals, $n = 6$ control animals).

Histological Analysis and Immunostaining

To evaluate β-gal expression, mouse organs were dissected and fixed in freshly made 0.2% paraformaldehyde (PFA) in 0.1 M PIPES (pH 6.9) at 4°C overnight or 4% PFA in phosphate buffered saline (PBS) for 1 hr, cryoprotected in 30% sucrose in PBS plus 2 mM $MgCl_2$ at 4°C overnight and then frozen in OCT (Miles, Elkhart, IL). Cryosections (7 μm) were cut using a Leica Cryocut 1800 cryostat, post-fixed in 0.2% PFA in 0.1 M PIPES (pH 6.9) at 4°C for 10 min, washed in PBS plus 2 mM $MgCl_2$, incubated in detergent rinse (0.1 M phosphate buffer [pH 7.4], containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% tergitol NP-40) for 10 min on ice, and placed in staining solution [detergent rinse, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 3H_2O$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; Fisher Biotech)] overnight in the dark at 37°C. The slides were post-fixed in 4% PFA in PBS at 4°C for 1 hr, dehydrated in graded alcohols to xylenes, and mounted with xylenes based mounting medium (Cytoseal XYL; Richard-Allen Scientific, Kalamazoo, MI). Cryosections of adrenal glands and some from testis were counterstained with eosin after the X-gal reaction.

To confirm the identity of cells that express β-gal in the $Ren1^d$-cre;R26R mice, slides were postfixed in Bouins for 5 min after the X-gal staining, rinsed in PBS, and then subjected to immunostaining (see below).

To evaluate GFP expression, organs were processed as previously described (Pentz et al., 2001).

Immunostaining was performed as previously described (Sequeira Lopez et al., 2001) using the antibodies described below and the Vectastain ABC kit (Vector Laboratories) to visualize the antibody binding. To identify renin-expressing cells, a polyclonal goat anti-rat renin antibody (1:10,000 dilution, kind gift of Dr. T. Inagami) was used, for smooth-muscle cell identification a monoclonal anti-α-SMA-specific antibody (isotype IgG2a, dilution 1:10,000; clone 1A4, lot no. 076H4843, Sigma, St. Louis, MO), and for Leydig cell detection a rabbit anti-rat cytochrome p450 side chain cleavage enzyme (dilution 1:500, Research Diagnostics, Flanders, NJ).

Statistical Analysis

Renin-positive cells in kidney tissue sections from 21-day-old $Atg^{+/+}$ ($n = 4$) and $Atg^{-/-}$ ($n = 5$) mice were counted and values compared using the Mann-Whitney Rank Sum Test in the SigmaStat program (SPSS, Chicago, IL).

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References

Bunting, M., Bernstein, K.E., Greer, J.M., Capecchi, M.R., and Thomas, K.R. (1999). Targeting genes for self-excision in the germ line. Genes Dev. *13*, 1524–1528.

Celio, M.R., Groscurth, P., and Inagami, T. (1985). Ontogeny of renin immunoreactive cells in the human kidney. Anat. Embryol. (Berl.) *173*, 149–155.

Chen, M., Harris, M.P., Rose, D., Smart, A., He, X.R., Kretzler, M., Briggs, J.P., and Schnermann, J. (1994). Renin and renin mRNA in proximal tubules of the rat kidney. J. Clin. Invest. *94*, 237–243.

Deschepper, C.F., Mellon, S.H., Cumin, F., Baxter, J.D., and Ganong, W.F. (1986). Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal, and pituitary of the rat. Proc. Natl. Acad. Sci. USA *83*, 7552–7556.

Egerer, G., Taugner, R., and Tiedemann, K. (1984). Renin immunohistochemistry in the mesonephros and metanephros of the pig embryo. Histochemistry *81*, 385–390.

el Dahr, S.S., Gomez, R.A., Gray, M.S., Peach, M.J., Carey, R.M.,

and Chevalier, R.L. (1990). In situ localization of renin and its mRNA in neonatal ureteral obstruction. Am. J. Physiol. *258*, F854–F862.

Gomez, R.A., and Norwood, V.F. (1995). Developmental consequences of the renin-angiotensin system. Am. J. Kidney Dis. *26*, 409–431.

Gomez, R.A., Chevalier, R.L., Sturgill, B.C., Johns, D.W., Peach, M.J., and Carey, R.M. (1986). Maturation of the intrarenal renin distribution in Wistar-Kyoto rats. J. Hypertens. *4*, S31–S33.

Gomez, R.A., Lynch, K.R., Chevalier, R.L., Everett, A.D., Johns, D.W., Wilfong, N., Peach, M.J., and Carey, R.M. (1988). Renin and angiotensinogen gene expression and intrarenal renin distribution during ACE inhibition. Am. J. Physiol. *254*, F900–F906.

Gomez, R.A., Lynch, K.R., Sturgill, B.C., Elwood, J.P., Chevalier, R.L., Carey, R.M., and Peach, M.J. (1989). Distribution of renin mRNA and its protein in the developing kidney. Am. J. Physiol. *257*, F850–F858.

Gomez, R.A., Chevalier, R.L., Everett, A.D., Elwood, J.P., Peach, M.J., Lynch, K.R., and Carey, R.M. (1990). Recruitment of renin gene-expressing cells in adult rat kidneys. Am. J. Physiol. *259*, F660–F665.

Hatano, O., Takakusu, A., Nomura, M., and Morohashi, K. (1996). Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. Genes Cells *1*, 663–671.

Kataoka, Y., Ikehara, Y., and Hattori, T. (1996). Cell proliferation and renewal of mouse adrenal cortex. J. Anat. *188*, 375–381.

Keeton, T.K., and Campbell, W.B. (1980). The pharmacologic alteration of renin release. Pharmacol. Rev. *32*, 81–227.

Kim, H.S., and Smithies, O. (1988). Recombinant fragment assay for gene targetting based on the polymerase chain reaction. Nucleic Acids Res. *16*, 8887–8903.

Kim, H.S., Krege, J.H., Kluckman, K.D., Hagaman, J.R., Hodgin, J.B., Best, C.F., Jennette, J.C., Coffman, T.M., Maeda, N., and Smithies, O. (1995). Genetic control of blood pressure and the angiotensinogen locus. Proc. Natl. Acad. Sci. USA *92*, 2735–2739.

Kim, H.S., Maeda, N., Oh, G.T., Fernandez, L.G., Gomez, R.A., and Smithies, O. (1999). Homeostasis in mice with genetically decreased angiotensinogen is primarily by an increased number of renin-producing cells. J. Biol. Chem. *274*, 14210–14217.

Kon, Y., Hashimoto, Y., Kitagawa, H., Sugimura, M., and Murakami, K. (1990). Renin immunohistochemistry in the adrenal gland of the mouse fetus and neonate. Anat. Rec. *227*, 124–131.

Morley, S.D., Viard, I., Chung, B.C., Ikeda, Y., Parker, K.L., and Mullins, J.J. (1996). Variegated expression of a mouse steroid 21-hydroxylase/beta-galactosidase transgene suggests centripetal migration of adrenocortical cells. Mol. Endocrinol. *10*, 585–598.

Naruse, M., Naruse, K., Inagaki, T., and Inagami, T. (1984). Immunoreactive renin in mouse adrenal gland. Localization in the inner cortical region. Hypertension *6*, 275–280.

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C.G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis *28*, 147–155.

Pentz, E.S., Lopez, M.L., Kim, H.S., Carretero, O., Smithies, O., and Gomez, R.A. (2001). Ren1d and Ren2 cooperate to preserve homeostasis: evidence from mice expressing GFP in place of Ren1d. Physiol. Genomics *6*, 45–55.

Sequeira Lopez, M.L., and Gomez, R.A. (2003). Studies of cell lineage in the developing kidney. Methods Mol. Med. *86*, 193–204.

Sequeira Lopez, M.L., Pentz, E.S., Robert, B., Abrahamson, D.R., and Gomez, R.A. (2001). Embryonic origin and lineage of juxtaglomerular cells. Am. J. Physiol. Renal Physiol. *281*, F345–F356.

Siril Ariyaratne, H.B., Chamindrani Mendis-Handagama, S., Buchanan, H.D., and Ian, M.J. (2000). Studies on the onset of Leydig precursor cell differentiation in the prepubertal rat testis. Biol. Reprod. *63*, 165–171.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. *21*, 70–71.

Taugner, R., and Hackenthal, E. (1989). The Juxtaglomerular Apparatus: Structure and Function. (Heidelberg, Germany: Springer Verlag).