Establishing the Role of Angiotensin-Converting Enzyme in Renal Function and Blood Pressure Control through the Analysis of Genetically Modified Mice

Kenneth E. Bernstein, Hong D. Xiao, Jon W. Adams, Kristen Frenzel, Ping Li, Xiao Z. Shen, Justin M. Cole, and Sebastien Fuchs

Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia


Angiotensin II is a vasoconstrictor and a hypertensive peptide that binds to the AT1 receptor and, through both direct and indirect mechanisms, induces salt reabsorption. Also, angiotensin II is thought to be a profibrotic and proproliferative peptide; abundant evidence now suggests that angiotensin II can stimulate cytokines and oxygen radicals, inducing an inflammatory response (1,2). Indeed, so much has been written about angiotensin-converting enzyme (ACE) and angiotensin II that it is legitimate to ask what new can be found concerning actions of the renin-angiotensin system (RAS). This review shows that, rather than being a subject without novelty, the ability to genetically manipulate mice gives scientists the power to investigate the many physiologic roles of angiotensin II with a level of understanding not previously available. At last, scientific tools are available to test some of the hypotheses and dogmas concerning RAS action. Perhaps you are not convinced and think that, in a system that has seen the publication of angiotensinogen, renin, and ACE knockout mice, how could even the powerful technology of mouse genetic targeting provide novel insight? Answering precisely this question is the subject of the review.

The technique of creating a knockout mouse is now widely appreciated (3). Cultured embryonic stem cells are manipulated by targeted homologous recombination to induce virtually any genetic change that can be imagined. The most common use of this method has been to eliminate a gene that, in a mice that are homozygous for the mutation, results in a knockout animal. In this method has been used to create a series of genetically altered mice that bear zero to four copies of the angiotensinogen gene. This celebrated study used gene targeting to create a series of genetically altered mice that bear zero to four copies of the angiotensinogen gene. This study used gene targeting to either eliminate or duplicate the angiotensinogen gene and showed that there was a gene dosage effect in which every additional copy of the angiotensinogen gene beyond one was responsible for an 8-mmHg rise in BP. These data, combined with human studies, suggest that genetic variability in angiotensinogen can account for some genetic differences in human BP (11,12).

In addition to gene elimination or duplication, targeted homologous recombination can create precise mutations within a protein. For example, our group used such an approach to study ACE. This enzyme is a single polypeptide chain but contains two zinc binding sites and two separate and independent catalytic domains (13). Although angiotensin I is a good substrate for both of the two catalytic sites of ACE, other peptide substrates show distinct preferences for only one of the ACE catalytic sites. An example is the ACE substrate acetyl-
SDKP that has been implicated as an antifibrotic and a bone marrow–suppressive peptide (14,15). Acetyl-SDKP is effectively hydrolyzed only by the N-terminal catalytic domain of ACE; ACE inhibition is associated with an elevation in blood levels of this peptide (16). As discussed, ACE null (knockout) animals have anemia characterized by hematocrits approximately 20% lower than that in wild-type mice. One hypothesis suggested that the anemia was a consequence of acetyl-SDKP build-up in the absence of ACE activity. To test this, our group introduced point mutations into the ACE gene that eliminated the ability of the N-terminal ACE catalytic domain to bind zinc and thus rendered this domain catalytically inactive (Figure 1) (17). The result was a mouse model called ACE 7/7, which makes normal amounts of an altered ACE protein that has only one catalytic domain. In the absence of the N-terminal domain, these animals have elevated serum levels of acetyl-SDKP but a normal BP as a result of the ability of the unaltered C-terminal ACE catalytic domain to effectively hydrolyze angiotensin I. Study of these mice found that they had no evidence of anemia. Thus, this is an example in which genetic targeting in mice produced an altered protein, as opposed to the complete elimination of that protein. This particular model allowed study of the physiologic effects of acetyl-SDKP accumulation in a setting free of the secondary effects of low BP, and ultimately it disproved the suggested hypothesis for the actions of acetyl-SDKP. Even with this study, it remains unclear why animals that lack all ACE (ACE knockout mice) are anemic. Most likely, the anemia reflects the inability to generate angiotensin II (as opposed to another peptide) because the infusion of angiotensin II into ACE null mice is associated with correction of the anemia (18).

Selected Tissue Expression of ACE

If one views ACE null mice as expressing an extreme phenotype, then it would be useful to have mouse models with a less severe genetic change. One approach might be to selectively eliminate ACE expression in a particular organ such as the kidney (Figure 2). This is technologically feasible using gene targeting but probably would not yield a phenotype in the resulting mice. ACE is widely distributed in animal tissues; large amounts of ACE are made by vascular endothelium, the kidney, areas of the gut, and activated macrophages and in...
parts of the brain. Given the highly regulated expression of renin, it seemed unlikely that the elimination of ACE expression in one target organ would have much effect. Our group has selected a different approach: To create a mouse model devoid of ACE activity, except for selected organs engineered to express this protein (Figure 2). Models of this type can be created by genetic manipulation of the promoter region of the ACE gene (the portion of the gene that regulates tissue and temporal expression). For example, consider the mouse model that is termed ACE.3, in which genetic engineering was used to position the albumin promoter in place of the natural ACE promoter (Figure 2B) (19). In these mice, ACE expression is controlled not by the ACE promoter but by the engineered albumin promoter, leading to ACE protein expression by hepatocytes within the liver. Because the coding portions of the ACE gene were not modified, the protein was transported to the hepatocyte cell surface, similar to its typical localization in endothelium. However, as the albumin promoter is active only in hepatocytes (and not in endothelium), this model resulted in mice with very restricted ACE expression. For example, wild-type mice normally produce large amounts of ACE in the lung as a result of the high content of endothelium within this tissue (Figure 3). In contrast, ACE.3 mice produced no ACE in the lung or by any endothelium throughout the mouse. Only in the kidney was there aberrant low-level recognition of the modified ACE promoter, leading to renal ACE levels approximately 15% those of wild-type mice. The low-level ACE found in the kidney was present in the proximal tubule and probably resulted from the use of a man-made albumin promoter to control ACE expression. However, even in the kidney, vascular expression of ACE was not present. Thus, a mouse model in which the widespread distribution of ACE in endothelium and epithelium was replaced by localized ACE expression on the surface of hepatocytes was created.

One advantage of this approach is that compound heterozygous mice can easily be created through simple breeding. For instance, our group refers to ACE knockout mice as ACE.1; a homozygous knockout mouse has an ACE genotype called ACE 1/1 to indicate that both ACE alleles contain the ACE.1 mutation (Table 1). These animals are null for all ACE expression. Likewise, a mouse homozygous for the ACE mutation that targets expression to the liver has a genotype termed ACE 3/3. A mouse with point mutations that inactivate the ACE N-terminal catalytic domain is termed ACE 7/7. These numbers merely refer to the order in which the mouse models were created, but they are useful in signifying the ACE genotype. For instance, an ACE heterozygous mouse with one ACE.1 allele and one wild-type allele is termed wt/1. If this animal is mated with an ACE 3/3 mouse, then half of the offspring will have the genotype ACE 1/3. In these mice, the “1” ACE allele is null, whereas the “3” ACE allele directs ACE expression to the liver. Because of the null allele, ACE 1/3 mice have half of the hepatic, renal, and plasma levels of ACE found in ACE 3/3 mice (20). As compared with wild-type mice, ACE 1/3 mice have only 7% normal renal ACE activity.

This promoter-swapping technique was used to create a series of mice with restricted patterns of ACE expression (Table 1). Rather than discuss each model individually, the major conclusions obtained from the combined data are presented below.

**Blood Pressure**

Mice that lack all ACE have a systolic BP of approximately 73 mmHg as opposed to a wild-type mouse with a systolic BP of approximately 110 mmHg (Figure 4). This marked reduction in the absence of all ACE belies that mice (and probably humans) are very tolerant of marked changes in tissue patterns and overall levels of ACE expression. For example, ACE 1/7 mice, which express only half of normal levels of an ACE protein that...
lacks enzymatic activity in one of the two ACE catalytic domains (the N-terminal domain), have a normal BP (17). ACE 1/3 mice, animals with no endothelial expression of ACE and renal levels approximately 7% those of wild-type mice, also have a normal BP (20). Even animals that are engineered to express ACE only in myocardium and lung smooth muscle (ACE 8/8) present with a BP not much different from that of wild-type mice (21). The ability to tolerate wide changes in ACE activity was postulated through a computer-based analysis of what our group observed experimentally. In the ACE 1/3 mouse, normal BP is maintained by an elevation of plasma renin. Even in a mouse, even modest total body loads of ACE positioned in tissues as diverse as either the liver or the heart are sufficient for renal compensation and maintenance of normal BP. Only when ACE activity falls to very low levels can the RAS not achieve compensation. These conclusions apply to otherwise healthy animals that are maintained in a relatively disease- and stress-free laboratory environment.

### Role of Bradykinin

In addition to producing angiotensin II, ACE is thought to play a major role in the degradation of the vasodilator bradykinin. To study this, we bred ACE knockout mice with a different line of mice that lack the bradykinin B2 receptor to generate double-knockout mice (23). It is the B2 receptor that is thought to mediate the cardiovascular effects of bradykinin (24). The experimental design was to compare mice that lack only ACE with the double-knockout mice that lack both ACE and the bradykinin B2 receptor. The double-knockout mice had a phenotype identical to single ACE knockout mice (very low BP, underdevelopment of renal medulla, and inability to concentrate urine). Although this study was somewhat incomplete in that it did not account for bradykinin B1 receptors, it suggests that the main pathologic defect in ACE null mice is a lack of angiotensin II and not a surplus of bradykinin.

### Local versus Systemic Angiotensin II

What about the question of local versus systemic production of angiotensin II? This discussion reflects the presence of various components of the RAS throughout the body. In turn, this has engendered a lively discussion as to the importance of local production of angiotensin II (tissue based) versus the systemic...

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### Table 1. Important characteristics of mouse lines with genetic changes to the ACE gene

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype</th>
<th>Description</th>
<th>Distribution of ACE</th>
<th>BP</th>
<th>Renal Development</th>
<th>Urine Concentration</th>
<th>Hematocrit</th>
<th>Male Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>wt/wt</td>
<td>Two normal ACE alleles</td>
<td>Vascular endothelium (lung), kidney, gut, brain, plasma, testis</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE.1</td>
<td>1/1</td>
<td>Null for all ACE</td>
<td>No ACE</td>
<td>Low</td>
<td>Medullar and papillary dysplasia</td>
<td>Dilute</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>ACE.2</td>
<td>2/2</td>
<td>Stop codon in ACE gene</td>
<td>No tissue ACE, 34% plasma ACE activity</td>
<td>Low</td>
<td>Normal renal papilla</td>
<td>Dilute</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>ACE.3</td>
<td>3/3</td>
<td>Albumin promoter controlling ACE</td>
<td>Hepatocytes ↑ ↑, kidney (44% of wild type), plasma, testis</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE 1/3</td>
<td>1/3</td>
<td>Compound heterozygote</td>
<td>Half the expression of 3/3</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE 1/3</td>
<td>1/3</td>
<td>Compound heterozygote</td>
<td>Half the expression of 3/3</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE.5</td>
<td>5/5</td>
<td>γ-GT promoter controlling ACE</td>
<td>&lt;6% kidney ACE, testis</td>
<td>Low</td>
<td>Most kidney present medullar and papillary dysplasia</td>
<td>Dilute</td>
<td>Low</td>
<td>ND</td>
</tr>
<tr>
<td>ACE.7</td>
<td>7/7</td>
<td>N-terminal catalytic domain inactivated</td>
<td>Same as wild type</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE 1/7</td>
<td>1/7</td>
<td>Compound heterozygote</td>
<td>Half the expression of 7/7</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td>ACE.8</td>
<td>8/8a</td>
<td>a-MHC promoter controlling ACE</td>
<td>Heart ↑ ↑, lung (40% of wild type), plasma (50% of wild type), testis</td>
<td>Nearly normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE 10</td>
<td>10/10</td>
<td>c-fms promoter controlling ACE</td>
<td>Macrophages ↑ ↑ plasma, testis</td>
<td>In progress</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE 11</td>
<td>11/11</td>
<td>THP promoter controlling ACE</td>
<td>Similar to ACE.4</td>
<td>Low</td>
<td>Medullar and papillary dysplasia</td>
<td>Dilute</td>
<td>Low</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ACE, angiotensin-converting enzyme; ND, not done. We measured serum angiotensin II levels in several of these lines. As would be anticipated, low BP is associated with very low plasma levels of angiotensin II. Most mouse models with normal BP (e.g., ACE 7/7 and ACE 8/8) have plasma angiotensin II levels that are not significantly different from wild-type mice. However, ACE 1/3 mice have a normal BP but also have threefold wild-type levels of plasma angiotensin II. Normal aldosterone levels in this model suggest that these mice may have normal end-organ concentrations of angiotensin II.*
Thus, our view of the controversy of local versus systemic generation of angiotensin II is that, at least for BP control, both of these systems contribute to normal regulation. In a normal animal, it is the final concentration of angiotensin II that is regulated; this peptide originates from both systemic and local production. If local production is genetically diminished, then systemic production is upregulated.

Renal ACE

In the kidney, ACE is associated with vascular endothelium and proximal tubular epithelium, particularly the distal (S3) portion of the proximal tubule. We now have examined two strains of mice with very low or no renal ACE. This is the ACE 1/3 mouse (7% normal renal ACE) and the ACE 8/8 mouse (no renal ACE) (20,21). In both strains, deprivation of water for 24 h resulted in urinary concentrations in excess of 3000 mOsm/L, which was indistinguishable from wild-type mice. Perhaps this is not surprising given that these animals retain a functional RAS with the ability to stimulate aldosterone production. However, it does indicate that the ACE within the kidney plays no irreplaceable physiologic role, at least as measured by renal concentrating ability.

We studied ACE 1/3 mice after a 2-wk diet that totally lacked NaCl (21). In the absence of all exogenous salt, mice maximally retained salt. Nonetheless, there is a small loss of urinary salt and a consequent small reduction of BP. A typical wild-type mouse will reduce systolic BP by 5 mmHg or less. Under the extreme stress of 2 wk without salt, we did observe a difference between the ACE 1/3 mice and wild-type animals: There was a small but significant additional loss of urinary sodium by the ACE 1/3 mice. This resulted in a greater reduction of systolic BP (approximately 10 mmHg). Nonetheless, it was remarkable that ACE 1/3 mice were able to tolerate easily this regimen and to maintain systolic BP in excess of 90 mmHg. An explanation for the remarkable behavior of the ACE 1/3 mice is the elevation of plasma renin levels (Figure 4B). These data show that, under basal conditions, the ACE 1/3 mice have an elevated renin level as compared with wild-type mice (it is this that maintains normal BP). In response to total salt deprivation, the RAS is activated and these mice respond with much higher plasma renin levels than control animals. Thus, the ACE 1/3 mice dramatically demonstrate that, given a normal kidney and its compensatory ability, animals can tolerate a marked change in both the quantity and the tissue expression patterns of ACE. Under conditions of salt deprivation, the ACE 1/3 mice maintain substantial homeostasis; it is only under extreme conditions that the selective lack of ACE expression by endothelium and within the kidney seems at all deleterious. We end this portion of the discussion with a caveat that our experiments were performed with young mice that were held under laboratory conditions. Clearly, the human response to the chronic injuries that are typical of old age are different paradigms. Although the ACE 1/3 mice have not yet been tested in these paradigms, they do underline the amazing ability of the RAS to compensate given normal renal function. In a sense, these data support the idea that abnormalities in human BP must be associated with some type of renal dysfunction.
Renal Angiotensin II

Mice with altered tissue patterns of ACE expression provide insight into the organ-specific generation and function of angiotensin peptides. In fact, an evaluation of angiotensin peptide levels was recently performed in mice that genetically lack ACE and in wild-type mice that were exposed to the ACE inhibitor lisinopril (27). These data proved that, in the mouse, ACE is the predominant pathway leading to angiotensin II formation. ACE is responsible for at least 90% of the conversion of angiotensin I to angiotensin II in the blood, kidney, heart, lung, and brain and at least 77% in the adrenal. In fact, evaluation of angiotensin II peptide levels in the kidneys of ACE null mice showed that this peptide was reduced by >97% as compared with wild-type mice. These data suggest that, at least in the mouse, chymase-like enzymes do not play an important role in angiotensin II formation. These data also completely refute the study by Wei et al. (28) reporting similar angiotensin I and angiotensin II levels in the organs of ACE knockout mice as compared with control animals. Angiotensin II is difficult to isolate under conditions that prevent the artificial conversion of angiotensin I (present in large amounts in ACE null mice) into angiotensin II. Our group took particular care to homogenize freshly isolated organs in 4 mol/L guanidine thiocyanate to prevent this artifact. Data from our group documenting markedly reduced tissue angiotensin II levels in ACE knockout mice seems very consistent with the striking reduction of BP measured by all groups who have studied ACE null mice (6,7).

An interesting aspect of the kidney is that wild-type mice have renal levels of angiotensin II that are significantly higher than those found in blood. This has engendered the idea that renal angiotensin II levels are the result of de novo formation in the kidney (29). Our data argue against de novo formation as being the major source of renal angiotensin II. ACE 8/8 mice have no ACE in renal tissue, instead having ACE within the heart and the blood. Despite no renal ACE, the kidney concentration of angiotensin II averaged 130 fmol/g. This was >16 times higher than the concentration observed in the plasma and even exceeded the level found in the lung of a wild-type mouse. The high residual angiotensin II peptide concentrations present in renal tissue that totally lacks tissue-bound ACE suggest that a significant percentage of total renal angiotensin II peptide levels must be due to absorption of the peptide from the blood.

ACE Selectively Expressed in Kidney

Our group has worked hard to create a mouse with ACE expression restricted to the kidney. It is our belief that the evaluation of this model or even mouse lines with ACE expression limited to different portions of the nephron will be informative as to the special role of the kidney in the maintenance of BP and electrolyte balance. Although animal models previously discussed indicate that renal concentrating ability is retained in the absence of renal ACE, it is important to remember that these models have other compensating sources of ACE. Wild-type mice do contain considerable ACE in the brush border of the proximal tubule and in renal vascular endothelium. Our hope is that animals with ACE expression restricted to the kidney will provide insight into the local, renal generation of angiotensin II and the renal and systemic effects of this peptide.

My group prepared three mouse lines that were engineered such that control of the ACE gene was under promoters that were previously described as specifically active in the kidney. These are the γ glutamyl transpeptidase (γ-GT), the kidney androgen regulated protein (KAP), and the Tamm-Horsfall promoters. Although published literature supported the use of these promoters in targeting gene expression to the kidney, our experience was very different (30–35). The KAP and Tamm-Horsfall promoters were inactive and gave rise to mice that were similar in BP and renal structure to the ACE null mice previously discussed in this article. Even an ACE 8/Tamm-Horsfall heterozygous animal, in which the “8” allele targets ACE to the heart and ensures normal renal tubular development, lacked renal expression of ACE (data not published). The γ-GT promoter did lead to small levels of ACE expression within the kidney, but these levels were only approximately 6% the normal ACE expression present in wild-type kidneys. The combination of low renal expression and the lack of ACE expression in other organs of the animal resulted in the γ-GT mice having a low BP equivalent to that of ACE null animals (data not published).

In one manner, these mice were different from an animal absolutely null for all ACE expression. These new strains of mice continued to express the testis isofrom of ACE. Because of this, the mice were fully fertile, despite low BP. This compares to ACE null mice, lacking testis ACE, in which fertility is markedly reduced in male mice. Our group now routinely uses mice that we term ACE 4/4 (homozygous for the mutation in which the KAP promoter controls ACE expression) as a substitute for ACE 1/1 null mice (36). Nonetheless, the lack of reliable renal-specific promoters was a disappointment and a source of frustration for our group. The development of tools for reliably expressing proteins selectively within the kidney is important. The lack of reliable renal-specific promoters will hinder the genetic manipulation of mice and the physiologic evaluation of protein expression in the kidney.

ACE and the Heart

The role of ACE and angiotensin II within the heart has been the subject of much discussion. Some authors have suggested that the local generation of angiotensin II within cardiac tissue may be deleterious, promoting pathologic development of cardiac fibrosis (37,38). To investigate this question, we made a mouse in which the cardiac-specific α-myosin heavy chain promoter was positioned to control the ACE gene (21). Our hope was that this animal would make ACE selectively in cardiac tissue, thus focusing production of angiotensin II specifically within the heart. In fact, precisely this occurred in these mice called ACE 8/8. Whereas wild-type mice have virtually no ACE expression by the myocardium, the ACE 8/8 animals have extensive ACE enzyme located on the surface of cardiac myocytes in both the atria and the ventricles. Ironically, vascular endothelium within the heart is completely absent of ACE expression. Although the α-myosin heavy chain promoter is often thought to be specific for the heart, we found that the
promoter also induced an unusual expression pattern in the lung, where some vascular smooth muscle expressed ACE. In addition, there was a patchy distribution of ACE within the lung parenchyma with the result that the lungs of ACE 8/8 mice have approximately 40% normal ACE activity. In contrast to the lung, ACE 8/8 mice totally lack renal expression of ACE; these animals are null for all renal epithelial and vascular expression. Indeed, endothelium throughout the animal produce no ACE.

We carefully evaluated cardiac levels of angiotensin II. Our hope was that these animals would increase the cardiac concentration of angiotensin II, and, in fact, we found levels of the peptide that were 4.3-fold greater than those of wild-type mice. Although the BP of the mice was near normal and renal concentrating ability was indistinguishable from wild-type mice, the ACE 8/8 animals definitely were not normal; the death rate of the mice was markedly increased such that only 64% of the animals were alive 66 d after birth (as opposed to 100% survival for wild-type mice). The increased mortality in this model was the result of two unusual findings: A marked increase in size of the atra and marked cardiac electrical abnormalities characterized by low voltage and atrial fibrillation (Figure 5). However, to our surprise, the ventricles of ACE 8/8 mice were in many ways normal. For example, histologic staining to identify collagen detected no increase of ventricular fibrosis. Both echocardiography and intraventricular catheterization (with a Millar catheter) failed to show any marked abnormalities of ventricular function despite greater than a fourfold increase of cardiac angiotensin II. Thus, this model questions the concept of an intrinsically deleterious effect of angiotensin II for myocardial function.

In contrast to the myocardial findings, the ACE 8/8 mice developed atrial enlargement between 2 and 3 wk of age through mechanisms that are under intense study. Although work continues to help us understand the phenotype of these mice, the findings in the ACE.8 model underscore that genetic manipulation of mice is a powerful method to test accepted concepts of pathology and disease etiology. For example, there is an extensive literature implicating angiotensin II as proinflammatory for disease processes such as vascular injury and even atherosclerosis (2,39). Our group is preparing mice that overexpress ACE by macrophages. Our hope is to use this and other mouse models to quantify precisely the role of angiotensin II in vascular injury.

Conclusion
Our group has created mouse models in which ACE expression is limited to certain small subsets of tissue. This was in response to the realization that a total ACE null mouse represents an extreme phenotype that, although very interesting, is intrinsically limited. Our new mouse models are examples of an approach to manipulate gene promoters and restrict/rearrange protein expression. Some of what we found was contrary to previously held concepts of tissue-specific RAS expression. This emphasizes the power of our approach and of genetic manipulation of the mouse as a tool to dissect the complex role of the RAS. We hope that these mouse models will be a resource for many other laboratories interested in the physiology and pathology of renal and cardiac disease.

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