Genetic Evidence That Lethality in Angiotensinogen-deficient Mice Is Due to Loss of Systemic but Not Renal Angiotensinogen* 

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Angiotensinogen (AGT)-deficient mice die shortly after birth presumably due to renal dysfunction caused by the presence of severe vascular and tubular lesions in the kidney. Because AGT is expressed in renal proximal tubule cells, we hypothesized that its loss may be the primary mediator of the lethal phenotype. We generated two models to test this hypothesis by breeding transgenic mice expressing human renin with mice expressing human AGT (hAGT) either systemically or kidney-specifically. We then bred double transgenic mice with AGT+/− mice, intercrossed the compound heterozygotes, and examined the offspring. We previously reported that the presence of the human renin and systemically expressed hAGT transgene complemented the lethality observed in AGT−/− mice. On the contrary, we show herein that the presence of the human renin and kidney-specific hAGT transgene cannot rescue lethality in AGT−/− mice. An analysis of newborns indicated that AGT−/− mice were born in normal numbers, and collection of dead 10-day-old pups revealed an enrichment in AGT−/− mice. Importantly, we demonstrated that angiotensinogen protein and functional angiotensin II was generated in the kidney, and the kidney-specific transgene was temporally expressed during renal development similar to the endogenous AGT gene. These data strongly support the notion that the loss of systemic AGT, but not intrarenal AGT, is responsible for death in the AGT−/− mouse model. Taken together with our previous studies, we conclude that the intrarenal renin-angiotensin system located in the proximal tubule plays an important role in blood pressure regulation and may cause hypertension if overexpressed, but may not be required for continued development of the kidney after birth.

Regulation of arterial blood pressure, renal hemodynamics, and fluid and electrolyte homeostasis are well-recognized functions of the renin-angiotensin system (RAS).1 The results of recent studies indicate that the RAS may also regulate renal growth and development. All the components of the RAS are detectable at the mRNA and protein level in the developing kidney. Angiotensinogen can be detected as early as embryonic day 17 (E17) in rodent kidney where it is expressed primarily in developing tubules (1). Renal angiotensinogen expression peaks around birth but declines thereafter (2). Renin can be found in rodents as early as E15 before obvious blood vessel and nephron formation occurs, and at E17 is expressed in the renal artery and its main branches. Its expression pattern gradually moves from vascular structures to mature juxtaplomerular cells as kidney development progresses (1, 3). Similar to angiotensinogen, renin expression reaches a peak around birth (4). In the rat kidney, ACE can be detected at E16, and AT-1 and AT-2 receptors are expressed throughout metanephric development (5–7). Consequently, all the components of the RAS are expressed during early kidney development in specific temporal-spatial patterns associated with nephrogenesis, suggesting the RAS may play an important role during renal development.

There is ample evidence showing that Ang-II has trophic or mitogenic effects on a variety of target cells and tissues in addition to its function as a regulator of volume and sodium homeostasis. Ang-II directly stimulates growth and proliferation of renal mesangial cells, proximal tubular cells, Henle’s loop cells, and vascular smooth muscle cells in vitro (8–10); Ang-II may also stimulate a cascade involving growth factors and oncogenes such as transforming growth factor β, basic fibroblast growth factor, platelet-derived growth factor, c-Fos, c-Myc, and c-Jun (11). Direct evidence implicating an important role of the RAS in renal development comes from studies using either pharmacological blockade or targeted disruption of the RAS. ACE inhibitor treatment during pregnancy (even during pre-eclampsia) is strongly contraindicated, because their infants have an increased incidence of renal tubular dysplasia, growth retardation, oligohydramnios, hypotension, hyponatremia, and death (12). Similarly, neonatal rats treated with captopril, enalapril, and losartan exhibit gross histopathologic changes in the kidney, including tubular atrophy and dilation, papillary atrophy, interstitial inflammation, and hyperplasia of renal vasculature (13). Similarly, mice with targeted mutations in the angiotensinogen, ACE, AT-1 receptor genes, and in both renin genes, have nearly identical phenotypes characterized by poor survival to weaning, low blood pressure, and abnormal kidney structure. Their kidneys de-

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1 The abbreviations used are: RAS, renin-angiotensin system; E, embryonic day; ACE, angiotensin converting enzyme; Ang-I and –II, angiotensin I and II; hREN, human renin; hAGT, human angiotensinogen; mAGT, mouse angiotensinogen; rAGT, rat angiotensinogen; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s).
the mouse angiotensinogen (mAGT) knockout background complemented (rescued) the lethality and renal lesions associated with the loss of angiotensinogen (18). However, the hAGT transgene used in that study was driven by its own endogenous promoter and expressed in multiple tissues such as liver, kidney, brain, and heart. Therefore, it is unclear whether restoration of the systemic RAS or intrarenal RAS was responsible for the rescue. Given that the primary defect in AGT-deficient mice may be the kidney, the aim of this study was to determine whether the intrarenal RAS plays a critical role in renal development by determining if restoring only the intrarenal RAS can rescue the lethal phenotype in mAGT-deficient mice. The results of this classic genetic complementation assay are reported herein.

Animal Breeding Strategy and Husbandry—The generation and characterization of hREN, hAGT, and KAP-hAGT single and double transgenic mice have been reported previously (19–21). The KAP-hAGT transgene segregates as an autosomal trait and is kidney-specifically expressed, whereas the hREN transgene is X-chromosome-linked and systemically expressed. The generation of mAGT-deficient mice has been described previously (14). All mice were maintained by backcross breeding to C57BL/6J, and were fed standard mouse chow (Teklad LM-485) and water ad libitum unless otherwise indicated. Care of mice met or exceeded the standards set forth by the National Institutes of Health in the Guidelines for the Care and Use of Experimental Animals. All procedures were approved by the University of Iowa Animal Care and Use Committee. Mice were killed by CO2 asphyxiation.

For the developmental study, KAP-hAGT single transgenic male mice were crossed with female C57BL/6J, and pregnancy was monitored by daily examination of vaginal plugs. Mouse fetuses were removed on E7.5 through E18.5. In addition, newborn through 8-week-old mice were also collected. For the genetic complementation study, hREN/hAGT transgenic mice were crossed with female C57BL/6J, and pregnancy was monitored by daily examination of vaginal plugs. Mice fetuses were removed on E7.5 through E18.5. In addition, newborn through 8-week-old mice were also collected. For the genetic complementation study, hREN/hAGT double transgenic females were mated to male mice heterozygous for the mAGT deletion (+/−) and the compound heterozygotes hREN/hAGT mAGT+/− were intercrossed. The resulting offspring immediately after birth and at 10 days and 3 weeks of age were collected. In addition, all cages were carefully monitored for dead pups, which were immediately taken for sampling and genotyping.

Genotyping—Genomic DNA was isolated from tail or placental biopsies as described previously (20). PCR analysis was performed to identify the hREN, hAGT, and KAP-hAGT transgenes using specific primer sets as reported (22, 23). To genotype the mAGT locus, different PCR primer sets were used: Primer a, 5'-GTATACATCCACCCCTCCTCA-3'; primer b, 5'-GGAGATGCACAAGTGGTAA-3'; primer c, 5'-TGACGGGTCTTCAAGGGATC-3'; and primer d, 5'-TAAGGGCGACGGCTGAGATC-3'. The set of primers a and b is specific to the wild-type mAGT locus and yields a 750-bp fragment, whereas primes c and d are specific to the targeted mAGT locus yielding a 1.3-kb fragment. Southern blot analysis was carried out to confirm the PCR results as previously described (18). Briefly, tail DNA was digested with XbaI, separated on 0.8% agarose gel, transferred to nitrocellulose membranes, and hybridized with a probe specific for intron 1 and part of exon 2 of the mAGT gene. After hybridization, the wild-type mAGT locus yields a 5-kb fragment, whereas the targeted locus yields a 6.5-kb fragment. The sex of fetuses and newborns was determined by PCR amplification of an sry gene fragment as described previously (24).

Gene Expression Analysis—RNase protection assay was used to compare the time course of expression of hAGT transgene with that of the endogenous mAGT gene during development. The hAGT probe was derived from exon 2 at nucleotides 302–819 relative to the transcription start site as described previously (21). The mAGT probe was derived by cloning a reverse transcriptase-PCR product from nucleotides 305–739. The oligonucleotides used to clone the mAGT cDNA probe were 5'-GCCGCGAAGAGTGATGATG-3' and 5'-TGCGAAGGAGGCGAGGGAAGAGAG-3'. This cDNA fragment was first cloned to pCR2.1 vector (Invitrogen) and then subcloned into pBluescript SK− to obtain the desired antisense orientation relative to the T7 promoter. The mouse 8-actin cDNA (mAct) probe was provided by Ambion (Austin, TX). Antisense RNA probe was prepared using an in vitro transcription reaction containing α-32P-UTP. Total RNA was isolated from whole fetuses from E7.5 to E18.5, and kidneys from E16.5 to 8-week-old mice as described previously (22). Total tissue RNA (20 μg) was hybridized to the probes and were treated according to the manufacturer’s protocol
for Hyb-Speed RNase protection assay kit (Ambion). The length of the full-length probe for hAGT, mAGT, and mAct are 630, 591, and 330 nucleotides, respectively, and the expected protected fragment are 518, 425, and 250 nucleotides, respectively.

Physiological Analysis and Statistical Analysis—Immunohistochemistry and measurements of urinary hAGT and blood pressure were described in detail previously (20, 21). Chi-square analysis was performed to compare the genotype frequencies with expected Mendelian ratios using the SigmaStat software package.

RESULTS

The purpose of this study is to genetically determine whether the intrarenal renin-angiotensin system alone can rescue the postnatal lethality observed in mAGT knockout mice. To accomplish this, we performed a complementation assay employing one transgenic mouse expressing hREN and two transgenic models expressing hAGT—one expressing hAGT systemically (hAGT or A+) and one expressing hAGT specifically in proximal tubule cells of the kidney (KAP-hAGT or KA+). In the systemic model, hAGT mRNA is abundant in the liver, kidney, heart, and brain, and elevated levels of hAGT protein are present in the systemic circulation (23). Double transgenic mice expressing both hREN and the systemic hAGT transgene exhibit a 4-fold elevation in plasma Ang-II and are chronically hypertensive (19). In the KAP-hAGT mice, hAGT mRNA and protein are restricted to the kidney as a consequence of being driven by the kidney-specific KAP promoter (20). Double transgenic mice containing both the hREN and KAP-hAGT genes do not have circulating hAGT protein, have normal plasma Ang-II levels, but are nevertheless hypertensive (20).

To perform the genetic analysis we first bred hREN mice with either hAGT or KAP-hAGT mice to generate systemic (R+/A+) and kidney-specific (R+/KA+) double transgenic mice (Fig. 1). Double transgenic females were then bred with male mice heterozygous for the mAGT deletion (+/−), and the compound heterozygotes (R+/A+ mAGT+/− or R+/KA+ mAGT+/−) were identified. These compound heterozygotes were then intercrossed, and the resulting offspring were genotyped by PCR and Southern blot analysis (Fig. 2). According to Mendelian genetics, the offspring would exhibit one of 12 different genotypes with different combinations of R+, A+, or KA+ transgenes and mAGT alleles (Table I). Our previous data indicated that single transgenic mice (R+/A− or R−/A+) have the same level of plasma renin activity, plasma Ang-II, and blood pressure as normal mice (R−/A−) and therefore can be stratified together as a single group (18, 19). This is because the enzymatic reaction between renin and angiotensinogen is species-specific, such that human renin cannot cleave mouse angiotensinogen and vice versa (25). However, Ang-I generated from the enzymatic reaction between human renin and human angiotensinogen can be cleaved to form Ang-II by mouse ACE, because Ang-I is evolutionary conserved in rodents and humans. Therefore, we stratified offspring from the third round of breeding into a double transgenic group (RA+ or RKA+), and a nondouble transgenic group (RA− or RKA−). The nondouble transgenic group consisted of mice that were either nontransgenic, single transgenic, but not doubly transgenic. This simplified the 12 original genotypes into 6 relevant genotypes (mAGT+/+, mAGT+/−, and mAGT−/− for each).

We previously genotyped 131 offspring from an intercross of R+/A+ mAGT+/− mice that survived to 3 weeks of age (18). In the absence of lethality, we expect 25% of offspring to be mAGT−/−. Only three of the nondouble transgenic mice that inherited both targeted alleles of mAGT survived to weaning. However, all double transgenic mice inheriting both targeted alleles of mAGT survived to weaning, demonstrating that systemic expression of hREN and hAGT can complement the lethality observed in AGT−/− mice. In fact, all R+/A+ mAGT−/− mice survived to at least 6 months of age, and the kidneys appeared histologically normal. The mice also exhibited slightly elevated blood pressure. Although we did not repeat this test breeding, we have since generated a fully homozygous inbred strain of R+/A+ mAGT−/− mice (Table III). To further determine whether the mAGT−/− mice were born at the appropriate ratio (25%) or died in utero, we performed a second set of breeding between R+/KA+ mAGT+/− parents and collected newborns from 27 litters. Genotype analysis of 159 offspring revealed appropriate proportions of all six genotypes as predicted by Mendelian segregation, including RKA− mAGT−/− and RKA+ mAGT−/− (Table III). To further determine when mAGT−/− mice died, we carefully examined offspring from newborn to 10 days of age and collected dead pups as they appeared. Similar to the findings at 3 week of age, of a total of 52 live 10-day-old offspring, none of them genotyped as mAGT−/− (data not shown), suggesting all homozygous mAGT−/− mice died before 10 days of age (generally between 4 and 5 days of age). Indeed, when we genotyped the dead pups, we found that 20 of 27 pups (12 of 16 RKA+ and 8 of 11 RKA−)

### Table I
Genotype of all 3-week and newborn hREN/KAP-hAGT mice

| mAGT | R+/KA+ | R+/KA− | R−/KA+ | R−/KA− |
|------|--------|--------|--------|--------|
| +/+  | 24     | 11     | 11     | 3      |
| +/−  | 39     | 22     | 12     | 6      |
| −/−  | 0      | 0      | 0      | 0      |

| mAGT | R+/KA+ | R+/KA− | R−/KA+ | R−/KA− |
|------|--------|--------|--------|--------|
| +/+  | 18     | 10     | 1      | 1      |
| +/−  | 50     | 31     | 5      | 2      |
| −/−  | 25     | 14     | 1      | 1      |

### Table II
Stratified genotype summary of hREN/KAP-hAGT mice at 3 weeks of age

| mAGT | RKA+/n (n = 63) | RKA−/n (n = 65) |
|------|----------------|----------------|
| +/+  | 24             | 18             |
| +/−  | 39             | 31             |
| −/−  | 0              | 16             |

### Table III
Stratified genotype summary of newborn hREN/KAP-hAGT mice

| mAGT | RKA+/n (n = 93) | RKA−/n (n = 66) |
|------|----------------|----------------|
| +/+  | 18             | 12             |
| +/−  | 50             | 38             |
| −/−  | 25             | 16             |

χ² analysis showed a highly significant difference between the numbers of mice observed and those expected based only on Mendelian inheritance assuming no lethal effects of the mAGT deficiency (p < 0.0001). To determine whether the mAGT−/− mice were born at the appropriate ratio (25%) or died in utero, we performed a second set of breeding between R+/KA+ mAGT+/− parents and collected newborns from 27 litters. Genotype analysis of 159 offspring revealed appropriate proportions of all six genotypes as predicted by Mendelian segregation, including RKA− mAGT−/− and RKA+ mAGT−/− (Table III). To further determine when mAGT−/− mice died, we carefully examined offspring from newborn to 10 days of age and collected dead pups as they appeared. Similar to the findings at 3 week of age, of a total of 52 live 10-day-old offspring, none of them genotyped as mAGT−/− (data not shown), suggesting all homozygous mAGT−/− mice died before 10 days of age (generally between 4 and 5 days of age). Indeed, when we genotyped the dead pups, we found that 20 of 27 pups (12 of 16 RKA+ and 8 of 11 RKA−)
were also mAGT−/−, a clear enrichin in that genotype (Table IV).

Because the expression of hAGT in the KAP-hAGT model is sexually dimorphic and androgen-responsive, we considered the possibility that females would have increased lethality as compared with males. We therefore genotyped mice at the sry locus located on the Y chromosome to distinguish between males from females (Fig. 2). There was no significant difference between the number of males and females in any group of mice, including those that died between birth and 1 week of age (Table V).

To ensure that the lack of complementation was not due to the absence of hAGT protein, we performed immunohistochemistry on kidney sections from nontransgenic and transgenic mice (Fig. 3). Along with our previous report showing expression of hAGT mRNA in proximal tubule cells (21), these results clearly demonstrate the production of hAGT protein in the kidney. Moreover, consistent with apical secretion of hAGT in proximal tubule cells (26), we detected hAGT protein in the urine of transgenic mice (5.01 ± 0.69 pmol/ml) but not nontransgenic mice (0.34 ± 0.14 pmol/ml, representing the background of the assay). Although we could not accurately measure intrarenal Ang-II in the KAP-hAGT model, double transgenic mice containing the hREN and KAP-hAGT transgenes exhibit hypertension (143 ± 5 versus 113 ± 3 mmHg in control mice) due to the production of Ang-II in kidney (20).

Studies using the Ang-II AT-1 receptor antagonist losartan confirmed that the blood pressure elevation was due to an intrarenal Ang-II-dependent mechanism (20).

Finally, we considered the possibility that, because hAGT is driven by the KAP promoter in KAP-hAGT transgenic mice, the inability to rescue the lethality may be due to the absence of transgene expression during late development. To examine this, we performed timed breeding and used RNase protection to compare the time course of hAGT transgene expression during gestation and neonatal life with that of the endogenous mAGT and KAP genes. As anticipated, the KAP-hAGT transgene was expressed at precisely the same developmental stages as endogenous KAP (Fig. 4B). Expression was evident in whole fetuses at E11.5 and in kidney beginning at E16.5. Transgene expression in kidney was evident in newborns and in mice 1 and 2 weeks of age. When compared with endogenous mAGT expression, it was evident that expression of the transgene tended to precede and was higher than expression of endogenous mAGT (Fig. 4A), although fetal expression of mAGT was evident on a longer exposure (data not shown). It is therefore unlikely that the inability of the kidney-specific transgene to rescue the lethal phenotype was related to the lack of transgene expression.

### DISCUSSION

The major finding of the present study is that transfer of both KAP-hAGT (localized to proximal tubule) and hREN transgenes onto the mouse angiotensinogen knockout (mAGT−/−) background is not sufficient to rescue the lethality associated with this model. Consistent with previous reports, mouse AGT knockout mice were born in predicted Mendelian ratios, but died soon after birth (14, 18). In the present study, no mAGT−/− mice survived past 10 days of age, regardless of the presence or absence of the KAP-hAGT and hREN transgenes. One possibility for the inability to rescue is that the transgenes were not active during a critical developmental period or perhaps they were expressed in inappropriate locations. However, an examination of temporal and spatial expression of the transgenes suggests that this explanation is unlikely. According to our data, the KAP-hAGT transgene was expressed in a similar temporal window as the endogenous gene. Moreover, we previously reported that the transgene is expressed in proximal tubule cells, the same cells that express mAGT endogenously in the kidney (21). Furthermore, unlike our observation in adult mice, hAGT is similarly expressed in both males and females during early stages of development. It is not until after 4 weeks of age that expression in males becomes much higher than in females.

Although all studies report significant mortality and renal abnormalities associated with deletion of RAS function, the severity (or penetrance) appears to be highly variable among models and laboratories. The perinatal mortality rate of mAGT−/− mice in this study (essentially 100%) is much higher than previously reported (60–70%). One explanation is that genetic background may contribute to the high mortality rate we observed. The heterozygous mAGT+/− mice used in our study were maintained by six generations of backcross breeding with inbred C57BL/6 mice. Similarly, the KAP-hAGT and hREN mice used in this study were maintained by 5 and 10 generations of backcross breeding with C57BL/6, respectively. Therefore, all the mice described herein are essentially C57BL/6 congenic. It is interesting to note that homozygous AT-1A receptor-deficient mice containing a mixed C57BL/6 and 129 background survive normally and exhibit normal kidney morphology, whereas homozygous AT-1A receptor-deficient mice maintained on a congenic C57BL/6 or inbred 129 background exhibit substantially reduced survival and abnormal kidney structure (27).

We reported that transfer of both the hREN and systemically expressed hAGT transgenes onto the mAGT knockout background restores survival and corrects the renal abnormalities caused by the mAGT deletion (18). In that study, human angiotensinogen was expressed not only in proximal tubule cells but also in other tissues such as liver, brain, and heart, etc., all normal sites for AGT expression. Because hAGT was expressed in the liver, plasma AGT and angiotensin II levels were elevated. This increase in plasma Ang-II was sufficient to not only rescue lethality but to reverse the hypotension and correct the renal lesions normally associated with mAGT−/− mice. This stands in contrast to the KAP-hAGT model used in the present study, in which circulating hAGT levels remain undetectable and will not have any circulating Ang-II when bred onto the mAGT−/− genetic background (20, 21). Unfortunately, the very early lethality of mAGT−/− observed in our study precluded any detailed renal histological analysis. Nevertheless, examination of several mAGT−/− kidneys from mice, which died before 10 days of age, did not show any clear evidence of renal lesions. This is not particularly surprising, because most of the lesions previously reported were in those rare mice that survived well past 3 weeks of age. This raises the intriguing possibility that circulating, rather than proximal tubule-derived Ang-II, is required for continued development of the kidney after birth. In agreement with this hypothesis, Kang et al. (28) reported that mAGT-deficient mice expressing the rat angiotensinogen (rAGT) only in the brain and liver did not exhibit renal lesions. These findings suggest that normaliz-
tion of circulating Ang-II alone is sufficient to rescue the renal lesions in mAGT-deficient mice and provide additional evidence of the crucial importance of circulating Ang-II in renal development.

In the present study, the use of the KAP promoter allowed for precise targeting of hAGT to the proximal tubule, the predominant site for AGT production in the kidney. The exact function of proximal tubule AGT production remains unclear; however, multiple lines of evidence suggest an important role in electrolyte balance and blood pressure regulation. The localization of AGT in proximal tubule, its polarized secretion into the proximal tubular lumen, and the presence of high levels of Ang-II in proximal tubular fluid have been clearly demonstrated (26, 29, 30). Ang-II has been reported to stimulate the sodium/hydrogen exchanger, an important regulator of proximal tubule sodium reabsorption, independently of systemic renin-angiotensin system in the proximal tubule may not be necessary for normal renal growth and development, and its loss is unlikely to be the cause of neonatal death in mAGT-deficient mice. Based on all available data, we conclude that the intrarenal renin-angiotensin system located in the proximal tubule plays an important role in blood pressure regulation and may cause hypertension if overexpressed but may not be required for continued development of the kidney after birth.

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