Antisense-mediated angiotensinogen inhibition slows polycystic kidney disease in mice with a targeted mutation in Pkd2

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Ravichandran K, Ozkok A, Wang Q, Mullick AE, Edelstein CL. Antisense-mediated angiotensinogen inhibition slows polycystic kidney disease in mice with a targeted mutation in Pkd2. Am J Physiol Renal Physiol 308:F349–F357, 2015. First published December 23, 2014; doi:10.1152/ajprenal.00478.2014.—Renal cyst enlargement is associated with the activation of both the circulating and intrarenal renin-angiotensin systems. Angiotensinogen (AGT) is the substrate for renin. The aim of the present study was to determine the effect of AGT inhibition on renal cyst enlargement. An AGT antisense oligonucleotide (ASO) that selectively inhibits AGT mRNA was injected once weekly in PKD2WS25 mice [an orthologous model of human autosomal dominant polycystic kidney disease (PKD) involving mutation of the Pkd2 gene] from 4 to 16 wk of age. The AGT ASO resulted in a 40% decrease in AGT RNA in the kidney, a 60% decrease in AGT RNA in the liver, and a significant decrease in AGT protein in the kidney and serum. The AGT ASO resulted in a significant decrease in kidney size, cyst volume density, and blood urea nitrogen. The AGT ASO resulted in a significant decrease in transforming growth factor-β and interstitial fibrosis in the kidney. Mice treated with the AGT ASO had a significant decrease in proinflammatory cytokines [chemokine (C-X-C motif) ligand (CXCL)1 and IL-12] in the kidney. Cluster of differentiation (CD)36 is a scavenger receptor found on tubular cells that can activate the renin-angiotensin system. Administration of a CD36 ASO had no effect on PKD and kidney function, suggesting that the effect of the AGT ASO is independent of CD36. In summary, AGT inhibition resulted in significant decreases in kidney size and cyst volume and an improvement in kidney function in PKD mice. The AGT ASO resulted in a decrease in transforming growth factor-β and interstitial fibrosis, and the proinflammatory cytokines CXCL1 and IL-12 in the kidney. Angiotensinogen; cytokines; fibrosis; polycystic kidney; polycystic kidney disease; renin-angiotensin system

THE RENIN-ANGIOTENSIN SYSTEM (RAS), through the production of ANG II, is a major regulator of organ function in both health and disease (42). In the RAS, angiotensinogen (AGT), which is thought to be principally derived from the liver, is cleaved by the peptidase renin to form ANG I (42). ANG I is converted to ANG II by angiotensin-converting enzyme (ACE) (42). ANG II is a potent vasoconstrictor peptide with a multitude of other effects, such as activation of transforming growth factor (TGF)-β (42). The effects of ANG II are mediated by binding to angiotensin receptors (42). All components of the RAS are found in the plasma (circulating RAS) and in organs (tissue RAS) (35, 53). Normally, the role of the RAS is to regulate blood pressure, renal hemodynamics, and fluid and electrolyte balance. ANG II is also found in the vascular compartment, heart, kidneys, and adrenal glands, where it can contribute to cellular proliferation, inflammation, and fibrosis, all of which are major factors in cystogenesis (35, 53). The amount of ANG II found in the kidneys is higher than in all other tissues (35). Although RAS inhibitors, such as ACE inhibitors, angiotensin receptor blockers (ARBs), and direct renin inhibitors, have been comprehensively studied in animals and humans (53), direct inhibition of AGT has been less studied due to a lack of pharmacological inhibitors that directly target AGT. Outside of the liver, AGT is synthesized by proximal tubules, adipocytes, and astrocytes (53). There is a paucity of studies that have investigated the impact of AGT inhibition in kidney diseases. An AGT antisense oligonucleotide (ASO) that selectively results in long-term inhibition of AGT mRNA was obtained. The first aim of the present study was to measure AGT in the serum (circulating RAS) and kidney (tissue RAS) in polycystic kidney disease (PKD) mice and determine the effect of long-term administration of an AGT ASO on AGT in the serum, liver, and kidney.

There is good reason to study inhibition of the RAS in PKD. The RAS is stimulated in hypertensive patients with PKD to a greater extent than in comparable patients with essential hypertension, likely due to renal ischemia caused by cyst expansion (6). Renal cyst enlargement in autosomal dominant PKD (ADPKD) is associated with activation of both the circulating and tissue RAS (40). There is ectopic renin production by the cyst epithelium (46). In addition to renin, AGT is produced by some cysts and dilated tubules (30). Also, ACE, ANG II, and ANG II receptors are present in cysts (30). There is evidence that the RAS plays a role in cyst growth as RAS inhibition using an ACE inhibitor or an ARB slows cyst growth in rodent models. ACE inhibitors enalapril or cilazapril or the ARB losartan slow cyst growth in the Han:SPRD rat model of PKD (25, 34, 55). However, in another study (56), enalapril had no effect on kidney function in Han:SPRD rats. The HALT-PKD study (7) has tested the effect of an ACE inhibitor or a combination of an ACE inhibitor and an ARB on cyst growth in human PKD. However, the effect of RAS inhibition has not been tested in a mouse model orthologous to human PKD. Based on the activation of the RAS in PKD kidneys and the known effect of ANG II on renal tubular proliferation, a major factor in cyst growth, we developed the hypothesis that AGT inhibition would slow cyst growth and improve kidney function in PKD. Thus, the second aim of the present study was to determine the effect of chronic administration of an AGT ASO on cyst growth and kidney function in Pkd2WS25/− mice, an orthologous model of human PKD.

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ANG II has proinflammatory and profibrotic actions (35, 38). In the kidney, ANG II activates renal fibroblasts to become myofibroblasts, stimulates the production of profibrotic TGF-β, and stimulates local chemokines and cytokines that may cause inflammation (31). ANG II results in an increase in proinflammatory cytokines and chemokines and leukocyte infiltration in the kidney, resulting in renal damage (44). We developed the hypothesis that AGT inhibition would result in a decrease in proinflammatory and profibrotic molecules. Thus, the third aim of the present study was to determine the effect of AGT inhibition on proinflammatory cytokines and TGF-β, an important profibrotic molecule, in Pkd2WS25/− mouse kidneys.

**METHODS**

**Animals.** The present study was conducted using Pkd2WS25/− mice and normal littermate control (+/+) mice. The Pkd2WS25/− mouse develops functional and histological features of ADPKD similar to those observed in patients. For example, by 16 wk of age, the cyst volume density is >50% of the kidney and there is renal failure compared with +/+ mice (13, 47, 54). A colony of Pkd2WS25/− mice was established in our animal care facility from a litter that was obtained from Stefan Somlo (Yale University). The study protocol was approved by the Animal Care and Use Committee of the University of Colorado at Denver. Mice had free access to tap water and standard rat chow. C57BL/6 Pkd2+/− and C57BL/6 Pkd2WS25/+/− mice were used as breeding pairs to generate Pkd2WS25/− mice for the study. Mice were genotyped by Southern blot analysis (2, 54). Pkd2WS25/− mice closely model the human condition by having one copy of Pkd2 knocked out and having a second recombinant-sensitive allele (i.e., WS25) that undergoes high rates of recombination to yield knockouts of the second copy of the gene in somatic cells during the lifespan of the animals.

**Experimental protocol.** Litters from Pkd2+/− mice crossed with Pkd2WS25+/− mice were weaned at 3 wk of age and then genotyped. In each litter, both male and female mice were randomly assigned to one of the following four treatments before results of genotyping were compared: 1) scrambled (Scr) ASO, 2) AGT ASO, 3) cluster of differentiation (CD)36 ASO, and 4) mammalian target of rapamycin (mTOR) ASO. The experiment described in the present study was part of a larger experiment that also included an mTOR ASO (37). The +/+ Scr ASO and Pkd2WS25/− Scr ASO groups in the present study were common to the published mTOR ASO study (37) and to both the AGT and CD36 ASO experiments in the present study.

The number of mice in each group (n) was as follows: +/+ Scr ASO, n = 11; +/+ AGT ASO, n = 14; +/+ CD36 ASO, n = 10; Pkd2WS25/− Scr ASO, n = 10; Pkd2WS25/− AGT ASO, n = 7; and Pkd2WS25/− CD36 ASO, n = 5.

Mice were treated with AGT or CD36 ASO, and littermate controls were given a control mismatch Scr ASO from 4 to 16 wk of age. Three of the Pkd2WS25/− littersmates were not treated with the Scr ASO. ASOs were administered daily via intraperitoneal injection at 100 mg·kg−1·wk−1 for the first 4 wk and then 50 mg·kg−1·wk−1 for 8 wk. ASOs were obtained from ISIS Pharmaceuticals (Carlsbad, CA).

The ASO dosing regimen was selected to produce rapid and sustained reductions of target of 50% or greater in the liver, kidney, and plasma. In healthy noncystic mice, 50–100 mg·kg−1·wk−1 of AGT and CD36 ASO treatments are well tolerated and produced significant liver and kidney target reductions (Fig. 2A). For the chronic study described in this report, a loading dose phase of 4 wk was incorporated to reach steady-state ASO tissue concentrations.

The AGT ASO, CD36 ASO, and Scr ASO were obtained from ISIS Pharmaceuticals. The ASOs are 20-mer second-generation 2′-me-thoxyethyl (MOE) gapmers of the 5-10-5 design. Specifically, the phosphorothioate oligonucleotides contain 2′-MOE modified ribonucleoside groups at positions 1–5 and 16–20 with 2′-deoxyxynucleosides at positions 6–15. The sequences of the AGT ASO, CD36 ASO, and Scr ASO were as follows: AGT ASO, 5′-TCTTCACCGTCA-CAGGCC-3′; CD36 ASO, 5′-GAAATGGATTTGTGAAACC-3′; and Scr ASO: 5′-CTCTCTGGAGTCTTCCTC-3′. AGT and CD36 ASOs were chosen after a screen of ~150 prospective leads in primary murine hepatocytes. The top in vitro leads were then tested in C57BL/6 mice, and the lead AGT ASO was chosen based on liver and/or renal activity and tolerability. The Scr ASO does not hybridize to any known target and was used to control for any ASO class effects that could impact cystogenesis and/or renal function.

**RT-PCR.** Total kidney mRNA was isolated from 100–200 mg kidney samples snap frozen in liquid N2 and then homogenized in Qiagen RLT buffer containing 1% β-mercaptoethanol. RNA was isolated using PureLink Kits (Invitrogen, Carlsbad, CA). RNA samples (50 ng) were subjected to quantitative RT-PCR analysis using commercial reagents (Invitrogen) and analyzed using an ABI StepOne Plus Sequence Detector (Invitrogen). TaqMan primers and probes were used for AGT (forward: 5′-AGGACCCTGAAGAAACTGCAT-3′, reverse: 5′-CTGCTTGGAGACCTCCCTGTG-3′, and probe: 5′-CAG-CACCATCAACCCTCCAAAAGGCC-3′) and GAPDH (forward: 5′-GGCAATTACCGCGACAGT-3′, reverse: 5′-GGGTTCTCGCTC-CTGGAAGAT-3′, and probe: 5′-AAGGCCGAGAATGGGAAGCT-TGTCAATC-3′). After 40 amplification cycles, absolute values were obtained with StepOne analysis software (Invitrogen). Values were normalized to total RNA, as measured by GAPDH, and reported as a fraction of the control group.

**Immunohistochemistry for detection of ASO in the kidney.** Formalin-fixed paraffin-embedded sections (4 μm) were deparaffinized, incubated for 10 min with an endogenous peroxide blocker (DAKO S2003), and then incubated 3 min with proteinase K. Next, sections were incubated with protein blocking solution (Cyto Q Background Buster) for 30 min before incubation with the anti-MOE ASO antibody for 1 h at 1:40,000 or the AGT ASO antibody for 1 h at 1:100. PBS-treated Pkd2WS25/− kidneys were used as a control and showed no staining. Sections were then incubated with a donkey anti-rabbit horseradish peroxidase-conjugated antibody for 30 min at 1:200 followed by diaminobenzidine chromagen for 5 min. Sections were next counterstained (Leica Autostainer), coverslipped, and digitally captured (Aperio ScanScope). Between each step, sections were washed with Tris-buffered saline with Tween buffer two times for 5 min.

The AGT ASO antibody is a pan ASO antibody, developed in a rabbit, capable of detecting the ASO regardless of the ASO sequence (26). The antibody was generated using keyhole limpet hemocyanin (KLH) conjugation to an ASO. ASOs are haptens that are only antigenic when conjugated to a protein; therefore, KLH conjugation makes an ASO antigenic in a rabbit. IgG antibodies were purified and also checked for specificity by performing negative controls as follows: in kidney tissue not exposed to the ASO, there was no staining; in tissue exposed to the ASO, there was no staining; in tissue exposed to the ASO, there was no staining when rabbit IgG was used instead of the ASO antibody.

**Cyst volume density in vivo.** Hematoxylin and eosin-stained sections were used to determine cyst volume density. This was performed by a reviewer blinded to the identity of the treatment modality using point counting stereology (8). In Pkd2WS25/− mice, 80% of the cysts originate from distal nephron segments (54). At least 10 areas of the kidney were randomly selected at each of 90, 180, and 270° from the hilum to guard against field selection variation.

**Proliferating cell nuclear antigen staining.** Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) staining was performed using an anti-PCNA antibody. Sections were incubated with alkaline phosphatase-labeled polymer (catalog no. K4016, DAKO EnVision System, DAKO, Carpinteria, CA) and visualized with the substrate chromogen (fast red). Negative control sections showed no staining. The number of PCNA-positive cells per cyst was counted using an Aperio scanner (Aperio Technologies, Vista, CA) at

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×20 magnification by an observer blinded to the treatment modality. To avoid confusion between noncystic tubules and small cysts as well as potential changes in tubular cells lining massive cysts, PCNA-positive tubular cells were counted in "medium-sized cysts" of ~50- to 250-μm diameter. The number of PCNA-positive cells in noncystic tubules (defined as tubules of <50-μm diameter) was expressed per high-power field and counted in areas of cortex devoid of cysts. At least 10 areas of the kidney were randomly selected at each of 90, 180, and 270° from the hilum to guard against field selection variation.

Sirius red staining. Sirius red staining was performed as previously described (17). Briefly, 4-μm formalin-fixed paraffin-embedded sections were rehydrated and then stained for 1 h with saturated picric acid containing 0.1% Sirius red F3BA (Sigma-Aldrich, St. Louis, MO).

For quantification of Sirius red staining, stained sections were scanned with an Aperioscope and analyzed using the Image Scope-positive pixel count algorithm, as previously described (17). Up to 10 areas of the kidney interstitium devoid of cysts, blood vessels, capsule, or any structures giving positive staining due to an "edge effect" were randomly selected at ×20 magnification. A pixel count was performed in each area, and the mean count was determined. Positivity was determined as the number of positive over the total (positive plus negative) and expressed as a percentage.

Measurement of cytokines. A multiplex sandwich immunoassay was used to measure eight inflammatory cytokines: IL-1, IL-4, IL-5, IL-12, chemokine (C-X-C motif) ligand (CXCL)1 (also known as IL-8 in humans and KC in mice), IL-13, interferon (IFN)-γ, and TNF-α using a multiarray electrochemiluminescence panel (catalog no. K15013C-1, Meso Scale Discovery, MULTI-SPOT Assay System, V-plex TH1/TH2 Panel-1 for mice, Rockville, MD).

TGF-β was measured using a mouse TGF-β1 immunoassay kit (catalog no. HB100B, R&D Systems, Minneapolis, MN).

AGT in the kidney, serum, and urine was measured using a mouse total AGT assay kit (catalog no. 27413, Immuno Biological Labs, Minneapolis, MN).

Protein quantitation of homogenized kidney samples for cytokine assays was performed using the Bio-Rad DC method as described by the manufacturer (Bio-Rad, Hercules, CA).

Chemistry. Blood urea nitrogen (BUN) was measured using quantitative colorimetric urea determination (QuantiChrom urea assay kit-DIUR-500, Bioassay Systems, Hayward, CA).

Statistical analysis. For all measurements, the mean of each group was calculated from different animals. The number of different animals in each group is shown in the tables and figures. Multiple group comparisons were performed using one-way ANOVA with a posttest according to Newman-Keuls (Figs. 4–6) or two-way ANOVA (GraphPad Prism, version 4; Tables 1–3 and Fig. 2). P values of <0.05 were considered statistically significant. Values are expressed as means ± SE.

RESULTS

AGT ASO penetrates the kidney and cyst epithelium. Mice were treated with the AGT ASO or Scr ASO from 4 to 16 wk of age, and kidney cross-sections were evaluated to determine ASO distribution. On immunohistochemistry, the distribution of AGT ASO accumulation throughout the kidneys demonstrated a high degree of renal ASO accumulation with a gradient of high to low accumulation from the renal cortex to the medulla in cystic kidneys (Fig. 1). Additionally, cystic epithelial cells also showed AGT ASO accumulation (Fig. 1).

AGT ASO reduced AGT RNA expression and AGT protein expression. The AGT ASO resulted in a 60% reduction of AGT RNA in the liver and a 40% reduction of AGT RNA in the kidney compared with Scr ASO (Fig. 2A). There was an increase in AGT protein in the kidney and serum in Pkd2WS25/− versus wild-type mice (Fig. 2, B and C). The AGT ASO resulted in a significant decrease in AGT protein in the kidney and serum in AGT ASO-treated versus Scr ASO-treated mice (Fig. 2, B and C). The mean value of AGT protein in the kidney and serum from six different animals in each group is shown in Fig. 2. B and C. AGT protein was detected in the urine. There was a wide variation in AGT urine values within each group without any statistical significance between

Fig. 1. Angiotensinogen (AGT) antisense oligonucleotide (ASO) distribution in the polycystic kidney. On immunohistochemistry, there was a high degree of renal ASO accumulation (brown) throughout the kidney with a gradient of high to low accumulation from the renal cortex to the medulla in the cystic kidneys. Additionally, cystic epithelial cells also showed AGT ASO accumulation.
groups (Fig. 2D). The mean value of AGT protein in the urine from three different animals in each group is shown in Fig. 2, B and C.

**AGT ASO decreases PKD and normalizes renal function in vivo.** The AGT ASO had no effect on body weight (Table 1). The two kidney weight and two kidney-to-total body weight ratio, which corrects for differences in body weight, were nearly double in Pkd2WS25/− mice compared with vehicletreated +/+ mice and normalized by the AGT ASO compared with the Scr ASO (Table 1). The cyst volume density was significantly increased in Pkd2WS25/− mice compared with +/+ mice and was decreased in AGT ASO-treated mice compared with Scr ASO-treated mice (Table 1). BUN was significantly increased in Pkd2WS25/− mice compared with +/+ mice and normalized by the AGT ASO compared with the Scr ASO (Table 1). Thus, the AGT ASO resulted in a normalization of kidney weight, two kidney-to-total body weight ratio (in %), and BUN in Pkd2WS25/− mice.

Representative kidney sections of Scr ASO-treated Pkd2WS25/− mice and AGT ASO-treated Pkd2WS25/− mice, stained with hematoxylin-eosin, at the same magnification, are demonstrated in Fig. 3. These representative sections showed that the percentage of the kidney that is occupied by cysts was significantly reduced in kidneys from AGT ASO-treated Pkd2WS25/− mice compared with Scr ASO-treated mice.

**Table 1. AGT ASO significantly reduced PKD and improved kidney function versus Scr ASO**

<table>
<thead>
<tr>
<th></th>
<th>Pkd2WS25/− Mice</th>
<th>+/+ Mice</th>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29.4 ± 2.2</td>
<td>29.4 ± 0.1</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.67 ± 0.07</td>
<td>0.35 ± 0.02</td>
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<tr>
<td>Two kidney-to-total body weight ratio, %</td>
<td>1.5 ± 0.1</td>
<td>1.2 ± 0.02</td>
</tr>
<tr>
<td>Cyst volume density, %</td>
<td>22 ± 2.9</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>34 ± 3</td>
<td>32 ± 3</td>
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</table>

Values are means ± SE; n, no. of mice/group. AGT, angiotensinogen; ASO, antisense oligonucleotide; PKD, polycystic kidney disease; Scr ASO, scrambled ASO; *P < 0.05 vs. +/+ mice; **P < 0.01 vs. +/+ mice; ***P < 0.0001 vs. +/+ mice; †P < 0.001 vs. Scr ASO-treated Pkd2WS25/− mice; ‡P < 0.05 vs. Scr ASO-treated Pkd2WS25/− mice; #P < 0.05 vs. Pkd2WS25/− mice; ¤P < 0.01 vs. Scr ASO-treated Pkd2WS25/− mice and not significant vs. +/+ mice.

Fig. 3. AGT ASO decreases polycystic kidney disease (PKD). Representative kidney sections of Scr ASO-treated Pkd2WS25/− mice and AGT ASO-treated Pkd2WS25/− mice (right), stained with hematoxylin-eosin, at the same magnification, show that kidney size and the percentage of the kidney that is occupied by cysts were significantly reduced in kidneys from AGT ASO-treated Pkd2WS25/− mice compared with Scr ASO-treated mice. The picture of the Scr ASO-treated kidney (left) is reproduced from our previous study (37) with permission.
Table 2. IL-12, CXCL1, and TGF-β are significantly increased in Pkd2WS25/− mice and decreased by AGT ASO

<table>
<thead>
<tr>
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<th>Scr ASO</th>
<th>AGT ASO</th>
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<tr>
<td></td>
<td>+/+</td>
<td>Pkd2WS25/−</td>
<td>+/+</td>
<td>Pkd2WS25/−</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>6</td>
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<tr>
<td>IL-1, pg/mg</td>
<td>0.28 ± 0.1</td>
<td>0.18 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>IL-4, pg/mg</td>
<td>0.08 ± 0.04</td>
<td>0.04 ± 0.03</td>
<td>0.34 ± 0.2</td>
<td>0.03 ± 0.01</td>
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<tr>
<td>IL-5, pg/mg</td>
<td>0.16 ± 0.05</td>
<td>0.08 ± 0.03</td>
<td>0.3 ± 0.14</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>IL-12, pg/mg</td>
<td>8.4 ± 1.2</td>
<td>9.3 ± 2.3</td>
<td>37.3 ± 16‡</td>
<td>8.8 ± 1.8§</td>
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<tr>
<td>CXCL1, pg/mg</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>3.4 ± 1.2*</td>
<td>0.6 ± 0.2†</td>
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<tr>
<td>TNF-α, pg/mg</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>0.8 ± 0.1</td>
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<tr>
<td>IFN-γ, pg/g</td>
<td>20 ± 6</td>
<td>10 ± 0.5</td>
<td>90 ± 50</td>
<td>90 ± 50</td>
</tr>
<tr>
<td>TGF-β, pg/mg</td>
<td>53 ± 7</td>
<td>40 ± 8</td>
<td>102 ± 29*</td>
<td>32 ± 5†</td>
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</table>

Values are means ± SE; n, no. of mice/group. CXCL1, chemokine (C-X-C motif) ligand 1; TGF-β, transforming growth factor-β. *P < 0.05 vs. vehicle-treated +/+ mice; †P < 0.05 vs. Scr ASO; ‡P = 0.07 vs. vehicle; §P = 0.07 vs. Scr ASO.

Next, differences in PKD between male and female Pkd2WS25/− mice were determined. Besides body weight, 2 kidney weight, two kidney-to-total body weight ratio, cyst volume density, and BUN were not significantly different between male (n = 7) and female (n = 3) mice in the Scr ASO-treated group. Body weight was 30 ± 0.8 g in male mice and 24.8 ± 3 g in female mice (P = 0.03). The two kidney weight was 0.65 ± 0.06 g in male mice and 0.7 ± 0.1 g in female mice. The two kidney-to-total body weight ratio was 2.1 ± 0.2% in male mice and 2.9 ± 0.3% in female mice. Cyst volume density was 27.4 ± 2.8% in male mice and 41.1 ± 7.6% in female mice. BUN was 39 ± 4 mg/dl in male mice and 47 ± 10 mg/dl in female mice.

Increase in IL-12 and CXCL1 in Pkd2WS25/− kidney. IL-1, IL-4, IL-5, IL-12, CXCL1 (also known as IL-8 in humans and KC in mice), IL-13, IFN-γ, and TNF-α were measured in kidney tissue homogenates as described in Methods. There was an increase in CXCL1 and IL-12 in Pkd2WS25/− mice (Table 2). The increase in CXCL1 and IL-12 was decreased by the AGT ASO.

AGT ASO decreased the number of PCNA-positive cells. The number of PCNA-positive cells per high-power field in noncystic tubules in the cortex was 0.4 ± 0.1 in +/+ mice, 0.8 ± 0.5 in Scr ASO-treated Pkd2WS25/− mice (P < 0.01 vs. +/+ mice), and 0.9 ± 0.5 in AGT ASO-treated Pkd2WS25/− mice (P < 0.01 vs. +/+ mice and P = not significant vs. Scr ASO-treated Pkd2WS25/− mice; Fig. 4A). Representative pictures of PCNA-positive cells in noncystic tubules are shown in Fig. 4, B–D. While PCNA-positive cells in noncystic tubules were increased in PKD kidneys, the AGT ASO did not have an effect on PCNA-positive cells in noncystic tubules of PKD kidneys.

Sirius red staining. Sirius red staining was quantitated in the interstitium in areas devoid of cysts. The percentage of Sirius red staining in the kidney was 17 ± 0.7 in +/+ mice, 32 ± 0.6 in Scr ASO-treated Pkd2WS25/− mice (P < 0.01 vs. +/+ mice), and 21 ± 0.5 in AGT ASO-treated Pkd2WS25/− mice (n = 4, P < 0.01 vs. AGT ASO-treated Pkd2WS25/− and P < 0.05 vs. +/+ mice; Fig. 6A). Representative pictures of Sirius red staining in +/+ , Scr ASO-treated Pkd2WS25/−, and AGT ASO-treated Pkd2WS25/− mice are shown in Fig. 6B. Sirius
red staining was seen surrounding kidney cysts as well as in the interstitium independently of kidney cysts.

Increase in TGF-β in the Pkd2WS25/− kidney. TGF-β in the kidney was increased in Pkd2WS25/− mice compared with +/+ mice and decreased in Pkd2WS25/− mice treated with the AGT ASO (Table 2).

Effect of CD36 ASO. The CD36 ASO reduced CD36 RNA expression by 90% in the liver and 70% in the PkdWS25/− mouse kidney. The CD36 ASO had no effect of PKD disease progression and kidney function in Pkd2WS25/− mice (Table 3).

DISCUSSION

Tissue ANG II contributes to kidney damage by hemodynamic factors, e.g., vasoconstriction, and nonhemodynamic factors, e.g., cell proliferation or growth, inflammation, immune responses, apoptosis, fibrosis, and scarring (31, 35, 38). Specifically, the mechanisms by which ANG II contributes to disease are generation of ROS, activation of NF-κB, endothelial dysfunction, cell hypertrophy, macrophage infiltration, fibroblast proliferation, excess extracellular matrix production, thrombosis, and atherosclerosis (35). Proliferation of cells lining the cyst, inflammation, and fibrosis are features of PKD. Thus, we determined the effect of AGT inhibition on proliferation, inflammation, and fibrosis.

Human and experimental data provide strong evidence that abnormal proliferation in tubular epithelial cells plays a crucial role in cyst development and/or growth in PKD (52). Genetic manipulations that induce the proliferation of tubular epithelial cells in mice cause cysts to form in the kidney (39, 49). In the present study, there was increased proliferation in noncystic tubular epithelium in Pkd2WS25/− mice compared with wild-type controls. In addition, there was proliferation in epithelial cells lining the cysts, although less than in other models of PKD, such as the Han:SPRD rat. However, the AGT ASO did not have a significant effect on proliferation, suggesting that...
the mechanism of decreased cyst growth by the AGT ASO is independent of proliferation. Pkd2WS25/* mice have been described as a model of progressive cystic epithelial cell dedifferentiation in which fluid accumulation in late-stage cysts is mediated by transepithelial secretion of Cl− (45). In addition to proliferation, other factors, like increased fluid secretion by cells lining the cysts, remodeling of the extracellular matrix (20), increased apoptosis (15), and suppressed autophagy (36), may play a role in cyst formation and growth. In another study (41), proliferation was found to unlikely be the sole mechanism underlying the rapid cystogenesis observed after injury in mice that lose cilia function in adulthood. Thus, the AGT ASO may decrease cyst growth by mechanisms other than inhibiting proliferation. The mechanism of effect of the AGT ASO may be related to decreased fluid secretion, decreased dedifferentiation, remodeling of the extracellular matrix, decreased apoptosis, or increased autophagy.

AGT inhibition reduced proinflammatory cytokines CXCL1 and IL-12 in PKD kidneys. CXCL1 is a neutrophil chemokine. In this regard, we have shown that neutrophil depletion is not protective in ischemic (33) and cisplatin-induced (18) acute kidney injury. The effect of neutrophil depletion in PKD is not known and represents an interesting subject of future study in PKD. Regarding inflammatory cells in PKD, macrophage depletion is protective in the Pkd1 knockout model of PKD (24). CXCL1 may have an effect that is independent of neutrophils. In liver cyst epithelial monolayers, CXCL1, via its receptor chemokine (C-X-C motif) receptor 2, promotes cell proliferation (1).

IL-12 is normally produced by dendritic cells, macrophages, and B cells in response to antigenic stimulation (23, 48). IL-12 stimulates T cells to produce IFN-γ and TNF-α (23, 48). In this regard, TNF-α is a mediator of cyst formation and/or growth in PKD, and inhibition of TNF-α is protective in Pkd2 knockout mice (27). There was an increase in IL-12 in PKD kidneys. It is possible that IL-12 produced by dendritic cells, macrophages, or B cells in PKD stimulates T cells to produce IFN-γ and TNF-α. However, while there was an increase in IFN-γ and TNF-α in Pkd2WS25/* kidneys (Table 2), the increase in IFN-γ and TNF-α in Pkd2WS25/* kidneys was not statistically significant. IL-12 also has antiangiogenic activity (11). IL-12 is being tested as an anticancer drug (11). Angiogenesis is increased in PKD (50). It is possible that there is an increase in IL-12 in PKD kidneys to counteract the increased angiogenesis. IL-12 can activate the JAK-STAT pathway (19). PKD1 directly activates JAK-STAT via a pathway that requires PKD2 (3). Polycystin 1 regulates STAT activity in a JAK2-dependent manner (43). In this regard, genetic and pharmacological methods of STAT3 or STAT6 inhibition have shown promising results in PKD (51). Thus, AGT ASO-induced inhibition of IL-12 may inhibit JAK-STAT signaling in PKD.

AGT inhibition reduced TGF-β in Pkd2WS25/* kidneys. TGF-β has been implicated in the vascular complications of PKD, such as aneurysms (5, 28). There is increased TGF-β expression in experimental Pkd1 knockout mouse kidneys and in human PKD kidneys (22). The pathophysiological role of the TGF-α/EGF receptor axis in murine PKD has been demonstrated, and TGF-α inhibition ameliorates PKD in the bpk mouse model (12). However, a mechanistic role of TGF-β in cyst formation and growth has not been demonstrated. In fact, in ADPKD cells from human kidneys in vitro, a system devoid of fibrosis, TGF-β inhibits cystogenesis (16). In the present study, it is possible that AGT inhibition-induced inhibition of TGF-β resulted in less cyst formation and growth.

AGT inhibition reduced TGF-β and fibrosis, as determined by Sirius red staining. Fibrosis is a pathological feature of all forms of chronic kidney disease (14). Therapeutic approaches to fibrosis that target the TGF-β pathway have received prominence in the treatment of kidney diseases (10). However, in PKD, it is proliferation and fluid secretion by cysts lining epithelial cells that play a primary role in cyst growth. It is believed that therapies for PKD should target cyst formation and growth before secondary factors like fibrosis occur (21). However, ADPKD cyst lining epithelium also demonstrates TGF-β-induced collagen type I and fibronectin expression (29). Fibrosis inhibition has not been directly tested in PKD. In the present study, while fibrosis may have played a role in the loss of kidney function in PKD, it is unlikely that AGT inhibition of fibrosis played a role in the inhibition of cyst growth in PKD.

CD36, also known as fatty acid translocase or FAT/CD36, is an integral membrane protein found on the surface of many cell types, including proximal tubular cells (4). CD36 is a member of the class B scavenger receptor family of cell surface proteins. CD36 has been implicated in hemostasis, thrombosis, inflammation, lipid metabolism, and atherogenesis. Advanced oxidation protein products activate the RAS via CD36 (4). As a RAS, activation and activation of the RAS are features of PKD. The effect of a CD36 ASO on PKD progression was determined. Inhibition of CD36 had no effect on PKD. Thus, it is likely that activation of the RAS in the Pkd2WS25/* mice model of PKD is independent of CD36.

There is a paucity of studies of the effect of AGT inhibition in kidney diseases. Chronic antisense therapy against AGT decreases blood pressure and cardiac hypertrophy in spontaneously hypertensive rats (32). AGT-deficient mice have a profound decrease in blood pressure (9). The effect of specific AGT inhibition on cyst growth and kidney function in PKD is not known. In the present study, the effect of AGT inhibition on cyst growth and kidney function was determined in PKD. In summary, AGT inhibition resulted in significant decreases in kidney weight, two kidney-to-total body weight ratio, and cyst volume density and an improvement in kidney function in PKD mice. The AGT ASO resulted in decreases in TGF-β, interst-
tional fibrosis, and the proinflammatory cytokotes CXCL1 and IL-12 in the kidney. ADPKD is a chronic disease that will require chronic therapy. Chronic AGT inhibition represents a possible future therapy for ADPKD.

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DISCLOSURES

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