

The expression of somatostatin receptor 2 decreases during cyst growth in mice with polycystic kidney disease.

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by progressive renal cyst formation and expansion. Several clinical trials show that somatostatin (SST) analogues halt cyst growth and progression of ADPKD by inhibiting **adenosine 3',5'-cyclic monophosphate (cAMP)** signaling. However, two studies suggest that the effect of the SST analogue octreotide on kidney growth during the first year of treatment is reduced in the subsequent follow-ups and the kidney enlargement resumes. We hypothesize that this biphasic change in kidney growth during octreotide treatment may be due to changes in SST receptor (SSTR) 2 expression. Here we analyzed expression of renal SSTR2 in various **polycystic kidney disease (PKD)** mouse models in which *PKD1* gene expression was disrupted on postnatal day 10 or 18 by tamoxifen. Using immunohistochemical analysis, we showed that the distribution of SSTR2 in murine kidneys is mainly in distal tubules and collecting ducts. In addition, in both PKD models, we observed a significant decrease in SSTR2 expression in epithelia of dilated tubules and cystic epithelia in mice with end stage of PKD compared to wild type mice. These findings were further confirmed by qPCR on mRNA levels of SSTR2. In conclusion, our data show that SSTR2 expression levels are reduced during kidney cyst growth, which may suggest reduced efficacy in long-term treatment with somatostatin analogues.

Key words: ADPKD, cystic kidney, somatostatin, cAMP

Impact Statement: Somatostatin (SST) analogues has been shown to halt cyst growth and progression of ADPKD by several clinical trials. However, two studies suggest that the effect of the SST analogue octreotide on kidney growth during the first year of treatment is reduced in the subsequent follow-ups and the kidney enlargement resumes. This biphasic change in kidney growth during octreotide treatment may be partially explained by alterations in SSTR2 expression. Here, we found that SSTR2 is mainly expressed in distal tubules and collecting ducts in murine kidneys and the expression of SSTR2 decreases during cyst growth in two PKD mice models. Our data may thus provide possible explanations for the lack of efficacy in long-term treatment with SST analogues.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most frequent hereditary disorder caused by a mutation in the *polycystic kidney disease (PKD)1* or *PKD2* gene[1,2]. The disease is characterized by progressive development of numerous fluid-filled kidney cysts accompanied by interstitial fibrosis formation, leading to destruction of the kidney architecture and ultimately kidney failure[1-3]. Extra-renal complications can occur during ADPKD, such as cysts in the liver and pancreas, hypertension and cerebral aneurysms[3]. Although tolvaptan has recently become available for selected ADPKD patients to slow progression of ADPKD, there is currently no cure[4-6]. In order to meet the urgent needs of patients, considerable effort has been made to explore the mechanisms that are essential for the development of ADPKD, allowing identification of several potential targets for therapeutic interventions[7].

Renal cyst formation and expansion during ADPKD are the results of excessive epithelial cell proliferation, continuous luminal fluid secretion as well as extracellular matrix remodeling [4,8]. Previous studies implicate that one of the mechanisms associated with these processes is enhanced intracellular cAMP signaling[5,9,10]. Therefore, the molecules that mediate cAMP signaling may be potential therapeutic targets for treating ADPKD. Indeed, the activation of vasopressin-2 receptor (V2R) induces generation of cAMP, and targeting V2R with its antagonist tolvaptan reduces cAMP levels in kidney epithelial cells limits cystogenesis in several PKD models[11-14]. Furthermore, several completed or currently active clinical studies under the TEMPO program indicate that tolvaptan shows a treatment effect in reducing total kidney volume[6,15,16]. In addition, somatostatin (SST), a cyclic 14

amino acid peptide hormone, exerts inhibitory effects on cAMP signaling through binding to its G-protein-coupled receptors (SSTR1-5)[17]. SST has been proven to inhibit vasopressin-induced cAMP formation in **Madin-Darby Canine Kidney (MDCK)** cells and diminishes the cAMP concentration in rat cortical and medullary collecting tubules[18,19]. Targeting SSTRs with SST analogues **inhibits** cell proliferation, cAMP levels and hepato-renal cystogenesis in rodent models of polycystic kidney and liver disease[11,20-22]. In consistence with these observations, a few completed clinical trials with the primary focus of inhibiting cAMP using SST analogues (octreotide or lanreotide) have indicated potential clinical benefit in patients with ADPKD[23-27].

The administration of octreotide for periods of 6–12 months effectively inhibits the increase in total kidney volume[25,26]. This has been confirmed in a 6-month clinical trial which demonstrated that lanreotide decreases total kidney volume in 43 ADPKD patients [27]. Interestingly, two relative small studies suggest that the efficacy of SST analogue treatment appears to reduce with longer follow-ups[23,24]. Long-acting octreotide halts kidney growth during the first year of treatment, but with ongoing treatment tolerance develops and the effect is reduced during year 2. In the future, more information on efficacy and tolerance of prolonged somatostatin treatment will come from an ongoing clinical trial with a period of 120 weeks[28]. Because of this biphasic change in kidney growth during SST analogues treatment, we propose that SSTRs might be progressively down-regulated with ADPKD development, leading curtailed long-term efficacy. Since both octreotide and lanreotide have the highest affinity for SSTR2 [29], we examined expression of this receptor in various PKD mouse models at different time points after disruption of the *PKD1* gene.

Materials and methods

Cell Culture

SV40 large T-antigen immortalized murine wild type (WT) proximal tubular epithelial cells (PTEC) were derived from a *Pkd1*^{lox,lox} mouse and cultured as previously described[30]. Briefly, PTEC cells were maintained at 37°C and 5% CO₂ in DMEM/F12 with GlutaMAX (Gibco, Fisher Scientific) supplemented with 100 U/ml penicillin/streptomycin (Gibco, Life Technologies), 2% Ultrosor G (Pall BioSeptra), 1x Insulin-Transferrin-Selenium-Ethanolamine (Gibco, Life Technologies), 25 ng/L Prostaglandin E1 (Sigma–Aldrich),

30 ng/L Hydrocortisone (Sigma–Aldrich). Murine collecting duct mIMCD-3 cells (American Type Culture Collection; ATCC) was maintained in DMEM/F12 with GlutaMAX (Gibco, Fisher Scientific) supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin (Gibco).

Animal Models of Polycystic Kidney Disease

The tamoxifen-inducible kidney-specific *Pkd1*-deletion (tam-KspCad-CreERT2;*Pkd1*^{lox2-11;lox2-11} or in short iKsp-*Pkd1*^{del}) mice and oral tamoxifen administration have been described previously[31,32]. The mice received 150 mg/kg tamoxifen at postnatal day PN18–PN19 (PN18 model), or 15 mg/kg tamoxifen at PN10–PN11 (PN10 model). PN18 mice were sacrificed at 4 or 12 (n=6) weeks after tamoxifen administration and PN10 mice were sacrificed at 1 or 3 weeks (n=5) after tamoxifen administration. Mice were bred at the animal care facility of the Leiden University Medical Center (LUMC). All experiments were approved by the local animal experimental committee of the LUMC and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture.

qPCR

Total RNA was isolated from cultured cells or kidney tissues with TRI Reagent (Sigma–Aldrich) following the manufacturer’s protocol. cDNA synthesis was done with the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s protocol. qPCR was done in duplicate on the LightCycler 480 II (Roche) using 2× FastStart SYBR-Green Master (Roche) according to the manufacturer’s protocol. The following primers were used: **hypoxanthine guanine phosphoribosyltransferase (HPRT)**: forward 5'-GGCTATAAGTTCTTTGCTGACCTG-3' and reverse 5'-AACTTTTATGTCCCCGTTGA-3'; SSTR2: forward 5'-TCCTCCGCTATGCCAAGAT-3' and reverse 5'-CAATGGCCAGGTTAAGGATG-3'; SSTR5: forward 5'-GCAAGGTCTTGGCCTTTATG-3' and reverse 5'-CAGTCTTCACCATGCGTCTG-3'; Data were analysed with LightCycler 480 Software, version 1.5 (Roche). Gene expression was calculated using the LinRegPCR method as described previously[33] and normalized to HPRT expression, giving the relative gene expression. Mean gene expressions and standard deviations of the different groups were calculated.

(Immuno)histochemistry

Kidney tissues were fixed in formalin, embedded in paraffin and 4- μ m-thick slides were subsequently stained with haematoxylin and eosin according to routine procedures. Segment marker staining was performed with sequential renal sections (4 μ m), using rabbit polyclonal anti-megalin (Pathology LUMC), goat polyclonal anti-Tamm-Horsfall protein (uromodulin; Organon Teknika-Cappel), and rabbit polyclonal anti-aquaporin-2 (Calbiochem) as previously described[34]. SSTR2 staining was performed with a rabbit monoclonal anti-SSTR2 antibody (Abcam, ab134152). A horseradish peroxidase (HRP)-conjugated polymer detection system was applied for visualization, using rabbit envision HRP (Dako) or rabbit anti-goat HRP (Dako) as the secondary antibody. Immune reactions were revealed using diaminobenzidine and counterstained with haematoxylin.

cAMP assay

Levels of cAMP were measured in mice control and PN10 cystic kidneys (n = 2 for each experimental condition). cAMP levels were determined by the cAMP enzyme immunoassay kit (Sigma-Aldrich Chemie N.V.).

Statistical analysis

Statistical comparisons between groups were performed using an unpaired *t*-test. *p* values less than 0.05 were considered significant.

Results

The expression pattern of SSTR2 in normal mouse kidneys

We investigated the expression pattern of SSTR2 in paraffin-embedded kidney sections of adult WT mice. Proximal tubules, distal tubules and collecting ducts were identified by staining for their specific markers megalin, Tamm-Horsfall protein and aquaporin-2, respectively (Figure 1A). SSTR2 was mainly detected in a large proportion of distal tubules and collecting ducts; In contrast, SSTR2 staining was almost absent in proximal tubules. To verify this observation, we subsequently examined the mRNA levels of SSTR2 and SSTR5 in PTEC and mIMCD-3 cells that derived from proximal tubules and collecting ducts respectively (Figure 1B). The mRNA level of SSTR2 was higher in the collecting ducts than in the proximal tubules, confirming the results of the SSTR2 immunostaining.

SSTR2 expression decreased during cyst growth in two PKD mouse models

We next examined SSTR2 expression in the *Pkd1*^{del} models during different phases of disease. Deletion of the *Pkd1* gene in mice of different age results in distinct PKD phenotypes[35,36]. Previous studies have indicated that inactivation of *Pkd1* at prenatal day 10 results in rapid cyst formation within 3 weeks and cysts **are** primarily from distal tubules and collecting ducts[36]. In contrast, the PN18 model **has** a much slower progression of PKD and **develops** polycystic kidneys within 3 months, with cysts derived from all tubular segments[36] (Figure 2).

At the early stage of PKD in the PN18 model (PN18+4weeks), SSTR2 expression was still observed in most of the dilated tubules originating from distal tubules and collecting ducts. At 12 weeks after tamoxifen administration, PN18 mice developed massive cystic kidneys and showed a **clear** loss of SSTR2 expression in almost all cysts compared with PN18 mice at 4 weeks (Figure 3A and also compare Figure 3A and B to Figure 1A). Similarly, PN10 model also showed reduction in SSTR2 levels along the disease progression compared with WT mice. SSTR2 expression was decreased in cystic epithelia at mild stage (PN10+1weeks) of PKD and was lost in the majority of cysts at the end stage of the disease (compare Figure 3C to Figure 1A). To verify this finding, we also analysed mRNA levels of SSTR2 in the animal kidney lysates, using SSTR5 as a comparison. The mRNA levels of SSTR2 from PN18+12 weeks mice was significantly lower compared to WT mice, whereas this reduction was not detected in PN10 mice (Figure 3D and E).

Discussion

ADPKD is a heterogenetic disorder caused by mutations in the *PKD1* or *PKD2* gene, but additional factors are also involved[1,2,8,36]. There is compelling evidence that aberrant cAMP signalling plays a critical role in renal and liver cystogenesis[5]. Several animal models (**including ours**) for cystic kidney disease demonstrate elevated cAMP content in kidneys (**Fig. S1A**), and show that reduced cAMP increase, by targeting SSTRs, limits the disease progression[5,11,20-22]. Based on these findings and the fact that SSTRs are expressed in human kidneys[37], SST analogues have been tested in several clinical trials for treating ADPKD. Although somatostatin analogues significantly halt cyst growth and progression of ADPKD during the first year of treatment, the effect then reduces, as observed for octreotide [23-28].

The reduced long-term treatment-effect of SST analogues may be partially explained by the data described in this study. Here, we show that renal SSTR2 expression is down-regulated during cyst growth in mice with ADPKD. In two PKD mouse models, expression of SSTR2 declined progressively in dilated distal tubules and collecting ducts, and was completely lost in almost all cystic epithelia at the end stage of the disease. The findings in immunohistochemistry were supported by data on corresponding mRNA expression of SSTR2 in PN18 mice, but not in PN10 mice. This may be due to the fact that PN10 mice were still in developmental stage and the proliferation indices varied between animals, so that the mRNA expression **could** not correctly reflect protein expression.

We also studied SSTR2 expression in human material in order to correlate our findings with human disease. In immunohistochemical analysis, we observed that SSTR2 is mildly expressed in all kidney segments, but high expression **is** mainly in proximal tubules. Importantly, cystic lesions of ADPKD patients also showed decreased expression of SSTR2 in epithelia of dilated tubules and cystic epithelia compared with healthy controls (Fig. S1B). Using antibodies that generated against the same immunizing peptide, ETQRTLLNGDLQTSI corresponding to residues 335–369 of the human SSTR2, other investigators detected renal SSTR2 expression in the same pattern as we observed in immunohistochemical experiment[38-41]. However, the results from immune-histochemical analysis are not in line with our qPCR analysis, which showed more SSTR2 expression in cells derived from human collecting duct than proximal tubules, similar as observed in mice (Fig. S1C); Therefore, we are not fully confident with the immunohistochemistry results from the human tissue. Further research to confirm SSTR2 expression pattern in human kidney using another experimental method is required.

Our findings, showing decreased SSTR2 expression in kidney cysts, are in line with the previous study reporting diminished expression of SSTR1 and SSTR2 in cystic cholangiocytes of animal models and patient of PKD[21]. The mechanism by which SSTR2 expression is down-regulated during cyst growth remains elusive. However, it has been shown that treatment with SST analogues (in response to octreotide and pasireotide) increases immunoreactivity of SSTR2 in cystic cholangiocytes[21]. Furthermore, it is suggested that SST has the ability to up-regulate SSTR2 at cell membrane[42]. Thus, it is tempting to speculate that the decrease in SSTR2 expression may be due to defective epithelial differentiation, which could be partially restored by SST analogues treatment during the progression of cystic kidney disease.

Several issues should be taken into consideration when interpreting our data. First, although our *Pkd1*^{del} models are well-established models to study ADPKD, they do not completely mimic the progression of cystic disease in patients with ADPKD. For example, the *Pkd1*^{del} model has relatively synchronized cyst formation, since deletions in the *Pkd1* gene can happen at the same time in much larger numbers of cells than in human ADPKD[8]. Therefore, our descriptive data obtained in animal models cannot fully represent the human situation. Second, although octreotide, lanreotide and pasireotide mainly target SSTR2, SSTR5 also shows relatively high affinity for SST analogues[29] and its expression should be investigated in normal and cystic kidneys. However, the commercially available antibody that is frequently used to detect SSTR5 in murine tissue failed to produce consistent and reproducible data [43] (data not shown), which may be explained by the fact that it is only rat-specific. Finally, our data suggested that the SSTR2 expression pattern may be **different** between human and murine kidneys, which should be kept in mind when targeting SSTR2 with SST analogues.

In conclusion, we present the distribution of SSTR2 in murine kidneys and irrespective of the potential clinical relevance, our data suggest that expression of SSTR2 decreases during the development of ADPKD. Reduced target expression may also be taken into account when targeting other trans-membrane receptors for treating ADPKD.

Statement of Author Contributions:

C. Lin conceived and designed the experiments, performed the experiments, analyzed the data and wrote main manuscript text; H. Happé conceived, designed and performed the part of the experiments; K. Veraar performed part of the experiments; M. Scharpfenecker designed the experiments and corrected manuscript text; D.J.M Peters conceived and designed the experiments, analyzed the data and wrote the paper. All authors reviewed the manuscript.

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Declaration of Conflict of Interest statement: None

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Figure legend

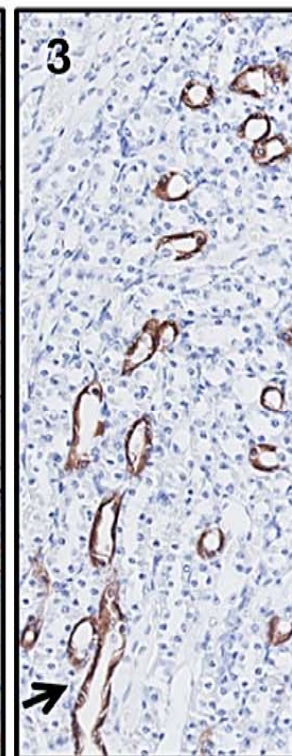
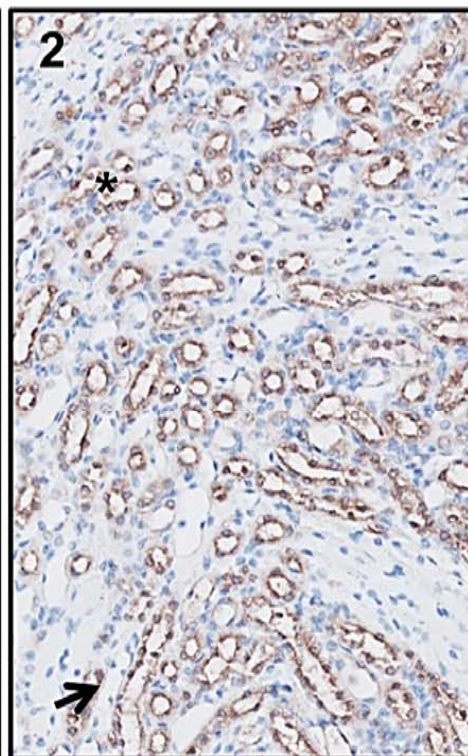
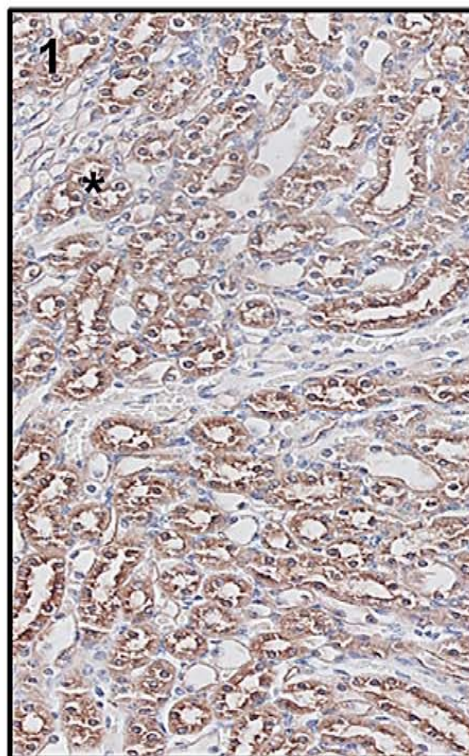
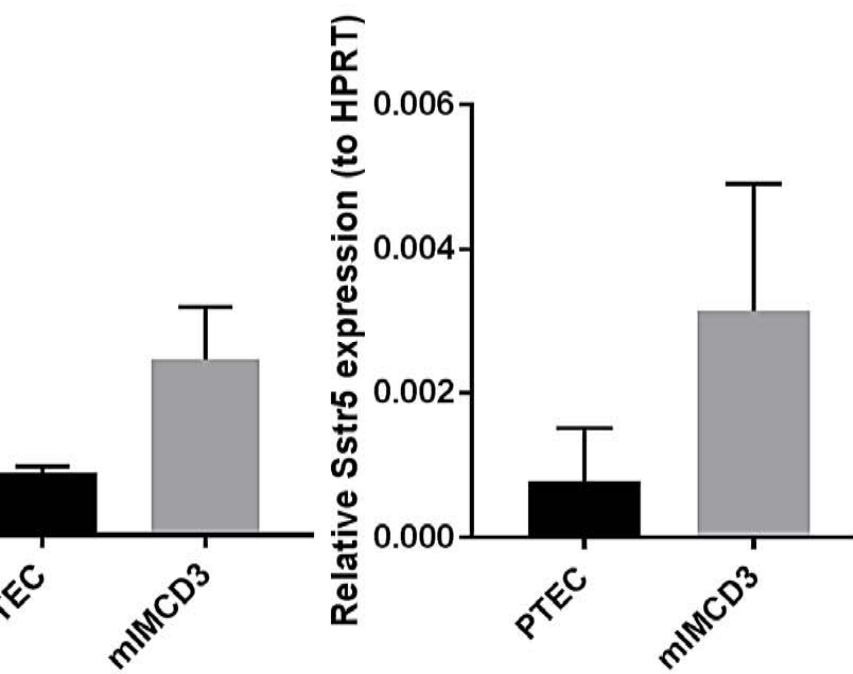
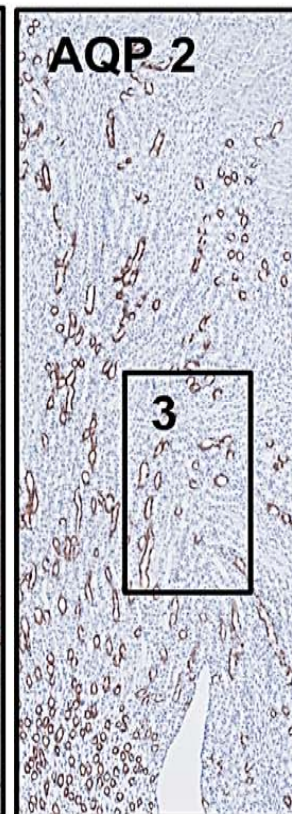
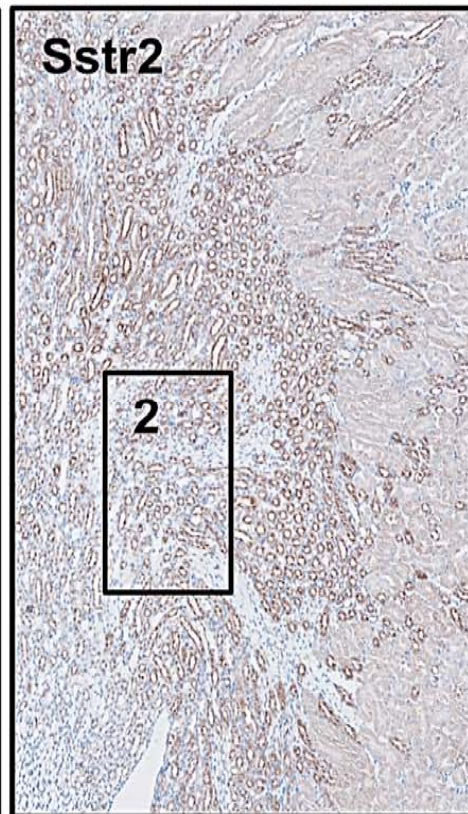
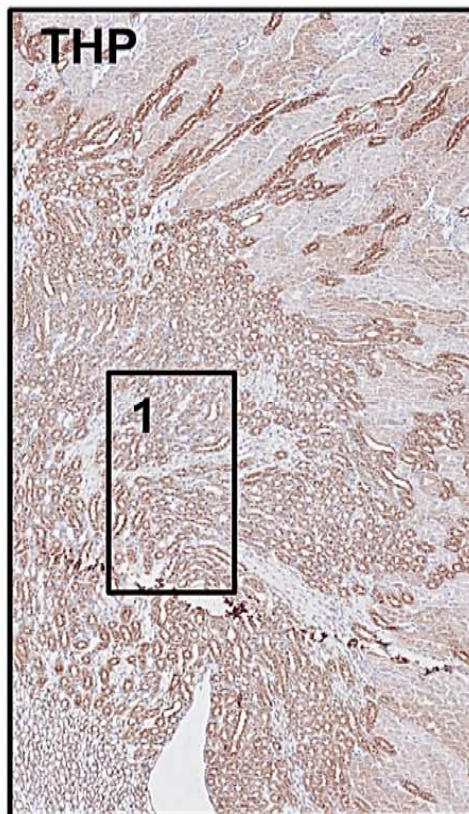
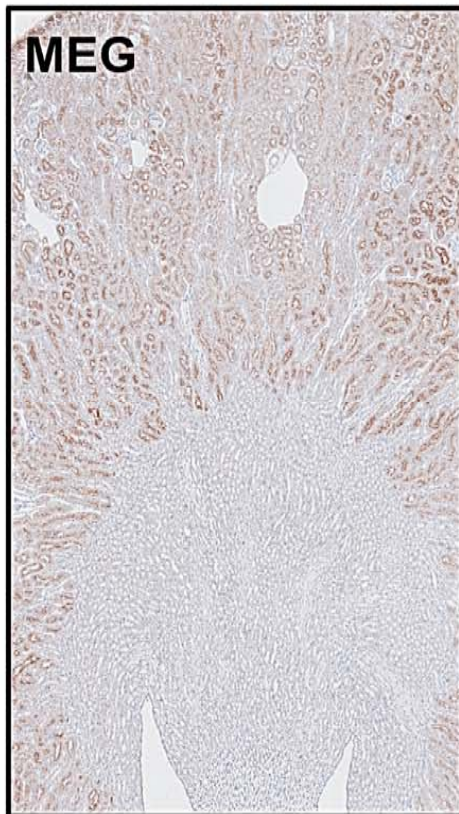
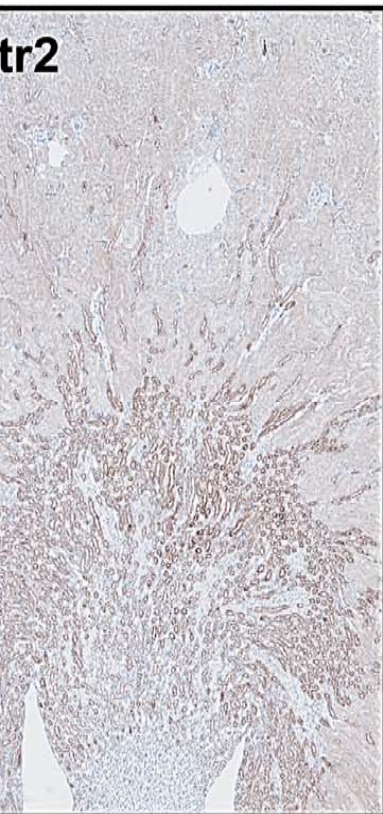
Fig. 1. SSTR2 expression pattern in WT adult mouse kidney. (A) Representative segment markers and SSTR2 antibody stained sections. MEG = megalin; THP = Tamm-Horsfall; AQP 2 = Aquaporin-2. Asterisks indicate the same area on sequential sections with THP (in enlarged picture 1) and SSTR2 staining (in enlarged picture 2). Also, arrowheads indicate the same area on sequential sections with AQP 2 (in enlarged picture 3) and SSTR2 staining (in enlarged picture 2). (B) SSTR2 and SSTR5 mRNA levels in PTEC and mIMCD3 cells were assessed by real-time reverse transcriptase PCR. Data are expressed relative to the housekeeping gene HPRT (n=3).

Fig. 2. Kidneys from P10 and P18 models after *PKDI* gene disruption. (A) Hematoxylin and eosin-stained sections of kidneys harvested from PN10 and PN18 mice at different time points after *PKDI* gene disruption.

Fig. 3. SSTR2 expression decreases during cyst growth in both P10 and P18 mice with ADPKD. (A) Image of renal section from PN18 mice at late stage (PN18+12wks) of PKD was immunostained using antibodies specific for SSTR2. The black frame indicates the area where non-dilated tubules with positive for SSTR2 staining. (B and C) Images of renal sections from PN18 and PN10 mice were immunostained using SSTR2 antibodies. Enlargements of SSTR2 images are shown in the lower panel. Arrowheads indicate dilated tubules or cysts with positive SSTR2 staining and asterisks indicate dilated tubules or cysts which show virtually negative SSTR2 staining. (D) and (E) SSTR2 and SSTR5 mRNA levels in PN18 and PN10 mice kidney tissue at different time points after *PKDI* gene disruption were assessed by real-time reverse transcriptase PCR. Data are expressed relative to the housekeeping gene HPRT. The comparison is between kidneys from WT and end stage of diseases, n=5-7, *P<0.05 (*t* test).

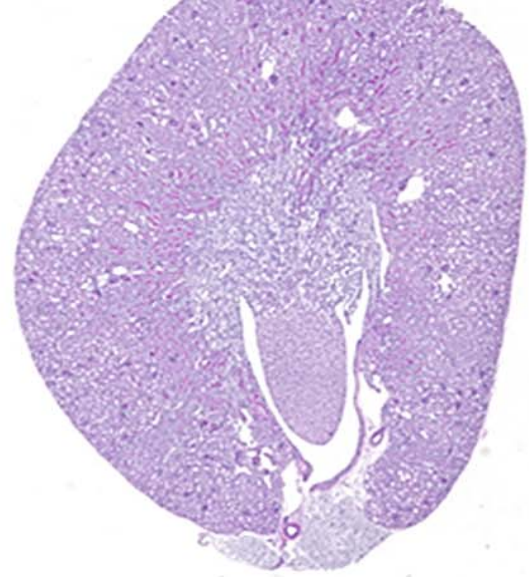
Fig. S1. Expression of SSTR2 in human kidneys. (A) cAMP assay was implemented showing a clear increase in cAMP levels in PN10 cystic kidneys compared to normal kidneys

(n=2). **(B)** Representative proximal tubules marker megalin and SSTR2 antibody stained sections. Left panel shows the same area on sequential sections from normal human kidneys with MEG and SSTR2 staining. Middle and right panels show the same area on sequential sections from kidneys of patient with ADPKD. **(C)** SSTR2 mRNA levels in HK2 and RCTEC cells were assessed by real-time reverse transcriptase PCR. Data are expressed relative to the housekeeping gene GAPDH (n=3)





+ 1 week



+ 4 weeks

Time after gene disruption

